

Durham E-Theses

Synthesis of retinoic acid analogues & investigations into their ability to induce stem cell differentiation

Caroline Emma Bridgens

How to cite:

Bridgens, Caroline Emma (2009) Sythesis of retinoic acid analogues & investigations into their ability to induce stem cell differentiation. Doctoral thesis, Durham University.

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a <https://etheses.durham.ac.uk/id/eprint/2068/> is made to the metadata record in Durham E-Theses
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the [full Durham E-Theses policy](#) for further details.

Synthesis of Retinoic Acid Analogues & Investigations into their Ability to Induce Stem Cell Differentiation

Caroline Emma Bridgens

**A thesis submitted for the degree of
Doctor of Philosophy**

The copyright of this thesis rests with the author or the university to which it was submitted. No quotation from it, or information derived from it may be published without the prior written consent of the author or university, and any information derived from it should be acknowledged.

**School of Biological and Biomedical Sciences
&
Department of Chemistry**



20 APR 2011

Abstract

Human embryogenesis and ensuing adult homeostasis are directed by the complex interplay of a wide range of exquisitely controlled signalling molecules and pathways. All-*trans*-retinoic acid (ATRA) is one such important hormone-like compound that regulates a wide range of processes. The endogenous effects of ATRA have the potential to be translated into treatment for numerous clinical indications; however administration at efficacious concentrations is associated with severe side effects. Consequently, a large group of synthetic molecules - known as retinoids - that are structurally and/or functionally analogous to ATRA have been prepared and tested *in vitro* in the search for a panacea and for use as pharmacological tools to elucidate the retinoid molecular pathway.

A small library of stable synthetic retinoids was prepared and their biological activity investigated using TERA2.cl.SP12 human embryonal carcinoma (EC) stem cells. Three compounds, CEB16, CEB17 and CEB18 were found to inhibit cellular proliferation and induce neural and non-neural differentiation. These effects were thoroughly characterised and quantified by monitoring phenotypic changes and the expression of established antigenic markers. Compared to the ability of ATRA and its geometric isomers 9-*cis*-retinoic acid and 13-*cis*-retinoic acid, the order of efficacy of induction of neural differentiation was found to be:

13-*cis*-retinoic acid > 9-*cis*-retinoic acid = ATRA > CEB18 > CEB17 >> CEB16.

The molecular mechanism of natural and synthetic retinoid action during TERA2.cl.SP12 differentiation was investigated by performing a detailed temporal analysis of the gene expression of eleven transcripts involved in retinoid transport, activation and metabolism. To date, limited data have been published on the effects of synthetic retinoids on the retinoid pathway during differentiation of human cells and no human studies have examined the activity of synthetic retinoids on the *HOX* genes. All of the retinoic acid inducible genes examined in this study were found to be modulated in TERA2.cl.SP12 cells in response to both ATRA and two isomeric synthetic retinoids, CEB16 and CEB17, albeit relatively slowly compared to other cell lines. These compounds are therefore believed to activate the same pathway as the natural metabolite in these cells and that this is responsible for at least some of the observed effects. Genes were regulated in a concentration and retinoid dependent manner and this modulation was often multi-phasic demonstrating the complex behaviour of the retinoid system. Interestingly, ATRA was not the most effective inductive agent in all gene analyses. For example 10 μ M CEB17 induced up-regulation of *RAR- β* more strongly than 10 μ M ATRA and 10 μ M CEB16 induced the greatest increase in *RAR- γ* transcripts.

Further experimentation is required to confirm an apparent relationship between the timing of addition of inductive agent and the expression pattern of several genes. This behaviour highlights the importance of retinoid degradation in culture, which is often overlooked. The three synthetic compounds described extensively herein should be more stable, and thus may be suitable and convenient alternatives for molecular biologists to use in place of ATRA.

Acknowledgements

Many thanks to my supervisors Dr. Stefan Przyborski, Dr. Andy Whiting and Professor Todd Marder for their guidance, inspiration and support. Thanks also to their three research groups, past and present, for day-to-day support. Special thanks are extended to Miss Vikki Christie for training in cell culture and flow cytometry, Dr. Stepan Fenyk for his assistance with western blotting, Ms. Bridie Murray for real-time PCR guidance and Dr. Ibraheem Mkhalid for training in inert atmosphere techniques and general synthetic methodology at the beginning of my project.

I wish to acknowledge Dr. Chris Redfern and his research group at the Northern Institute for Cancer Research, Newcastle University for performing tests on the SH-SY5Y neuroblastoma cell line, the kind donation of a sample of retinoid ligand LG100268, statistics advice and helpful discussions. Dr. Tolu Fasina prepared the tolan and BPEB compounds. Jonathan Barnard kindly prepared additional samples of CEB16 and CEB17.

Thanks also to the Chemistry Department analytical services, including Dr. David Albesa-Jové and Dr. Andrei Batsanov for conducting X-ray crystallographic analysis of compounds **4** and **14**, and **5** and **17**, respectively.

TABLE OF CONTENTS

Abstract.....	i
Acknowledgements	ii
Abbreviations	vi
CHAPTER 1 : THE RETINOIDS	1
1.1 Introduction.....	2
1.2 Functions of endogenous retinoids.....	3
1.3 Discovery, deficiency and excess.....	5
1.3.1 Discovery of “vitamine A”	5
1.3.2 Vitamin A deficiency & hypervitaminosis A	5
1.4 Absorption, transportation and metabolism of retinol.....	6
1.5 Molecular mechanism of retinoid action.....	11
1.5.1 Retinoid receptor complexity	14
1.5.2 Retinoid receptor independent gene regulation and non-genomic effects.....	16
1.6 Therapeutic applications	17
1.7 Limitations and adverse effects.....	19
1.8 Conclusions	20
CHAPTER 2 : SYNTHESIS OF RETINOIC ACID ANALOGUES	21
2.1 Design and synthesis of retinoids.....	22
2.1.1 Introduction	22
2.1.2 Basic structural requirements for retinoid activity	22
2.1.3 Evolution of retinoid structure.....	25
2.1.4 Synthetic approaches to the preparation of retinoids.....	25
2.2 Experimental overview	26
2.3 Preparation of TTNPB and its isomers	27
2.3.3 Vinyl C-H activation using rhodium Vaska’s catalyst	31
2.4 Preparation of 3-Me TTNPB series of compounds.....	32
2.5 Synthesis of a naphthoic acid and two acrylic acid retinoids.....	33
2.6 Crystal structures.....	34
2.7 Conclusions	37
CHAPTER 3 : BIOLOGICAL SCREENING AND CHARACTERISATION OF NATURAL & SYNTHETIC RETINOIDS IN HUMAN EC STEM CELLS	38

3.1	<i>In vitro</i> retinoid screening	39
3.1.1	Introduction	39
3.1.2	Stem cells.....	40
3.1.3	Retinoid induced cell differentiation	43
3.1.4	Retinoid (in)stability.....	44
3.1.5	Chapter aims	45
3.2	Methodology and compounds investigated.....	46
3.3	Observations of changes to cell phenotype.....	49
3.3.1	Results	49
3.4	Flow cytometry: quantitative determination of cell fate	55
3.4.1	Results	56
3.5	Proliferation.....	61
3.5.1	Results	61
3.6	Immunocytochemical (ICC) localisation of antibodies.....	64
3.6.1	Results	65
3.7	Protein expression profiling by western blot.....	83
3.7.1	Results	84
3.8	Synergism with a retinoid X selective agonist.....	87
3.8.1	Results	87
3.9	Retinoid effects on SH-SY5Y neuroblastoma cells	94
3.9.1	Neurite outgrowth.....	94
3.9.2	Fenretinide induced apoptosis	96
3.10	Discussion	97
3.11	Conclusions	107

CHAPTER 4 : INVESTIGATING THE MOLECULAR MECHANISM OF SYNTHETIC AND NATURAL RETINOID ACTION.....110

4.1	Introduction	111
4.2	Retinoid-binding proteins: mediators of retinoid activity	112
4.2.1	Cellular retinol-binding proteins.....	112
4.2.2	Cellular retinoic acid-binding proteins	113
4.2.3	CYP26: A family of retinoic acid specific P450 enzymes	114
4.2.4	Retinoic acid receptors	117
4.3	<i>HOX</i> gene expression	118
4.4	POU5F1 (OCT4) transcription factor	121
4.5	Experimental objectives	122
4.6	Results and discussion.....	123

4.6.1 Observation of changes to cell phenotypes.....	123
4.6.2 Induction of differentiation – POU5F1 expression.....	126
4.6.3 Regulation of retinoid-binding protein expression	128
4.6.4 Analysis of retinoic acid receptor mRNA expression	137
4.6.5 Analysis of HOXB expression.....	145
4.6.6 Agarose gel electrophoresis of rt qPCR products.....	150
4.7 Conclusions.....	152

CHAPTER 5 : FINAL CONCLUSIONS & RECOMMENDATIONS FOR FUTURE WORK.....157

5.1 Conclusions.....	158
5.2 Further work.....	160

CHAPTER 6 : EXPERIMENTAL SECTION.....161

6.1 Chemistry	162
6.1.1 Synthetic scheme	162
6.1.2 General.....	163
6.2 Biological testing	172
6.2.1 Cell culture techniques	172
6.2.2 Morphological changes.....	175
6.2.3 Immunofluorescent flow cytometry	175
6.2.4 Tetrazolium proliferation assay	176
6.2.5 Immunocytofluorescence.....	177
6.2.6 Western blots	179
6.2.7 Real-time PCR.....	181
Bibliography	184
Appendix 1: Structures of natural and synthetic retinoids and RAMBAs discussed in text	202
Appendix 2: Crystallographic data	204
Appendix 3: Typical standard curve generated during rt qPCR analysis.	205

Abbreviations

9- <i>cis</i> -RA	9- <i>cis</i> -retinoic acid
13- <i>cis</i> -RA	13- <i>cis</i> -retinoic acid
ATRA	all- <i>trans</i> -retinoic acid
B ₂ pin ₂	bis(pinacolato)diboron
BPEB	1,4-bis(phenylethynyl) benzene
BSA	bovine serum albumin
c.	concentrated
cDNA	complementary DNA
CNS	central nervous system
COE	η^2 -cylcooctene
CRBP	cellular retinol binding protein
CRABP	cellular retinoic acid binding protein
Cy	cyanine
CYP / Cyp	cytochrome
δ	chemical shift
d	doublet
DCM	dichloromethane
dd	doublet of doublets
dH ₂ O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMEM-FGA	Dulbecco's modified Eagle's medium supplemented with FCS, L-glutamine and antibiotics
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dppf	1,1'-bis(diphenylphosphino)ferrocene
dtbpy	4,4'-di- <i>tert</i> -butyl-2,2'-bipyridine
EC cell	embryonal carcinoma cell
EI	electron impact
eq.	equivalents
ES	electrospray
ES cell	embryonic stem cell
ether	diethyl ether
FITC	fluorescein isothiocyanate
FC	flow cytometry
FCS	foetal calf serum
GC-MS	gas chromatography – mass spectrometry

<i>HOX</i>	human homeobox gene
HPLC	high performance liquid chromatography
hu	human
ICC	immunocytochemi-cal/stry
Ig	immunoglobulin
IR	infra-red
m	multiplet
M	molar
MAP-2	microtubule associated protein-2
Me	methyl
mol	moles
mp	melting point
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium, inner salt
mu	murine
MW	molecular weight
NF	neurofilament
NMR	nuclear magnetic resonance
NSE	neuron specific enolase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pin	pinacolato (OCMe ₂ CMe ₂ O)
PNS	peripheral nervous system
PPh ₃	triphenylphosphine
QSAR	quantitative structure-activity relationship
RalDH	retinal dehydrogenase
RAMBA	retinoic acid metabolism blocking agent
RAR- α,β,γ	retinoic acid receptors; α , β and γ isotypes
RARE	retinoic acid response element
RoLDH	retinol dehydrogenase
RBP	retinol binding protein
RNA	ribonucleic acid
RT	reverse transcription
rt qPCR	real-time PCR
Rf	relative front
rpm	revolutions per minute
RT	room temperature

RT	reverse transcription
RXR- α,β,γ	retinoid X receptors; α , β and γ isotypes
RXRE	retinoid X response element
s	singlet
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSEA-3	stage specific embryonic antigen-3
t	triplet
TBAF	tetrabutylammonium fluoride
THF	tetrahydrofuran
tlc	thin layer chromatography
tris	tris(hydroxymethyl)methylamine
TTNPB	(<i>E</i>)-4-[2-(5,6,7,8-tetrahydro-5,5',8,8'-tetramethyl-2-naphthalenyl)propen-1-yl] benzoic acid
UV	ultraviolet
vis	visible
v/v	volume per volume
WB	wash buffer
wrt	with respect to
w/v	weight per volume

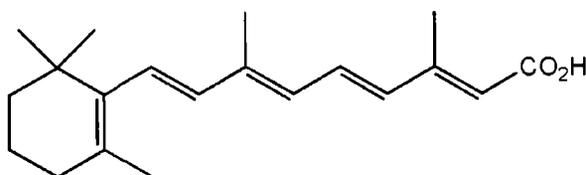
CHAPTER 1: THE RETINOIDS



1.1 Introduction

Vitamin A has long been established as an essential micronutrient for normal embryonic development and for a wide range of physiological processes in the adult. The major active metabolite of vitamin A is all-*trans*-retinoic acid (ATRA, also known as tretinoin), a small, lipophilic, isoprenoid molecule consisting of a trimethylcyclohexenyl group and an all-*trans* conjugated tetraene side chain terminated by a carboxylic acid group.

The biological roles of ATRA make it an obvious drug candidate for many clinical indications, however the administration of efficacious doses is associated with undesirable side effects. These observations have led to the design, synthesis, biological evaluation and – in some cases – clinical trials of a large group of molecules that are structurally and/or functionally analogous to ATRA. These synthetic compounds and their natural counterparts are known as “retinoids”. More than 4000 retinoids have been prepared by a large number of academic research groups and commercial organisations searching for safe, effective and stable alternatives to ATRA



all-*trans*-retinoic acid,
(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraen-1-ol

The structures of retinoids discussed in the text are presented in Appendix 1.

1.2 Functions of endogenous retinoids

The term “retinoid” was proposed by Sporn in 1976 to broaden the base of thinking about the vitamin A family of molecules beyond nutrition and vision, and to emphasize the potential biological and mechanistic relationships between metabolites of retinol and steroids.¹ This description evolved into a formal IUPAC structure-based definition, “A class of compounds consisting of four isoprenoid units joined in a head-to-tail manner.”² Standard usage of the term has subsequently been extended to describe a family of compounds that includes natural dietary vitamin A (retinol), its metabolites and several thousand synthetic analogues that are structurally related to retinol and/or can elicit specific biological responses by binding to one or more of the retinoid receptors.^{3,4} However, the IUPAC has advised that retinoic acid analogues which are unable to replace the full range of biological activities of vitamin A should be distinguished as “retinoate analogues”.⁵

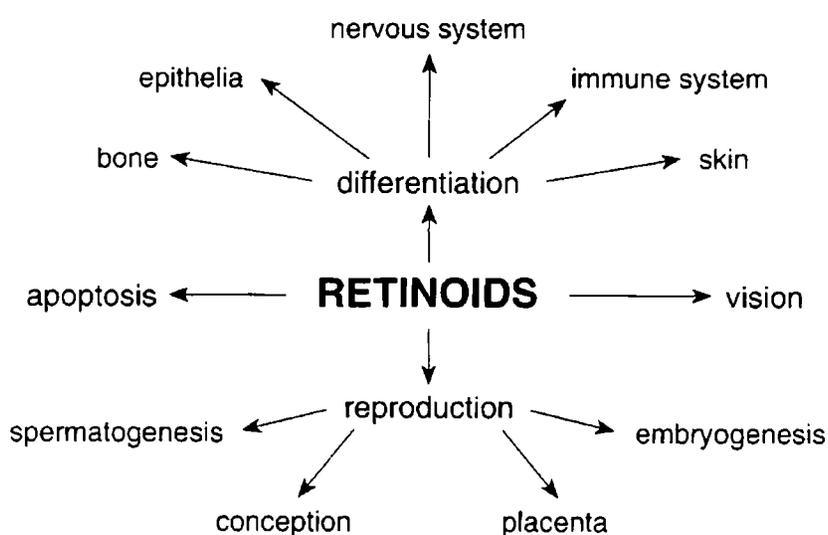


Figure 1-1: Functions of naturally occurring retinoids. From Napoli, 1996.⁶

Endogenous retinoids are distributed through many tissues *in vivo*,⁷ and regulate a wide range of essential processes during chordate embryogenesis and adult homeostasis including embryonic development,⁸ vision,⁹ reproduction,¹⁰ immune competence¹¹ and cellular differentiation, proliferation and apoptosis¹² (Figure 1-1). The role of 11-*cis*-retinal in the transduction of light into a visual signal is well established,^{13,14} but our understanding of the other functions of retinoids remains to be fully elucidated and represents an area which continues to be actively investigated.

During early embryogenesis, the antero-posterior (AP, head-tail) and dorso-ventral (DV, back-front) axes are established within the embryo. These axes specify what has been described as Cartesian coordinates that predict the overall body plan of the developing embryo,¹⁵ through the position-specific expression of developmental control genes.¹⁶ Studies in the chick limb bud and *Xenopus* and zebrafish embryos have established the requirement for a number of synergistic morphogenic signals in determining positional identities along the AP axis of the vertebrate nervous system.¹⁷⁻¹⁹ ATRA, together with a number of other molecules including Sonic hedgehog homologue (SHH), fibroblast growth factors (FGFs), transforming growth factor beta (TGF- β) ligands and WNT proteins become established in concentration gradients along the AP and DV axes in humans.²⁰⁻²⁵ Different combinations of morphogen concentrations and duration of signalling cause the induction of specific genes, which in turn specify the spatial organisation of cell types.²⁵

The ATRA gradient is precisely controlled by synthesis, facilitated by retinal dehydrogenase 2 (RaldH2) at the posterior end of the embryo, and expression of *CYP26A1* and *CYP26C1* metabolising enzymes at the anterior mesoderm.²⁶⁻³⁰ The small magnitude of the concentration gradient (2.5-fold) suggests the existence of amplification mechanisms, such as the activation of retinoic acid receptors (RARs) which may also be present in a gradient along the axis.³¹ Cells in the embryo respond differently to ATRA depending on their exposure, thus influencing pattern formation, limb development^{32,33} and development of the central nervous system.^{8,24,34-38}

The ability of ATRA to regionalise the entire posterior central nervous system (CNS) is mediated, at least in part, by its direct action on the spatial regulation of the homeobox *Hox* family of genes. *Hox* genes are putative master regulators of development, responsible for specifying the antero-posterior axis and controlling segmental patterning during embryogenesis.^{34,39-44} ATRA is also required beyond the initial stages of neural induction for the continued development of the nervous system in the embryo and regeneration and maintenance of the CNS in the adult.^{38,45}

Defects in the normal functioning of any part of the retinoid pathway may result in defects of varying severity. For example, aberrant expression or regulation of the RARs,⁴⁶⁻⁴⁸ *HOX* genes⁴⁹⁻⁵⁴ and cellular retinol-binding protein-I (CRBP-I)^{55,56} are all associated with the development of human cancers. Retinoid therapy offers the potential to ameliorate aberrations of the retinoid pathway.^{13,47}

1.3 Discovery, deficiency and excess

1.3.1 Discovery of “vitamine A”

Long before the concept of “vita-amines” was defined,⁵⁷ the ancient Egyptians realised that night blindness could be cured by feeding the patient liver,⁵⁸ a symptom that is now recognised as one of the earliest signs of vitamin A deficiency. Throughout the early 1900s deprivation studies in laboratory animals were used to isolate and identify a number of vitamins. Vitamin A was first identified in 1913 as an essential fat-soluble nutrient present in butterfat and cod liver oil. It was initially named “fat-soluble A” but subsequently re-designated “vitamine A”.¹¹

The molecular structure of vitamin A was determined using a combination of observations and classical techniques: β -carotene was demonstrated to be converted into vitamin A *in vivo*; the products formed by oxidative degradation of β -carotene and retinol were examined and then, by applying the isoprene rule,⁵⁹ the correct formulae of β -carotene (eight isoprene units) and retinol (four units) were established.⁶⁰

Once the nutritional requirement for vitamin A became evident, retinol was initially distilled from fish liver oil, whilst synthetic preparations were developed to provide vitamin A and β -carotene for use as food and feed supplements and as colourants in cosmetic and pharmaceutical preparations. The synthesis of ATRA was first published in 1946,⁶¹ en route to the synthesis of retinol.⁶² It was at this time that the biological properties of ATRA and other retinoids were first recognised.

1.3.2 Vitamin A deficiency & hypervitaminosis A

ATRA is an essential morphogen during embryogenesis and disruption of the precisely controlled gradient of ATRA along the antero-posterior axis of the developing central nervous system therefore results in a range of congenital deformations.^{10,35} Vitamin A deficiency (VAD) during pregnancy results in a spectrum of developmental malformations known as VAD syndrome;⁶³⁻⁶⁵ whereas exposure to excess retinoid is teratogenic and may result in spontaneous miscarriage or a range of malformations referred to as retinoid embryopathy.^{65,66} Therapeutic doses of retinoids are therefore contraindicated during pregnancy.^{10,67-69} Furthermore, the teratogenic effects of ethanol (foetal alcohol

* Wallach’s “isoprene rule” was based on extensive natural product research and suggested that isoprene units can be linked together “head to tail” to form linear chains or rings. Although since superseded, this rule was extremely useful in predicting the structures of unknown plant products.

syndrome) and birth defects associated with smoking have been suggested to result from their ability to disturb vitamin A metabolism or its signalling pathways.⁷⁰⁻⁷²

Vitamin A deficiency is a problem largely in developing countries. It can lead to night blindness and xerophthalmia, growth retardation, keratinisation of epithelia, impaired hearing, taste and smell, increased susceptibility to infection, increased child mortality and reduced male fertility.^{35,73,74} Deficiency in the developed world is usually limited to those with absorption difficulties, increased susceptibility to opportunistic infections, chronic liver disease and alcoholics.⁷³ Vitamin A deficiency or dysfunctional regulation of the retinoid genetic pathway may be associated with the development of neurological disorders, including late onset Alzheimer's disease and schizophrenia.⁷⁵ The observed symptoms of hypervitaminosis A include weight loss, irritation and desquamation of skin, hair loss, bone fractures, osteoporosis and liver toxicity.⁷⁶

1.4 Absorption, transportation and metabolism of retinol

Higher eukaryotic organisms depend on the dietary supply of vitamin A for a range of essential biological processes (Figure 1-1). In the UK, the recommended daily dietary allowance for adults is 600 mg for women and 700 mg for men.⁷⁷ Humans are not capable of *de novo* vitamin A production, so are obliged to derive it either in active form as retinol or retinyl esters from animal sources (for example, dairy products, oily fish and liver) or as carotenoid provitamins from fruit and vegetables containing yellow, orange and dark green pigments. The absorption, transport and metabolism of retinol are summarised in Figure 1-2.

After ingestion, carotenoids are absorbed through the enterocytes of the small intestine and oxidised by enzymes including carotene-15,15'-mono-oxygenase and carotene-9'10'- mono-oxygenase to give retinaldehydes (known simply as retinal, except in ocular studies) and then reduced to retinol by as yet unidentified retinal reductases.⁴ Dietary retinyl esters are hydrolysed to retinol in the intestinal lumen prior to uptake in the enterocytes.⁷⁸ Within the enterocytes, retinol is converted to retinyl esters (retinyl palmitate/oleate and retinyl stearate) by two enzymes: free retinol is esterified by acyl-CoA retinol acyltransferase (ARAT) whereas lecithin-retinol acyltransferase (LRAT) utilises both free retinol and retinol bound to cellular retinol-binding protein II (CRBP-II, also known as RBP2).⁷⁹ CRBP-II is a specific retinol binding protein localised to the enterocytes.^{80,81} In common with other retinoid binding proteins, the function of CRBP-II is to solubilise its lipophilic ligand, protect it from metabolism and regulate its transport, metabolism and action.^{79,82,83} Retinol may also be converted to retinyl esters by lipoprotein lipase (LPL), the physiological role of this process may be to increase uptake of retinoids by adipocytes, the second largest retinoid store in the body.⁸⁴ The majority of retinyl esters are then

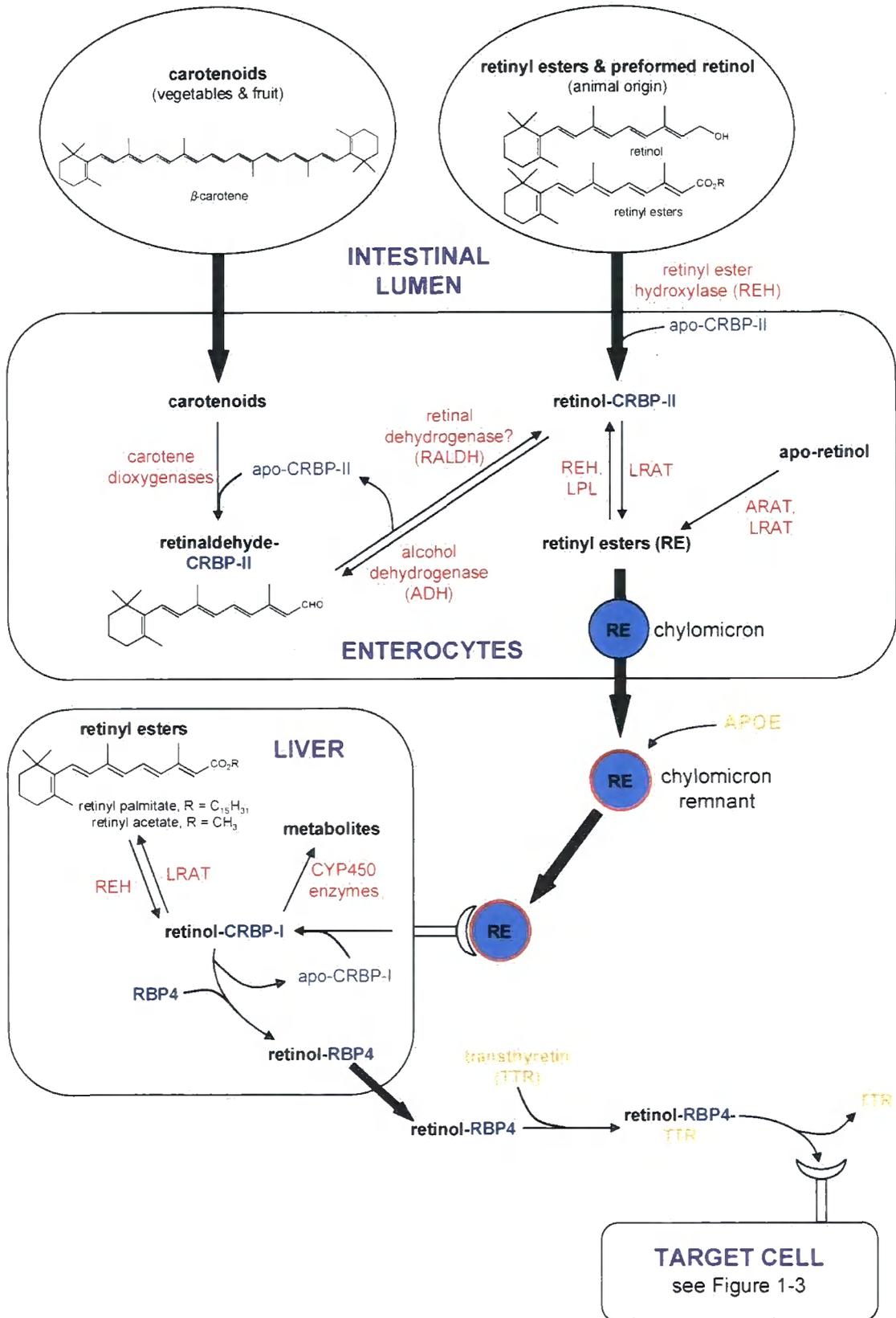


Figure 1-2: Schematic diagram of vitamin A absorption, transportation and storage, significantly adapted from Goodman, 2006.⁷⁵ For extensive reviews see Blomhoff *et al.*, 1991 and 2006.^{4,85} Abbreviations: APOE = apolipoprotein E, ARAT = acyl-CoA retinol acyltransferase, LPL = lipoprotein lipase, LRAT = lecithin-retinol acyltransferase, REH = retinyl ester hydroxylase and TTR = transthyretin.

incorporated into chylomicrons,⁸⁶ exocytosed into the intestinal lymph and then moved into the general circulation where they are formed into chylomicron remnants.⁸⁷ The majority of chylomicron remnants are delivered to the parenchymal cells (hepatocytes) of the liver, though a significant proportion are taken up by extra-hepatic tissues.⁸⁵

The liver plays a pivotal role in maintaining blood retinol levels within a narrow range, by storing retinoids when in dietary excess and releasing retinol when dietary intake decreases.^{88,89} Upon delivery to the liver, retinyl esters are hydrolysed to retinol by retinyl ester hydrolase⁹⁰ and bind to a second cellular retinol-binding protein (CRBP-I, also known as RBP1), which is highly homologous to CRBP-II yet expressed in multiple tissues.⁹¹ Retinol is then processed for either hepatic storage or secretion into blood plasma. The liver is the main store for dietary retinoids with approximately 50-90% of total mammalian body vitamin A stored in the hepatic stellate cells (Ito cells) as retinyl esters, predominantly retinyl palmitate/oleate and retinyl stearate, depending on vitamin A status.^{92,93} Retinol associates with plasma retinol-binding protein (RBP4) in a 1:1 ratio and is thus secreted from the liver.⁹⁴ The levels of CRBP-I and RBP4 vary as required in order to maintain retinoic acid homeostasis in the body.^{82,83,95} RBP4 is able to carry retinol alone, but the major carrier of retinol from the liver is as RBP4-transthyretin complex; the association of this second protein prevents the glomerular filtration of RBP4.⁹⁶ At the target tissue, this complex breaks up and retinol is released from TTR and RBP4.⁹⁷ However, the majority of plasma retinol that leaves the general circulation is returned to it without undergoing irreversible metabolism, a process known as retinol recycling.⁸⁵ RBP4 is also able to bind to ATRA, but this protein has no affinity for 9-*cis*-retinoic acid (9-*cis*-RA, structure **B** in Appendix 1), 13-*cis*-retinoic acid (13-*cis*-RA, **C**) or synthetic retinoids, which are transported directly to target cells bound to serum albumin.⁹⁸

Two mechanisms are proposed to transport retinol into cells: simple diffusion and/or through binding with a specific receptor on the target cell membrane. Retinol and other retinoids are hydrophobic and readily partition into the lipid phase of the membrane, whereupon they may be transferred by diffusion to intracellular binding proteins.⁹⁹⁻¹⁰¹ A specific glycoprotein cell membrane receptor, STRA6, has been identified in target epithelium that binds plasma RBP4 at the extracellular surface and facilitates the transfer of retinol to cytoplasmic cellular retinol-binding proteins (CRBPs).¹⁰²⁻¹⁰⁵ The importance of this receptor in the delivery of retinol to cells is shown by the observation that patients with STRA6 mutations present malformations consistent with vitamin A deficiency.¹⁰⁶ There is little information available in the literature regarding how synthetic retinoids enter cells/tissues from the circulation,¹⁰¹ the most likely route is therefore *via* indiscriminate passive diffusion.

Within the cell, all-*trans*-retinol can be oxidised in a two step process to ATRA, the major active metabolite of vitamin A (Figure 1-3). The spatiotemporal concentration of ATRA is regulated, to prevent toxicity and maintain normal gene functions, by the concerted control of synthesis and

degradation. This is co-ordinated by a number of specific binding proteins and enzymes, including CRBPs, cellular retinoic acid-binding proteins (CRABPs), retinol dehydrogenases (RoLDHs), retinal dehydrogenases (RalDHs) and cytochrome P450 enzymes. Specific ocular binding proteins are also known, including cellular retinal-binding protein (CRALBP).^{83,107}

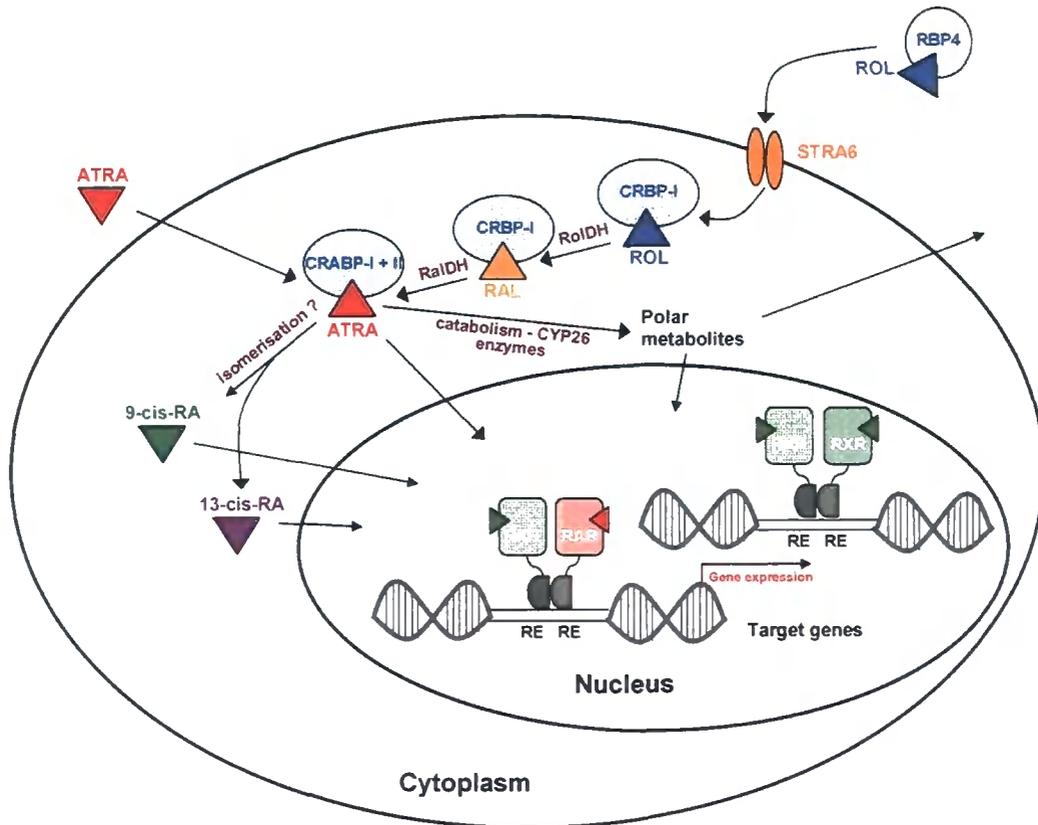


Figure 1-3: Schematic representation of the extra-visual retinoid signal transduction pathway. Abbreviations: ROL = retinol, RAL = retinal, RBP4 = serum retinol-binding protein, RoLDH = retinol dehydrogenase, RalDH = retinal dehydrogenase, CRBP-I = cellular retinol-binding protein I, CRABP-I + II = cellular retinoic acid-binding proteins I and II, RE = DNA response element. Adapted from Klaassen and Braakhuis, 2002.¹⁰⁸

Intracellularly, retinol is bound to cellular-retinol binding proteins of which four human CRBP proteins have currently been identified, CRBP-I, -II, -III and -IV.^{80,102,109,110} In addition to its homeostatic roles, CRBP facilitates the reversible oxidation of retinol to retinal by microsomal RoLDHs¹¹¹⁻¹¹³ and secondly the irreversible and rate-limiting oxidation of retinal to ATRA, its major active metabolite, by retinal dehydrogenases (RalDH).^{36,114-116} The CRBPs possess both high specificity and binding affinity for retinol and retinal, but discriminate against ATRA and *cis* forms of retinol.¹¹⁷ ATRA binds strongly to the cellular retinoic acid-binding proteins (CRABP-I and -II), which serve to transport, stabilise, and protect their ligand.⁶ Furthermore, the CRABPs regulate the level of intracellular ATRA available for binding to the nuclear receptors, acting by soaking up free ATRA and modulating metabolism, thus protecting the cell from ATRA-excess.^{82,118,119} CRABP-II is

responsible for delivering ATRA to the retinoid receptors in the nucleus¹²⁰⁻¹²² and CRABP-I is associated with regulating the metabolic fate of ATRA, by delivering it to the CYP26 family of enzymes.^{120,121,123-125}

ATRA is metabolised and subsequently deactivated by the specific and non-specific activity of cytochrome P450 (CYP) enzymes.¹²⁶ The major CYP enzymes involved in the degradation of ATRA *in vivo* are the ATRA hydroxylases, CYP26,¹²⁷ currently divided into three subfamilies in humans: CYP26A1,^{128,129} CYP26B1¹³⁰ and CYP26C1.¹³¹ The CYP26 enzymes play essential roles in the regulation of ATRA exposure during embryogenesis and in adult tissues.¹³²⁻¹³⁷ Metabolites of CYP26A1 include 4-hydroxy-ATRA, 4-oxo-ATRA, 18-hydroxy-ATRA and 4-oxo-retinol.^{129,138-140} These intermediates can be further hydrolysed before they are converted to glucuronides and eliminated.^{6,141} The majority of retinoid metabolites are inactive in the retinoid pathway, however exceptions include 4-oxo-retinol,¹⁴²⁻¹⁴⁴ 4-hydroxy-ATRA, 18-hydroxy-ATRA and retinoyl β -glucuronide.^{28,145,146} 9- and 13-*cis*-RA are similarly metabolised to their respective 4-hydroxy- and 4-oxo-metabolites (Figure 1-4).¹⁴⁷

The retinoid-binding proteins and CYP26 enzymes are reviewed in detail in Section 4.2.3.

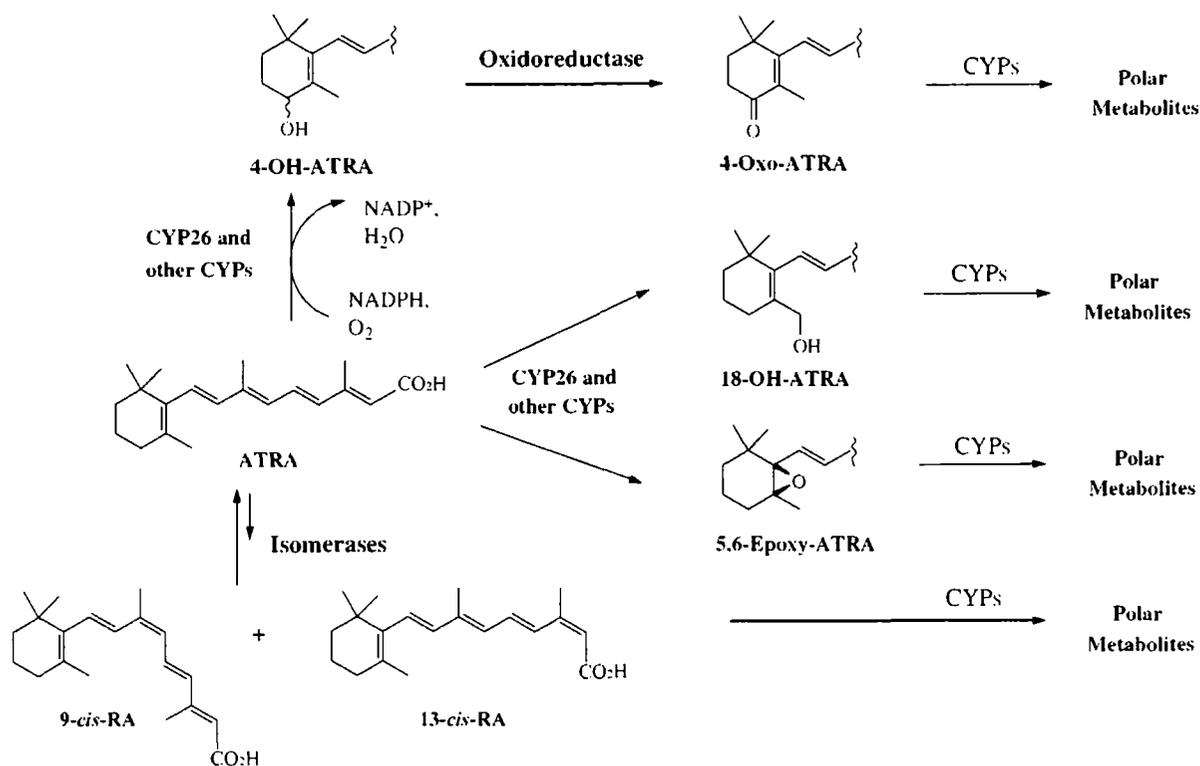


Figure 1-4: Metabolism of ATRA, 9-*cis*-RA and 13-*cis*-RA. Figure adapted from Bruon and Njar, 2007.¹⁴⁸

1.5 Molecular mechanism of retinoid action

The precise regulation of retinoid biological activities and ability to induce pleiotropic effects *in vivo* would appear to be related to the complex molecular pathway of combinatorially diverse elements that convey and modulate its effects.^{149,150}

The mechanism by which vitamin A functions in the visual cycle was the first of its actions to be elucidated, by Wald in 1968.⁹ In the retina of the eye 11-*cis*-retinal acts as the chromophore of rhodopsin: one molecule of 11-*cis*-retinal is isomerised by one photon of light to all-*trans* retinal, which initiates a nerve impulse that is perceived as light. In vertebrates, all-*trans* retinal is then enzymatically re-isomerised to 11-*cis*-retinal to complete the cycle.^{9,151}

Progress in extra-visual retinoid research, beyond the systematic medicinal chemistry approach of designing, synthesising and testing libraries of compounds in the search for enhanced therapeutic efficacy, was previously impeded by a lack of knowledge of the nature of the receptors that mediate the biological activity of the retinoids. Specifically, how is it possible for such structurally simple molecules to exert such pleiotropic effects? The first key member of the retinoid signalling pathway, a “Retinoic Acid Receptor” (now known as RAR- α_1) was identified in 1987, and heralded a greater understanding of retinoid activities *in vivo*. The retinoid receptors have subsequently been classified into two subfamilies, each composed of at least three isotypes – the retinoic acid receptors RAR- α ,^{152,153} RAR- β ^{154,155} and RAR- γ ,^{156,157} and the retinoid X (or rexinoid) receptors RXR- α , RXR- β and RXR- γ .¹⁵⁸⁻¹⁶⁰ This categorisation is based upon differences in amino acid structure and ability to modulate gene expression of various target genes, and is exemplified by their responsiveness toward natural and synthetic ligands: ATRA binds and activates all three RAR isoforms, while 9-*cis*-RA activates both the RARs and RXRs.¹⁶¹⁻¹⁶³

In the absence of a ligand, RAR-RXR heterodimers bind to retinoic acid response elements (RARE) in the promoter regions of target genes and bind with co-repressor proteins which prevent transcriptional activation. Though rarely considered, gene silencing is itself essential for some developmental processes.¹⁶⁴ Upon binding of agonists for both RAR and RXR, the receptors undergo conformational changes that cause the co-repressors to be displaced, facilitating the recruitment of co-activators, which initiate transcription (Figure 1-5).¹⁶⁵⁻¹⁷² Agonists and competitive antagonists bind at the same site within the core of the ligand binding domain (LBD), however antagonists appear to either disrupt the receptor structure to prevent co-activator binding^{173,174} or alternatively stabilise co-repressor binding in order to silence gene transcription.^{168,175}

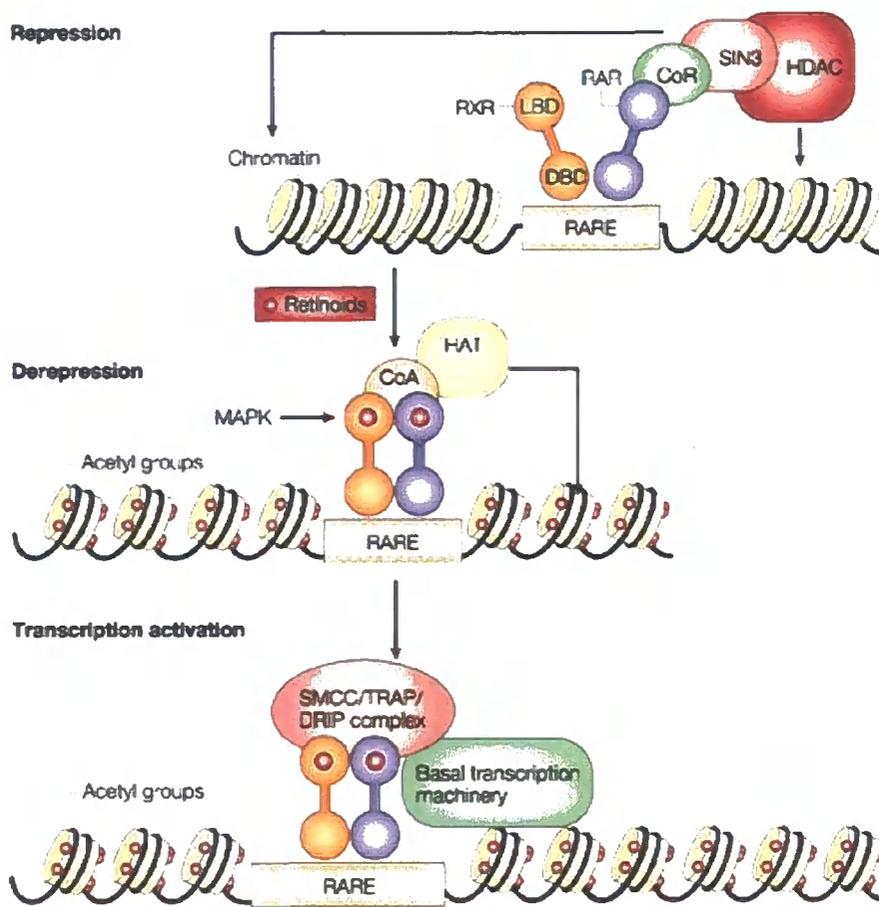


Figure 1-5: Mechanisms of transcriptional repression and activation by RAR–RXR heterodimers. In the absence of agonists, co-repressor complexes bind to the RAR–RXR heterodimer. The co-repressors (CoRs) NCoR or SMRT link the heterodimer to histone deacetylases (HDACs) through SIN3. HDACs remove acetyl groups from nucleosomal histones, resulting in chromatin condensation and gene silencing. Binding of an agonist to the ligand-binding pocket induces an allosteric conformational change in the ligand-binding domain, including altered position of helix H12. This structural transition destabilises the interface with the co-repressor and allows interaction with co-activators (CoAs) of the p160 family (SRC1, TIF2 and AIB1 in humans). CoAs recruit (or pre-exist in a complex with) histone acetyltransferases (HATs), such as CBP (CREB-binding protein) or p300. HAT action results in acetylation of histone amino-terminal tails, thereby inducing nucleosomal repulsion and chromatin decondensation. A third multi-subunit complex, variously termed thyroid-hormone-receptor-associated protein (TRAP), vitamin D receptor-interacting protein (DRIP) or Srb and mediator protein-containing complex (SMCC) appears to establish contact with the basal transcription machinery, leading to increased frequency of transcription initiation. However, the order of formation, receptor selectivities and requirement for (or involvement in) transactivation of these complexes remain elusive.¹⁷⁶ Abbreviations: RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X (rexinoid) receptor. Adapted from Altucci and Gronemeyer, 2001.¹⁶⁷

The retinoid receptors are members of the super-family of steroid hormone receptors that function as regulators of gene transcription.¹⁷⁷ In excess of 500 genes have been identified that are direct or indirect regulatory targets of ATRA.¹⁷⁸ Nuclear receptors are ligand-inducible transcription factors which share a common architecture constituted of a DNA-binding domain and a ligand-binding domain (LBD) united by a short flexible hinge region (Figure 1-6).¹⁷⁹ The amino acid sequences of the C and E regions are highly conserved within RAR or RXR isotypes, whereas those of the A/B and D regions are less well conserved.¹⁴⁹ *In vitro*, RARs predominantly bind to specific cognate response elements as heterodimers with RXRs,¹⁸⁰ which optimises their binding.^{149,181,182} RAR/RXR heterodimers have been established as the functional units that transduce retinoid signals *in vivo*.^{183,184} The RXRs, which can also bind *in vivo* to certain DNA response elements as homodimers,^{182,185} are heterodimerisation partners for a number of other nuclear receptors including the thyroid hormone receptors,^{159,186-188} vitamin D receptor^{186,189} and peroxisome proliferator-activated receptor γ (PPAR- γ).^{190,191} Apo-RXR (*i.e.* not bound to ligand) has been observed to form high-affinity homo-tetramers in solution,¹⁹² which dissociate rapidly upon ligand binding.¹⁹³ The biological significance of this phenomenon is unclear, though it is proposed that the tetramers could represent a storage form of RXR in the cell.¹⁹⁴

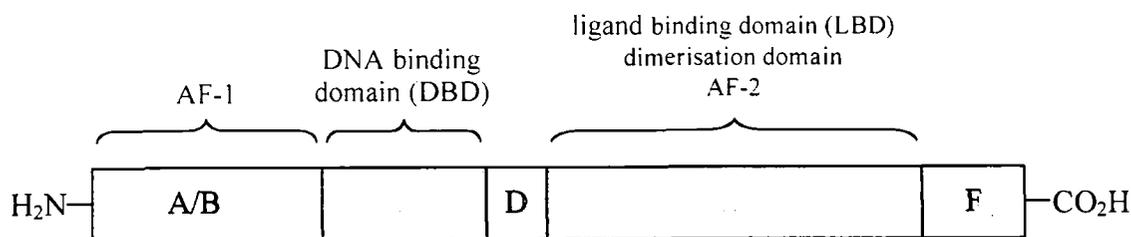


Figure 1-6: A schematic representation of the six generic regions (A-F) present in steroid receptors. The NH₂ terminal region (A/B) harbours a ligand-independent transcriptional activation function (AF-1). Region C is the core DNA-binding domain, containing two highly conserved zinc finger motifs that target the receptor to specific DNA sequences known as hormone response elements. The hinge region (D) permits protein flexibility to allow for simultaneous receptor dimerisation and DNA binding. The ligand binding domain (E) includes a ligand-dependent activation function (AF-2) and dimerisation interface, it is adjoined to the CO₂H terminal region (F). Adapted from Chawla *et al.*, 2001.¹⁷⁹

RAR-null and RXR-null mutant mice exhibit body-wide abnormalities, demonstrating the necessity of both types of receptor during development.¹⁹⁵ Although gene disruption studies have revealed some functional redundancy among the different RAR isotypes, each RAR isotype performs unique functions in development and differentiation which cannot be entirely replaced by the actions of the other isotypes.^{74,195-198} The phenotypes observed are similar to those observed in vitamin A-deficient animals confirming their role in this pathway.^{35,74}

In the absence of an RAR agonist, RXR agonists are unable to induce transcription. A ligand-bound RXR (*holo*-RXR) is unable to dissociate the CoR from *apo*-RAR and in this way the transcriptional activity of RXR is described as subordinate to that of its RAR partner.¹⁹⁹⁻²⁰² This phenomenon plays a crucial role *in vivo* in allowing RXRs to act concomitantly, within the same cell, as heterodimerisation partners for repression and for activation events in which they are transcriptionally active.²⁰³ Furthermore, RXR ligands have the ability to synergistically increase the potency of RAR ligands.^{199,200,204-206}

1.5.1 Retinoid receptor complexity

The retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are each divided into three isotypes (- α -, - β and - γ) which are encoded by genes on separate chromosomes and are differentially expressed within tissues. RAR- α is ubiquitous, RAR- β is common in heart, lung and spleen tissue and RAR- γ is confined to lung and skin.^{152,154,157,207} RAR- α , β and γ are more homologous to each other than the three RXR isotypes,²⁰⁸ and the interspecies conservation of a given member of the RAR subfamily (either α , β or γ) is much higher than the conservation between the three receptors within a given species.¹⁵⁶ These observations indicate that each of the RAR isotypes may perform specific functions. Each isotype can be further divided into a number of different isoforms with distinct regulatory and functional properties by the differential use of two promoters and alternative splicing. There are two major isoforms for RAR- α (α_1 and α_2) and for RAR- γ (γ_1 and γ_2), and five major isoforms for RAR- β (β_1 to β_5). Similarly, at least two isoforms have been identified for RXR- α (α_1 and α_2), RXR- β (β_1 and β_2) and RXR- γ (γ_1 and γ_2).^{74,149,209,210} Furthermore, a group of receptors which lack identified ligands, termed retinoic acid-related orphan receptors (RORs), are known.²¹¹ This receptor diversity, as part of a complex retinoid pathway, may go some way towards explaining the pleiotropic effects of retinoids.²⁰⁹

The endogenous ligands for these receptors are ATRA, its natural geometric isomers 9-*cis*-RA and 13-*cis*-RA and a number of its metabolites. A large number of synthetic retinoids have been prepared and many of these bind to one or more retinoid receptors, although some possess some functionality independent of the retinoid pathway.²¹² The RARs and RXRs show differing affinity towards individual ligands, which may also be concentration dependent, providing further evidence that the receptors may possess individual physiological roles that are yet to be fully understood.²¹³

Activation of the RARs appears to be involved in mediating the systemic toxicity and teratogenicity of ATRA, TTNPB (structure **D**, Appendix 1) and several other synthetic retinoids. RXR-exclusive agonists elicit a minimal or much weaker teratogenic response than RAR agonists.²¹⁴ RXR activation also confers alternative biological activity to that of RAR agonists, by virtue of their ability to heterodimerise with other receptors. Retinoids are able to inhibit cell proliferation and induce

differentiation but, unlike RAR agonists, are themselves poor inducers of apoptosis.²¹⁵ These properties have led to the development of synthetic agonists that selectively activate RXR without influencing the activity of the RARs.^{216,217} Compounds such as Targretin[®] (bexarotene, LGD1069, Ligand Pharmaceuticals, E) and LG100268 (Ligand Pharmaceuticals, F) are being investigated as potential therapies for the treatment of cancer, diabetes and other metabolic disorders.^{218,219}

Numerous receptor-selective agonists and antagonists have been designed, synthesised and tested in an attempt to delineate the structural biology of the RARs and RXRs and their physiological roles.^{47,213,220,221} It was hoped that targeting the activation of individual receptors would separate the multiple biological effects of the natural retinoids and thus increase therapeutic efficacy whilst minimising the adverse effects that result from non-specifically activating all receptors.²²⁰ However, this approach has yet to yield clinical results: the complexity of the retinoid molecular pathway is not so easily overcome.

Early *in vitro* experiments identified 9-*cis*-RA as a ligand for RXR and also found it in animal tissues.^{161,162} Although generally accepted as the endogenous ligand for RXR, subsequent studies performed at low levels of detection and quantitation have failed to detect 9-*cis*-RA,²²² leading to uncertainty.^{4,213,223} Its initial discovery may be attributable to the extreme sensitivity of ATRA towards spontaneous isomerisation.^{224,225} The heterodimeric partner for RAR is RXR, but it appears possible that a ligand other than 9-*cis*-RA may be responsible for RXR activation *in vivo*. Other low affinity endogenous ligands for RXR have been identified, including phytanic acid²²⁶ and unsaturated fatty acids, particularly linoleic, linolenic and docosahexanoic acids.^{227,228} The isomerase involved in the visual pathway has been identified^{229,230} and it has been inferred that this mechanism is also involved in controlling RAR/RXR transduction pathways.^{162,231} There is evidence that 9-*cis*-RA can be converted to ATRA in mammalian cells,²³² a thermodynamically favoured process,²²⁴ but the reverse isomerisation was not observed.²³² CRBP-I and CRBP-II also recognise 9-*cis*-retinal, though not 13-*cis*-retinal,²³³ which may suggest that 9-*cis*-RA could be derived from dietary 9-*cis*-retinoids naturally present in human foodstuffs, since no evidence supporting an enzyme-catalysed isomerisation of ATRA to 9-*cis*-RA has been reported.^{224,234,235} A specific *cis*-retinol dehydrogenase for the hepatic oxidation of 9-*cis*-retinol to 9-*cis*-retinal has been identified and it has also been shown that 9-*cis*-retinol can be esterified for storage and subsequently hydrolysed by retinyl ester hydrolase(s).²³⁶ Although these data are from *in vitro* models, the controversy surrounding the existence, mode of synthesis and function of 9-*cis*-retinoic acid appears to be approaching a conclusion.

1.5.2 Retinoid receptor independent gene regulation and non-genomic effects

In addition to modulating the retinoid receptor pathway, as described above, a number of synthetic retinoids have also been found to activate additional pathways, which may enable wider therapeutic application.²³⁷ For example, AGN 193109 (structure **G**) a pan-RAR antagonist, and two very weak RAR-agonists, AGN 190730 (**H**) and CD437 (AGN 192837, AHPN, **I**) induce the aryl hydrocarbon (AhR) signal transduction pathway at pharmacological concentrations.²³⁸ The AhR pathway is involved in mediating response to xenobiotics, liver development and immune function.²³⁹⁻²⁴¹

A number of compounds described as retinoid-related molecules (RRMs) or atypical retinoids possess some RAR-activating ability but also exert growth regulatory or apoptotic activity that is retinoid receptor independent.²⁴² Fenretinide (4-hydroxy-phenylretinamide or 4-HPR, **J**), CD437 (**I**) and ST1926 (**K**) are three of the most effective retinoids included in this classification.^{12,237,243,244} Fenretinide is a modest but highly selective agonist of RAR- β ^{245,246} and RAR- γ ,²⁴⁷ but also has some retinoid pathway independent functionality.²⁴⁸⁻²⁵⁴ It is an effective inducer of apoptosis of cancer cells,²⁵⁵ with good clinical results for a number of malignancies including neuroblastoma.^{256,257}

An important feature of RXR specific agonists is their ability to heterodimerise with a number of different nuclear receptors which permits them to affect multiple signal transduction pathways. For example, activation of the PPAR- γ or thyroid hormone receptor modulates energy metabolism.^{218,258} Targretin[®] (LGD1069, bexarotene, Ligand Pharmaceuticals, **E**) and LG100268 (Ligand Pharmaceuticals, **F**) modulate many regulatory networks to inhibit cell proliferation or to induce differentiation.²¹⁵ These properties help to explain the powerful chemotherapeutic activity that such compounds have demonstrated against a wide range of cancers, including breast, prostate, non-small cell lung cancer and cutaneous T-cell lymphoma.^{215,219} Retinoids are also able to synergistically enhance the activity of RAR ligands.^{200,205,259}

1.6 Therapeutic applications

Due to their ability to modulate cellular proliferation and differentiation, and in some cases to exhibit anti-inflammatory effects, natural and synthetic retinoids have been used successfully in the treatment of a variety of skin disorders, including acne vulgaris and psoriasis, for many years.²⁶⁰⁻²⁶³ Topical application of tretinoin (ATRA), isotretinoin (13-*cis*-RA, Accutane[®], Roche Laboratories and generics), adapalene (Differin[®], Galderma Laboratories, CD271, L) and tazarotene (Zorac[®], Allergan Ltd., M) are all currently licensed in the UK for one or both of these conditions. The adamantane group of adapalene confers a number of beneficial properties for dermatological therapy. The compounds are found to have high melting points, resulting in low flux through the skin and hence low systemic toxicity, and low solubility which further sustains high levels of the drug in the skin.²⁶⁴ Treatment with oral retinoids, including acitretin (Neotigason[®], Roche Products Ltd., N), tretinoin and isotretinoin, may be more effective, but also results in more severe side-effects.^{262,265} Topical treatment with 9-*cis*-RA (alitretinoin) is approved for AIDS-related Kaposi's sarcoma.¹⁶⁷ More recently, retinoids have also been used in 'cosmeceuticals', to induce non-genomic dermatological effects.²⁶⁶

The only selective retinoid currently approved for therapy is Tamibarotene (Am80, P), an RAR- α/β agonist used to treat relapsed or refractory acute promyelocytic leukaemia (APL) in Japan.²⁶⁷ APL is associated with a chromosomal translocation of RAR- α , which typically fuses with a gene known as promyelocytic leukaemia (*PML*) silencing the activity of the retinoic acid receptor.^{268,269} Treatment with ATRA or Tamibarotene induces apoptosis of APL cells and the breakdown of the *PML-RAR- α* chimeric gene, allowing normal differentiation and function to be regained.²⁷⁰⁻²⁷² While the RAR- α selectivity and physical stability of Tamibarotene may play a role in its efficacy, the most important features of its clinical profile are that patients do not develop resistance,²⁷³ unlike ATRA-treatment which induces hyper-metabolism,²⁷⁴⁻²⁷⁶ presumably *via* the CYP26 enzymes, and that it is less toxic than ATRA partially due to poor binding affinity for RAR- γ .^{267,277}

The strong association between cancer development and vitamin A is well established and has led to extensive research into the potential of retinoids to be used for chemotherapy and chemoprevention. Early studies established a link between the development of some cancers and a low dietary intake of retinoids^{1,48} and aberrant expression of the RARs and retinoid metabolism has been found in a number of malignant tissues.^{46,48} Retinoic acid can inhibit proliferation and induce differentiation in a variety of human cell lines (Section 3.1). Promising pre-clinical data¹⁶⁷ led to a large number of clinical trials to evaluate the preventative or therapeutic ability of retinoids with mixed success.²⁷⁸ Despite all the hype, only a limited number of indications have shown results that counter-balance the adverse effects.⁴⁷

ATRA, 13-*cis*-RA and fenretinide are three retinoids that have been used with some success as chemotherapeutic agents in the treatment of neuroblastoma.^{256,279} However, the search continues to find a treatment with improved pharmacokinetic properties and improved clinical results. 9-*cis*-RA, 13-*cis*-RA and ATRA all readily isomerise in biological systems.²⁸⁰ ATRA is the most thermodynamically stable isomer and is the major isomer at equilibrium.²²⁴ The activity of 13-*cis*-RA in neuroblastoma differentiation therapy and a number of other fields is believed to be due to isomerism to ATRA.^{261,281} 13-*cis*-RA thus acts as a pro-drug for ATRA, enabling a controlled synthesis of the active isomer and resulting in a reduction of the toxic events induced by treatment with ATRA directly.^{256,281} Furthermore, 13-*cis*-RA is better absorbed from the intestine than either 9-*cis*-RA or ATRA,²⁸² and is metabolised more slowly than 9-*cis*-RA, which results in greater exposure to the ATRA.²⁸³ Despite promising *in vitro* results for 9-*cis*-RA in neuroblastoma cells,²⁸⁴⁻²⁸⁶ *in vivo* studies revealed elevated levels of toxicity.^{287,288}

The atypical retinoid fenretinide induces apoptosis in a wide range of cancer cell lines including neuroblastoma,²⁸⁹⁻²⁹¹ myeloid leukaemia (HL60)²⁹² and breast cancer cell lines.²⁴⁸ Promising pre-clinical data suggested an improved therapeutic index relative to standard retinoid therapy for a wide range of tumours.⁷⁵ While some cancers did not respond in clinical trials,²⁹³ a number of phase III trials for a number of cancers including breast, prostate and bladder are ongoing.²⁹⁴ Fenretinide has also received orphan drug status[†] for Ewings's sarcoma family of tumours, primary malignant bone tumours and neuroblastoma.²⁹⁵

As previously discussed, pre-clinical results using rexinoids such as LG100268 and Targretin[®] show the potential to treat metabolic syndromes including diabetes and obesity.^{218,258} Targretin[®] has been approved for use in cutaneous T-cell lymphoma and has shown good results in clinical trials for non-small cell lung cancer and breast cancer.^{167,219,296}

Retinoids are also prime candidates for cancer chemoprevention since they regulate cell proliferation and differentiation, and cancer is associated with abnormal growth and with the loss of differentiation.²⁹⁷ Cancer prevention by retinoids was first demonstrated in the 1970s,^{298,299} and is effective in suppressing the development of several types of tumours in animal models.³⁰⁰ However, clinical trials using natural and synthetic analogues have generally not been successful, for as yet unknown reasons.³⁰¹ Furthermore, where promising results have been obtained, the therapeutic index is so strongly balanced on the risk side, with the side-effects that accompany long-term treatment generally outweighing perceived benefit, that retinoids have failed to enter common use in this field.³⁰²

[†] Sponsors of orphan drugs are provided with financial incentives to assist the development of treatments for rare diseases or conditions that would otherwise be prohibitively expensive and/or un-profitable to develop.

It is hoped that the new generation of atypical retinoids may be more successful;²⁴³ for example fenretinide has been shown to provide long-term prevention against secondary breast cancer.³⁰³

Retinoids can also augment the activity of other biological and chemotherapeutic agents, offering potentially effective combination regimens.^{304,305} Successful applications include overcoming tolerance to breast cancer treatments,³⁰⁶ enhancing the sensitivity of tumours to cytotoxic agents and overcoming drug resistance.^{307,308} These synergistic effects have provided the impetus for synthesis and evaluation of mutual prodrugs, *i.e.* a hybrid drug linking two active compounds.³⁰⁹

1.7 Limitations and adverse effects

Pharmacological nuclear receptor ligands may serve as important therapeutic agents. However these exogenous ligands may alternatively or additionally transactivate other nuclear receptors and pathways with potentially deleterious effects.³¹⁰ Systemic retinoid therapy is limited by severe toxicity, teratogenicity and other side-effects which are collectively defined as retinoic acid syndrome (RAS). These adverse effects present with wide inter-patient variation and are potentially life-threatening.³¹¹ Whilst the etiology of RAS is poorly understood, it is generally believed to stem from the pleiotropic nature of ATRA, regulated by its pan-RAR activational properties. Indeed, retinoid therapy is generally better tolerated than treatment with RAR agonists.²¹⁴ Some selective retinoids are also better tolerated than ATRA, for example the administration of RAR- α selective Am80 for AML²⁷⁵ and acne treatment with adapalene, which is moderately selective for RAR- β/γ .^{312,313} Patient-specific dosing, adjusted in response to clinical response, allows for differential pharmacokinetic responses and enables optimal plasma drug levels to be achieved whilst minimising the risk of adverse events.³¹⁴

Furthermore, the most widely used and studied retinoid, oral 13-*cis*-RA for acne treatment, is associated with human psychiatric disorders,³¹⁵⁻³¹⁷ (confirmed by mice developing depression related behaviours,³¹⁸ although this has not been observed in rat studies³¹⁹) causes varying degrees of loss of night vision,³²⁰ may increase risk factors of atherosclerosis,³²¹ cause altered brain function and decreased brain metabolism,³²² induce insulin resistance,³²³ and result in decreased neurogenesis and learning ability in exposed mice.³²⁴

The other major problem with retinoid therapy is the development of resistance. Continuous ATRA-treatment may induce the up-regulation of CYP26A1 resulting in hypercatabolism and lower drug plasma levels, leading to reduced drug sensitivity and resistance.^{274,325-328} Employing a high-dose, intermittent regimen, rather than continuous low-dose treatment is one way to improve clinical outcomes.^{314,329} Resistance to ATRA-treatment in APL can be overcome by the administration of Am80, which is not metabolised by the CYP26 enzymes.^{275,330} Another approach is to administer

retinoic acid metabolism blocking agents (RAMBAs) which specifically block CYP26 activity.³³¹ RAMBAs may permit an increase in endogenous retinoid levels for therapeutic benefit,³³² or alternatively act synergistically with ATRA or 13-*cis*-RA to inhibit the development of ATRA-resistance.³³³ This approach may yield effective agents for the treatment and/or prevention of cancers and dermatological diseases and appears to circumvent the extreme toxicities and resistance to traditional retinoid therapy.

The bioavailability of orally administered retinoids is poor and erratic, which may limit the usefulness and practicality of this route.²⁸² This is partly attributable to the poor aqueous solubility of retinoids and a tendency to self-associate in aqueous environments.³³⁴ ATRA may be stabilised by incorporation into lipid based carriers: this approach also improves aqueous solubility, maintains a more constant concentration of ATRA in plasma and may permit targeted administration to reduce systemic toxicity. However, a clinical trial of an ATRA-liposomal formulation to treat APL failed to show a significant improvement on traditional therapy.^{335,336}

Improving drug formulations and administration protocols may increase the clinical benefit of existing and novel retinoids. Several patents have recently been issued for topical preparations including microemulsion and foam formulations and gelatine microsphere encapsulation. These preparations may increase the rate of drug release and penetration, and microspheres are additionally able to protect ATRA from photo-degradation.³³⁷⁻³³⁹

1.8 Conclusions

A large amount of research has been conducted to investigate the wide ranging and essential *in vivo* effects of ATRA and attempt to harness the perceived potential therapeutic applications that retinoids offer. Members of this family are used successfully in the treatment of dermatological disorders, APL and neuroblastoma. However, even with the development of selective synthetic retinoids, promising *in vitro* data and pre-clinical trials have generally not been translated into clinical treatments. Of particular note are the fields of chemotherapy and chemoprevention, of growing significance to modern medicine, where it would appear that alternative (for example non-classical retinoid activity) or synergistic approaches may be required. Further work is clearly required to fully understand the molecular basis of retinoid activity and to expand the scope of retinoid therapy.

CHAPTER 2: SYNTHESIS OF RETINOIC ACID ANALOGUES

2.1 Design and synthesis of retinoids

2.1.1 Introduction

The preparation of synthetic analogues of all-*trans*-retinoic acid (ATRA) was initiated in 1968 when Roche prepared a series of compounds by modifying the structure of retinol with the objective of identifying compounds with a better therapeutic ratio than the parent compound.³⁴⁰ Over the past forty years, more than 4000 retinoids have been prepared and tested in an attempt to realise the enormous clinical potential of the retinoid pathway and to provide pharmacological tools to investigate the endogenous functions of ATRA and its receptors, the retinoic acid receptors (RARs).

ATRA is characterised by its pleiotropic nature: its ability to induce multiple effects in the embryo and adult by modulating a large number of genes.^{149,178} Ligands that are able to strongly activate individual receptors may elicit more targeted activity *in vivo*, whilst minimising the adverse effects that may derive from the non-specific activation when ATRA is used therapeutically. A further aspect of retinoid design is to engineer a molecule with greater physical stability than ATRA, which is notoriously labile.

2.1.2 Basic structural requirements for retinoid activity

The relationship between structure and activity of different retinoids was first recognised in the 1970s.³⁴¹ It was quickly realised that each component of the basic structure of ATRA could be modified in order to achieve an improved biological response whilst also reducing adverse effects.²⁹⁹ Since then the iterative modification of putative key portions of the structure of ATRA and concurrent biological evaluation of the resulting retinoids, has gradually established the ATRA pharmacophore and acceptable bioisosteric replacements. (See reviews by Altucci *et al.*,⁴⁷ Kagechika *et al.*^{277,342} and Dawson.²¹³)

The crystal structures of many of the RAR and retinoid X receptor (RXR) ligand-binding domains, both unbound (*apo*) and bound (*holo*) to ligands have also been determined. (See Greschik and Moras, 2003³⁴³ for review.) This information reveals the interactions between ligand and receptor, identifying the important structural features of effective ligands, which has permitted molecular models to be developed for *in silico* modelling and analysis of quantitative structure-activity relationships.³⁴⁴⁻³⁴⁷ Such methods are ideal for the rational design of agonists and antagonists,³⁴⁸ and are particularly useful for screening large numbers of potential ligands.³⁴⁵

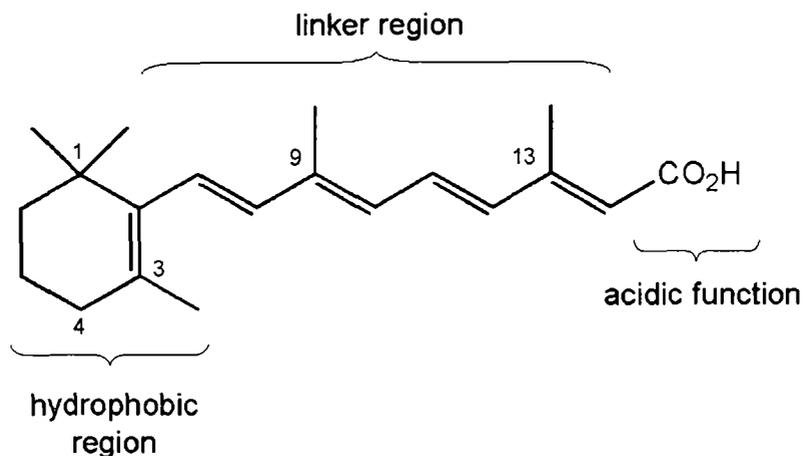


Figure 2-1: A molecule of ATRA annotated to show the three general regions that define the pharmacophoric groups necessary to confer binding of retinoids to RAR and/or RXR.

The pharmacophore of a molecule is defined as the essential features of a ligand that are required for biological activity and includes electronegativity, steric size and lipophilicity. Three key structural components are required for RAR and/or RXR binding affinity, as depicted in Figure 2-1:

- 1 A relatively planar lipophilic cyclohexene, annular or polycyclic group with alkyl substituents. This bulky group is relatively hydrophobic and forms many van der Waals interactions with the surrounding amino acid residues.
- 2 Conjugated polyene or conformationally restricted electron-rich “linker” unit. When heteroatoms are incorporated into this region they may confer isotype specificity. This unit must also provide the correct spacing and orientation between the two other regions to enable the molecule to fit the ligand binding domain (LBD).³⁴⁹
- 3 A carboxylic acidic functional group or appropriate bioisostere. This moiety is involved in a hydrophobic network including salt bridges and hydrogen bonds.³⁵⁰ Where a phenyl ring is present, optimal activation is achieved with groups which are *para* with respect to the rest of the molecule,³⁵¹ whereas *ortho* substitution results in a complete loss of activity, as the resulting compounds are unable to bind in any of the RAR or RXR binding pockets.³⁴⁹ *Meta* isomers may have relatively reduced retinoidal activity or even induce a different pattern of differentiation.³⁵²

Rational modification of this general motif can be achieved by replacing single atoms or groups with bioisosteres that elicit similar biological activity due to common physicochemical properties.³⁵³ The building blocks that have been successfully utilised to generate RAR and RXR ligands are summarised by Altucci *et al.*⁴⁷ Lipinski’s Rule of Five, a model used to predict the absorption or permeation of a compound, and hence its likely bioavailability, cannot be applied to the retinoids. According to this model, both ATRA and its geometric isomer 9-*cis*-retinoic acid (9-*cis*-RA, structure **B** in Appendix 1) are predicted to be poorly absorbed.³⁵⁴

ATRA binds to all three RAR isotypes (RAR- α , RAR- β and RAR- γ), whereas 9-*cis*-RA is a pan-RAR and pan-RXR agonist.^{161-163,355,356} Analysis of *holo*-receptor crystal structures has revealed the complexity of interactions that retinoid ligands make with their LBD, as exemplified by Figure 2-2. The LBDs of *apo*-RAR and *apo*-RXR are globally similar, although they share only 27% sequence homology.³⁵⁰ As might be predicted from comparison of the molecular structures of ATRA and 9-*cis*-RA, the binding pocket of the RXRs is L-shaped whereas the RAR LBD is more elongated and linear.^{201,357,358} Therefore only flexible ligands, such as 9-*cis*-RA, possess pan-agonist properties.²²¹

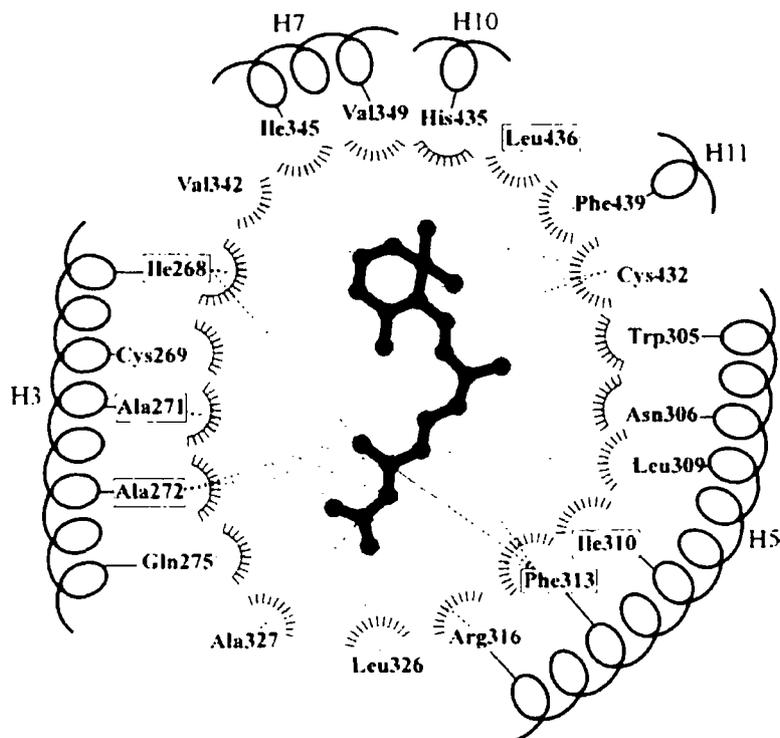


Figure 2-2: Depiction of the interactions between the amino acids of the human RXR- α ligand-binding domain and 9-*cis*-RA. Figure from Love *et al.*, 2002,²⁰¹ using co-ordinates from entry 1FBY.pdb³⁵⁹ in the RCSB Protein Data Bank.³⁶⁰

There are only three variable amino acid residues in the ligand-binding pockets of RAR- α , β and γ ; these residues and the interactions they form with isotype specific retinoids have been reviewed by Zusi *et al.*²²⁰ and de Lera *et al.*²²¹ This divergence explains the differences in the binding affinities of each RAR isotype for a ligand and is useful for the design of receptor-specific retinoids. Accordingly, a number of receptor subtype retinoids have been prepared for all three RAR isotypes and a number of different isoforms, although RXR subtype specificity is more elusive due to the highly conserved nature of the RXR LBDs.²²¹ Specific retinoid and rexinoid antagonists have also been designed, prepared and tested.^{213,221} One approach to the design of antagonists is based upon inhibiting the folding of helix 12 (H12), which normally seals the ligand cavity and permits recruitment of co-

activators to permit transcription (refer to Section 1.5 for further detail).^{173,201,361} These compounds are useful for inhibiting the activity of individual receptors in order to investigate their biological role and that of any co-administered retinoids.²¹³

2.1.3 Evolution of retinoid structure

Retinoid development can be organised into a series of generations, charting the structural divergence from ATRA to compounds which superficially bear little resemblance to the parent compound. The structures presented in Appendix 1 illustrate the structural diversity of this family.

The first generation of synthetic derivatives were based very closely on ATRA and include 13-*cis*-retinoic acid (13-*cis*-RA, structure **C** in Appendix 1) and fenretinide (4-hydroxy-phenylretinamide or 4-HPR, **J**). Substituting the ATRA 2,6,6'-trimethylcyclohex-1-enyl group for the aromatic 4-methoxy-2,3,6-trimethylphenyl group generated acitretin (**N**), the pharmacologically active metabolite of etretinate. The third generation consists of polyaromatic compounds, in which part or all of the labile polyene chain is incorporated into one or more aromatic rings. Included in this group are arotinoids (for example TTNPB, **D** and Targretin[®], **E**)³⁶² and heteroarotinoids (for example Tazarotene[®], **M** and Tamibarotene, **O**).^{277,342,363} These retinoids have greater physical stability and the restrained conformations engender receptor selectivity. A fourth generation of compounds could be said to be comprised of retinoid related molecules, which are also known as atypical retinoids, including CD437 (**I**), ST1926 (**K**) and Adapalene (CD271, **L**). These compounds possess retinoid receptor dependent and independent functionality and show great chemotherapeutic potential.³⁶⁴

2.1.4 Synthetic approaches to the preparation of retinoids

The first synthesis of ATRA was reported in 1946.⁶¹ Since then, the arsenal of reagents and reaction procedures available to the synthetic chemist has evolved substantially and more rigorous separation and characterisation techniques have been developed.³⁶⁵ In addition to the continued preparation of novel retinoids, improved syntheses of ATRA and clinically or commercially interesting compounds continue to exploit these new technologies.^{366,367} The synthetic methodologies employed to prepare retinoids have been thoroughly reviewed over the years,^{60,368-371} and most recently and extensively by Domínguez *et al.*³⁶⁵

Further developments in understanding both the biological mechanism of action of ATRA and synthetic retinoids and the therapeutic requirements for efficacious retinoids, coupled with superior modelling and novel synthetic methodologies, provide continued opportunity for the design and synthesis of novel retinoids.

2.2 Experimental overview

A number of synthetic retinoid analogues were identified as targets to establish the retinoid research theme and demonstrate proof of concept: that retinoids can be successfully synthesised and used to induce differentiation in the human TERA2.cl.SP12 embryonal carcinoma (EC) cell line. For this reason, the molecules chosen closely resemble known ligands of the retinoic acid receptors (RAR) and/or retinoid X receptors (RXR); the targets are shown in Figure 2-3. Compounds with a *para* carboxylic acid group are structurally similar to all-*trans*-retinoic acid (ATRA), while *meta*-CO₂H isomers more closely resemble the structure of 13-*cis*-retinoic acid (13-*cis*-RA). Similar receptor binding properties and biological activities to the natural retinoids are therefore expected, namely ATRA binds and strongly activates all RAR receptors, whilst 13-*cis*-RA is a strong pan-RAR and pan-RXR agonist. These structures are also ideal targets to apply new synthetic methodology developed in the Marder and Whiting research groups.

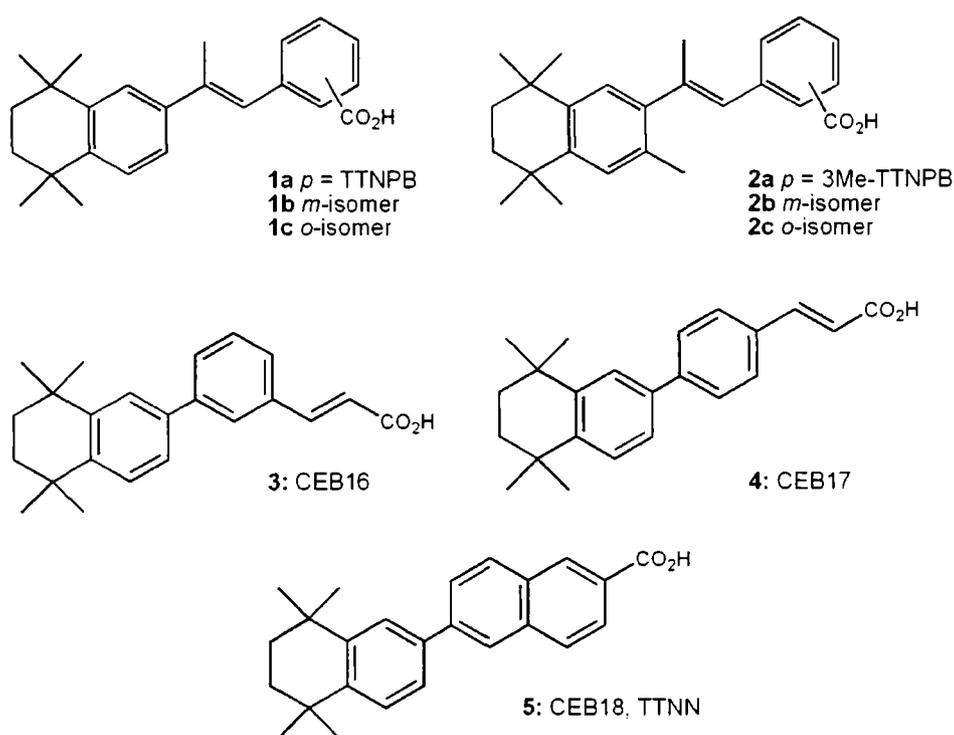


Figure 2-3: Target molecules: **1a**, **1b** and **1c**: (E)-4, 3 and 2-[2-(5,5',8,8'-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acids. **2a**, **2b** and **2c**: (E)- 4, 3 and 2-[2-(3,5,5',8,8'-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acids. **3-4**: 3-[3 and 4-(5,5',8,8'-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl) phenyl]-acrylic acid respectively. **5**: 6-(5,5',8,8'-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)naphthalene-2-carboxylic acid.

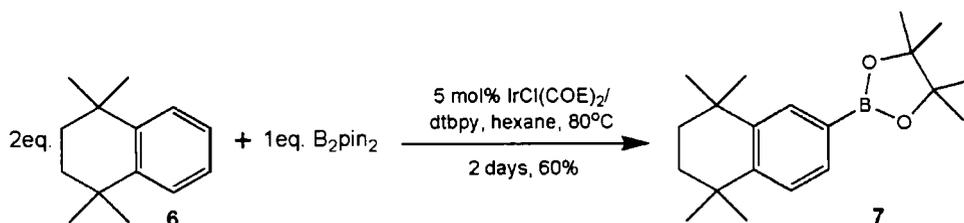
Compounds **3**, **4** and **5** were prepared and carefully purified by the author. The preparation of molecules **1a-c** and **2a-c** built upon previous work within Professor Marder's research group. Due to problematic synthesis, and a change in project direction, only partial synthesis of these compounds is presented.

The compounds are based on a 1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene or 1,2,3,4-tetrahydro-1,1',4,4',6-pentamethylnaphthalene lipophilic core. These cyclic moieties have been used extensively in the preparation of synthetic retinoids, detailed in a recent extensive review by Dawson,²¹³ as a bioisostere for the trimethylcyclohexyl group of ATRA. Extensive structure activity relationship studies have shown that minor modifications and substitutions to this structure are tolerated without loss of biological activity; the two geminal dimethyl groups at positions 1 and 4 confer optimal activation and activity.²¹⁷ The dimethyl groups in the 4-position block catabolism by hydroxylation by the major CYP enzymes involved in the degradation of ATRA.¹²⁷ The labile polyene chain of ATRA is replaced by cyclic bioisosteres constraining the structure and conferring optimal activation, selectivity and stability on the synthetic retinoids.^{213,362}

2.3 Preparation of TTNPB and its isomers

Commencing with the commercially available 1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene **6**, a four-step reaction sequence of alternating C-H activation and Suzuki-Miyaura coupling reactions was one step away from yielding TTNPB and its *ortho*- and *meta*-isomers (**1a**, **1b** and **1c**). Each step required optimisation, yields were often low and all reactions problematic to purify.

2.3.1 C-H activation using iridium (I)/dtbpy



Equation 2-1: Iridium(I)/dtbpy catalysed borylation of **6**.

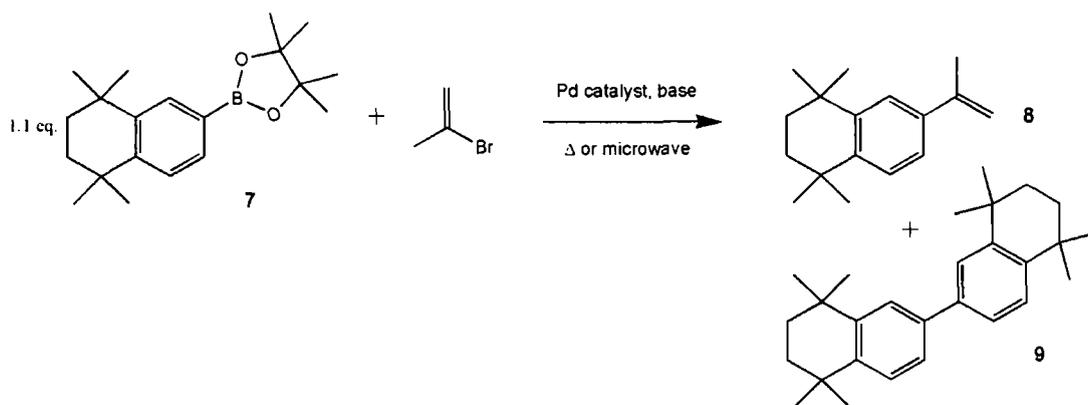
The first reaction in this sequence utilises the C-H activation methodology developed within the Marder group³⁷²⁻³⁷⁴ and an iridium (I) catalyst system devised by Ishiyama *et al.*³⁷⁵⁻³⁸⁰ with the di-boron reagent bis(pinacolato)diboron (B_2pin_2). Di-boron reagents including B_2pin_2 , bis(catechol)diboron (B_2cat_2) and bis(neopentylglycolato)diboron (B_2neop_2) can be used to functionalise a range of organic substrates.³⁸⁰⁻³⁸² Significant features of these reaction conditions

include: the use of a non-polar solvent to facilitate faster reaction rates than more coordinating solvents such as DME and DMF³⁷⁵ and a strongly electron donating ligand, 4,4'-di-*tert*-butyl-2,2'-bipyridine (dtbpy), which is significantly more efficient than alkylphosphine ligands in Ir-catalysed aromatic C-H borylation. dtbpy enertes a relatively soluble iridium complex and, in combination with Ir(Cl)(COE)₂ as a catalyst precursor, enables high-yielding and efficient room-temperature reactions.^{375,377,378}

This reaction was performed using a high catalyst loading (20 mol%) to ensure that the reaction would achieve complete consumption of the starting material. This was achieved within 24 hours, and after work-up and purification the isolated yield was 90%. The reaction was subsequently repeated using a standard catalyst loading of 5 mol%: under these conditions, only 55% conversion of starting material was achieved.

Small-scale test reactions using two different solvents (THF and cyclohexane) gave satisfactory consumption of starting material and apparently clean product, as determined by GC-MS analysis of the reaction mixtures. However, when the reaction was performed on a larger scale (3 g) a by-product was observed (approximately 10% of the final product, as estimated by GC-MS and ¹¹B NMR). From the mass ion of 238 *m/z* and fragmentation pattern, the identity of this product was determined to be COE-Bpin, from reaction of B₂pin₂ with excess catalyst. Silica gel chromatography (1:1 hexanes:DCM) failed to remove this impurity. The first preparation of 7 was, therefore, used without further purification. Small-scale re-crystallisation from hot hexane subsequently yielded pure white crystalline material for characterisation.

2.3.2 Optimising the Suzuki-Miyaura coupling reaction between alkylboronate 7 and 2-bromopropene



Equation 2-2: Overview of Suzuki-Miyaura coupling of 7 with 2-bromopropene.

Suitably functionalised, the next step was to couple **7** with 2-bromopropene to create the linker region of the arotinoid. C-C bonds can be formed using a wide range of different catalysts and reaction conditions,³⁸³ however, the palladium-catalysed Suzuki-Miyaura cross-coupling reaction³⁸⁴⁻³⁸⁷ is particularly well suited to the synthesis of thermally unstable retinoids due to the mild reaction conditions.³⁸⁸ In total, six different experimental conditions were attempted to try to minimise formation of **9**, an oxidatively coupled homodimer. Catalyst, base, solvent and energy source were all varied in the five conditions described in Table 2-1.

Table 2-1: Reaction variables for Suzuki-Miyaura coupling of **7** and 2-bromopropene.

Reaction	Catalyst system	Base	Solvent(s)	Reaction Conditions	Inert atmosphere & degassed solvents?	Approx. % of products *
i	5 mol% Pd(PPh ₃) ₂ Cl ₂	3 equiv. K ₃ PO ₄	1:6 H ₂ O:dioxane	microwave: 150 °C, 300 W, 60 min	no	TM = 40 HC = 25 O = 35
ii	5 mol% Pd(PPh ₃) ₂ Cl ₂	3 equiv. K ₂ CO ₃	1:6 H ₂ O:dioxane	microwave: 100 °C, 300 W, 30 min then: 150 °C, 300 W, 60 min	no	SM = 65, TM = 10 HC = 25 SM = 20, TM = 15 HC = 50, O = 15
iii	10 mol% Pd(OAc) ₂ / PPh ₃	2.5 equiv. K ₃ PO ₄	1:5 H ₂ O:dioxane	thermal: 80 °C, 19 h	yes	TM = 25 HC = 50 O = 25
iv	5 mol% Pd(dppf)Cl ₂	1.5 equiv. K ₃ PO ₄	1:5 H ₂ O:DMF	thermal: 80 °C, 19 h	yes	SM = 5, TM = 55 HC = 15, O = 25†
v	5 mol% Pd(dppf)Cl ₂	2.5 equiv. K ₃ PO ₄ ·2H ₂ O	DMF	thermal: 80 °C, 19 h	yes	TM = 80 HC = 5 O = 15

*% of each component in reaction mixture estimated by GC-MS:

SM = starting material (**7**)

TM = target molecule (**8**)

HC = homo-coupled product (**9**)

O = other un-determined by-product(s)

†% of each component in crude product estimated by GC-MS

Microwave irradiation has been favourably reviewed as a convenient, rate-enhancing method of conducting a variety of reactions, especially palladium catalysed couplings.^{389,390} Using conditions (i) and (ii), notably without excluding oxygen, significant amounts of a by-product were produced, which was identified as the homo-coupled product **9** by MS, without isolation. GC-MS analysis of the reaction mixtures was used to determine the product composition of the test reactions to compare the

different conditions. It should be noted that these GC-MS yields have not been standardised, so should only be viewed as an approximation. GC peak area is a function of the total ions detected by the MS, which is dependent upon the relative ionisation abilities of the different components of a sample.

Using an inert atmosphere and thermal heating in (iii) produced less homo-coupling. Changing the catalyst and solvent, and reducing the quantity of base (iv) did reduce the quantity of **9** generated, but also appeared to increase the formation of other by-products. It was therefore decided to try an alternative approach and replace the water added to the organic solvent with a hydrated base, to provide the trace amount of water required for the reaction. The results obtained in (v) were a significant improvement, so were used on a one gram scale reaction. However, work-up and purification were problematic, such that the isolated yield was only 45%.

Next, the microwave and thermal reaction conditions were compared directly using the best catalyst/base/solvent system (v). Both reactions were prepared in the glove box using the same starting materials, yet it can be seen from Table 2-2 that the two different conditions gave distinctly different product mixtures.

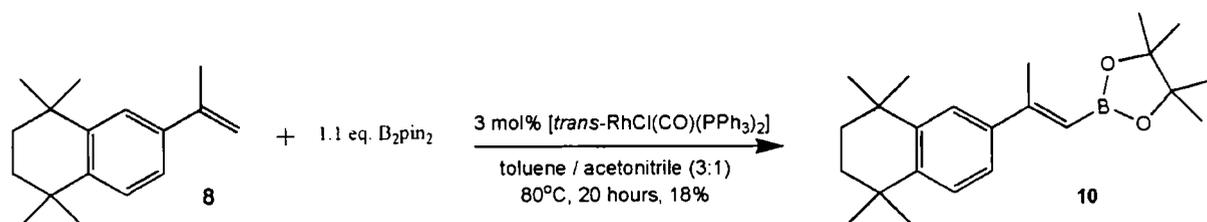
Table 2-2: Direct comparison of microwave and thermal Suzuki-Miyaura reactions.

Energy Source		Microwave	Thermal	
Reaction conditions		60 min 150 °C, 150 W	4 hours 80 °C	23 hours 80 °C
Approx. % of products	SM	0	20	10
	TM	30	50	50
	HC	10	15	25
	O	60	15	15

In the microwave experiment all starting material was consumed within 60 minutes and the quantity of homo-coupled product generated was low, but the yield of desired product was poor. Thermal reaction conditions produced more homo-coupling than previously observed and product yield was also unsatisfactory.

Finally, reaction conditions published for the coupling of 5,6-dibromoacenaphthene with 4-methoxy phenyl boronic acid were attempted. These were much more successful with only 2% of **9** detected in the reaction mixture. Using 3-4 mol% Pd(PPh₃)₄ as catalyst and 2.5 equivalents of Ba(OH)₂·8H₂O in a mixture of *N,N*-dimethylacetamide / H₂O (5:1)³⁹¹ improved the quaternerisation of the boron atom, favouring formation of **8** (isolated yield 95%, purity >95%).

2.3.3 Vinyl C-H activation using rhodium Vaska's catalyst

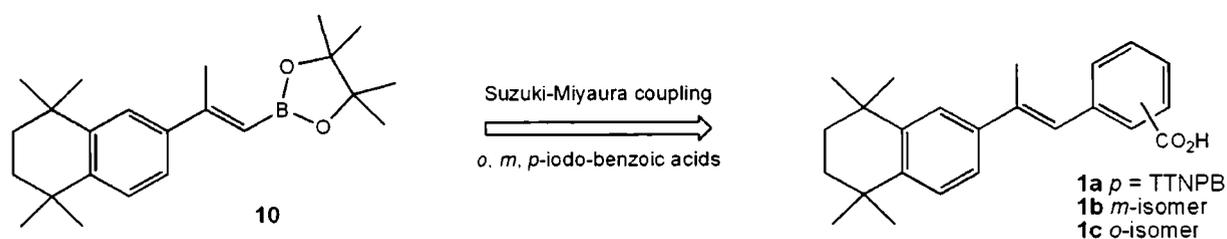


Equation 2-3: C-H activation of **8** to give vinylboronate **10**.

Preparation of the second pinacolboron, **10**, was achieved *via* another transition metal catalysed borylation to activate the propene for further reaction. 3 mol % of rhodium Vaska's catalyst [*trans*-Rh(Cl)(CO)(PPh₃)₂],³⁹² was used and the reaction was complete in under 43 hours. Attempted purification by silica gel chromatography yielded an impure yellow oil, containing residual B₂pin₂. Washing with methanol solidified the product, and recrystallisation from hot hexane gave an impure off-white solid. Only one alkenic proton was observed by ¹H NMR, indicating that only one isomer of **10** was formed. Due to the steric bulk of the pinacol group this was expected to be the *E* isomer, which was confirmed by X-ray crystallography.³⁹³

2.3.4 Proposed final step – preparation of retinoids

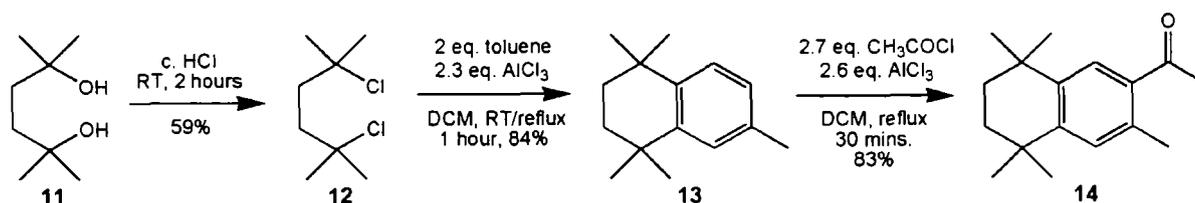
Insufficient alkeneboronate (**10**) was available to attempt the final coupling steps: three Suzuki-Miyaura couplings with *o*-, *m*- and *p*-iodobenzoic acids to give (*E*)-2, 3 and 4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acids, **1c**, **1b** and **1a**, respectively (Equation 2-4).



Equation 2-4: Proposed synthesis of **1a**, **1b** and **1c**.

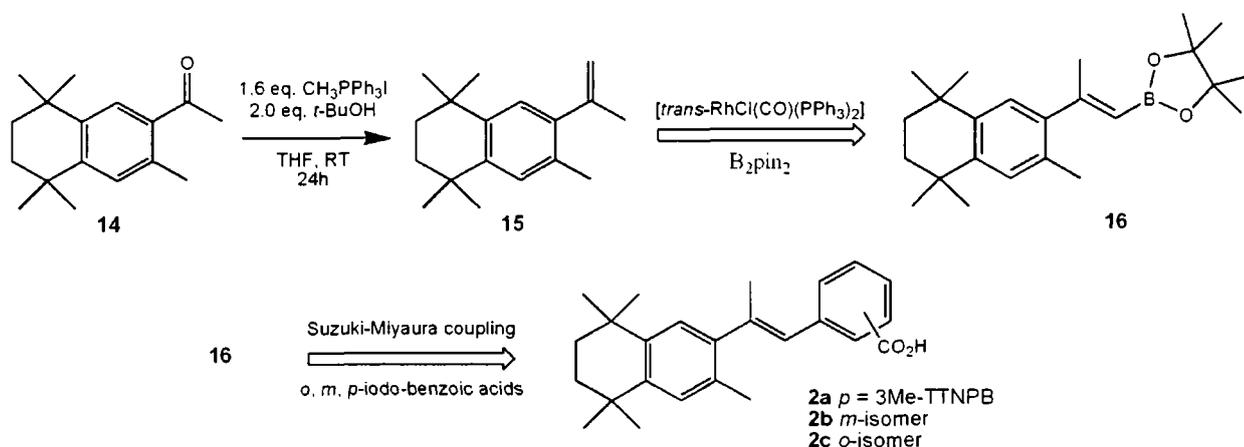
2.4 Preparation of 3-Me TTNPB series of compounds

The initial steps used to prepare 3-Me TTNPB and its *ortho*- and *meta*-isomers (**2a**, **2c** and **2b** respectively) are well established and use classical reactions (Scheme 2-1).³⁹⁴⁻³⁹⁶ The Ir(I)/dtbpy borylation used to prepare **7** cannot be used with **13** as the substrate because the reaction is sterically disfavoured.



Scheme 2-1: Initial reactions towards the preparation of **2a**, **2b** and **2c**.

The commercially available diol starting material **11** was cleanly and easily converted to the di-chloro alkane **12**. Friedel-Crafts alkylation of **12** to give **13** was followed by Friedel-Crafts acylation with acetyl chloride to give a good yield of ketone **14**. The alkylation reaction produced a by-product, identified as 1,2,3,4-tetrahydro-1,1',4,4',6,7-hexamethylnaphthalene by GC-MS, formed by the reaction of **12** with trace amounts of xylene that were present in the toluene. Xylene is more reactive than toluene in this reaction and therefore magnified a trace impurity to a notable by-product.

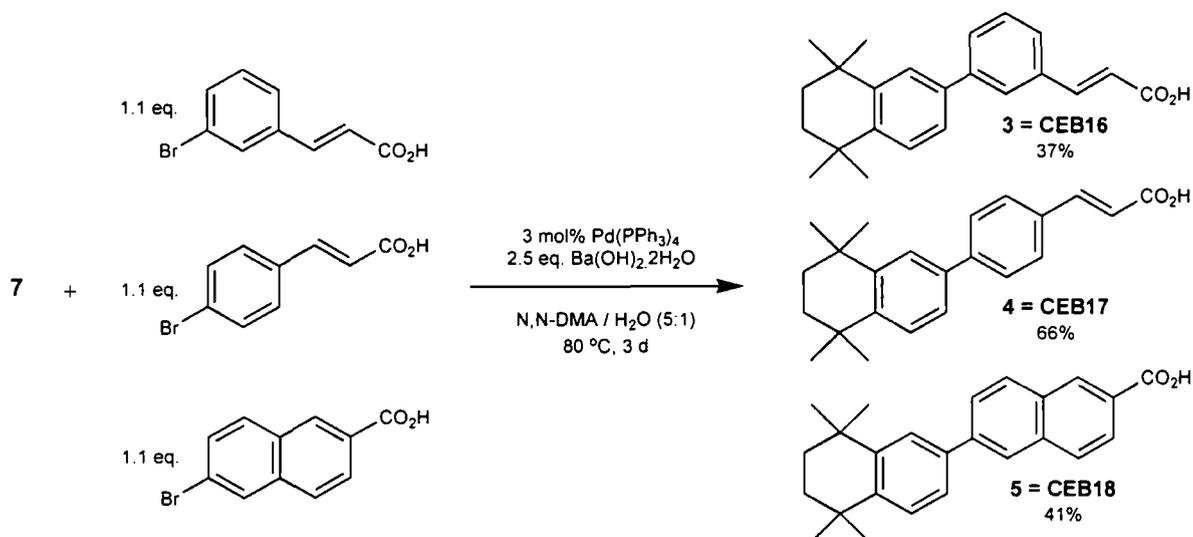


Scheme 2-2: Proposed synthetic route to complete the preparation of 3Me-TTNPB and its isomers.

The final proposed reaction steps to complete the synthesis of **2a**, **2b** and **2c** are detailed in Scheme 2-2. Subjecting **14** to a Wittig reaction produced olefin **15**, though these reaction conditions produced large quantities of PPh_3O which were difficult to remove from the product.³⁹⁶ (Product **15** was worked up by Dr. Ibraheem Mkhaliid.) The final proposed reactions are a rhodium catalysed dehydrogenative borylation to produce the vinylboronate ester **16** and finally Suzuki-Miyaura coupling of iodo- or bromo-benzoic acids to give **2a**, **2b** and **2c**.

2.5 Synthesis of a naphthoic acid and two acrylic acid retinoids

Three retinoid analogues, known as CEB16, CEB17 and CEB18 (**3**, **4** and **5** respectively) were prepared in one step from alkylboronate (**7**). The Suzuki-Miyaura coupling reaction was used to react **7** with 3- and 4-bromocinnamic acids and 6-bromo-2-naphthoic acid to produce retinoids **3**, **4** and **5** respectively (Scheme 2-3).



Scheme 2-3: Preparation of CEB16 (**3**), CEB17 (**4**) and CEB18 (**5**).

Analysis of the reaction mixtures by GC-MS showed complete consumption of **7**, but little or no product was detected. ¹H NMR analysis confirmed that the major products were the target molecules. The crude products of **4** and **5** were contaminated with approximately 25 and 10 mol% respectively of their respective bromide starting materials. The *trans*-geometry of the double bonds of **3** and **4** was established by ¹H NMR; the spectra of both products displayed two doublets with coupling constants of ~16 Hz for the vinylic protons whereas the *Z*-isomers would be expected to have smaller coupling constants of ~12.5 Hz.

A major problem with the handling and purification of these products was their low solubility, which resulted in low yields. During work-up, excess EtOAc was used to dissolve and extract all the products. During subsequent purification, EtOAc was used to dissolve **3**, toluene to dissolve **4**, and acetone to dissolve **5**. The use of such solvents is not ideal, as they tend to dissolve anything else they come into contact with, and purification on the basis of differences in solubility is poor.

All three products were purified by passing through a silica pad (deactivating the silica by washing with dilute acid may have helped to prevent the compounds sticking) to remove the catalyst from the crude product. Column chromatography failed to remove residual starting material from **4** and **5**. Final

purification was achieved by multiple re-crystallisations from EtOAc (**3**), toluene (**4**), or acetone (**5**), to yield white crystalline products. Crystals suitable for diffraction were grown during re-crystallisation of **4** and **5**. Final yields (of completely pure material suitable for biological testing) were 37% (CEB16, **3**), 66% (CEB17, **4**) and 41% (CEB18, **5**).

2.6 Crystal structures

A number of both the intermediate compounds and target retinoids synthesised thus far have been found to be crystalline. Single crystals suitable for X-ray diffraction have been grown for four of the compounds described (**4**, **5**, **20** and **26**). The crystal structures of compounds **11**, **15**, **18** and **21** had been solved previously.³⁹³ The results of the crystallographic refinements are summarised in Appendix 2.

Crystal structures of the retinoids provide valuable data about the orientation of the molecule in the solid state and, in combination with the structures of RAR and RXR nuclear receptor ligand-binding pockets, may enable a greater understanding of the molecular interactions involved in successful binding. The crystal structures of many of the RAR and RXR ligand-binding domains bound to agonists and antagonists have been determined.^{47,213} This information will guide the rational design of ligands,³⁴⁸ and permit *in silico* modelling of receptor-ligand complexes – a particularly useful technique for screening large numbers of potential ligands.³⁴⁵

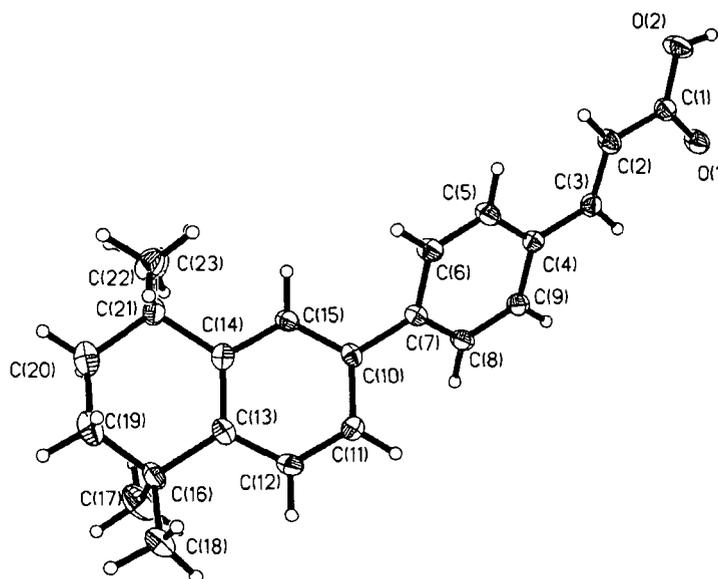


Figure 2-4: Single molecule of 4-[4-(5,6,7,8-tetrahydro-5,5',8,8'-tetramethyl-2-naphthalenyl)phenyl] acrylic acid (CEB17, **4**). Triclinic crystals (space group P-1) were grown from a concentrated solution of CEB17 in toluene at low temperature. The dihedral angle between the 1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene and cinnamic acid groups is 39.5°. The crystals show disorder over two crystallographically independent positions with occupancy of 37% and 63%.

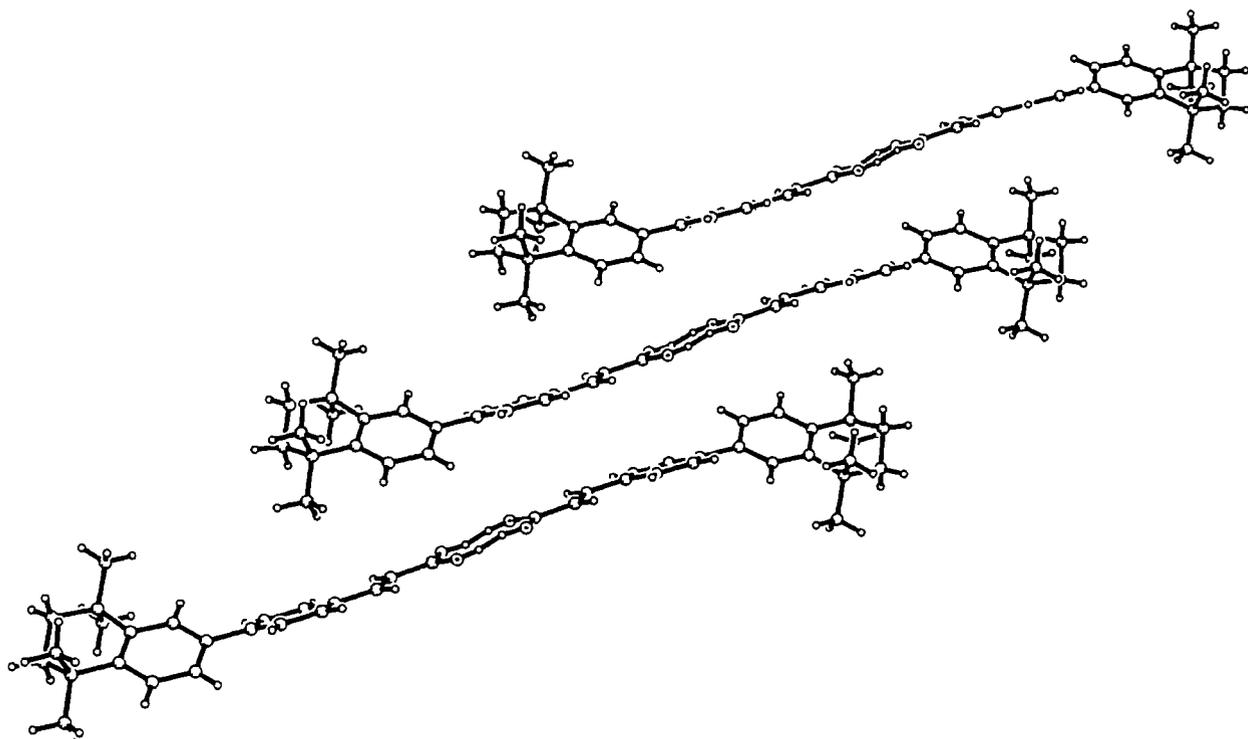


Figure 2-5: Six molecules of CEB17 (4). The packing motif consists of parallel stacks of dimers, which are formed through intermolecular O-H...H hydrogen bonds.

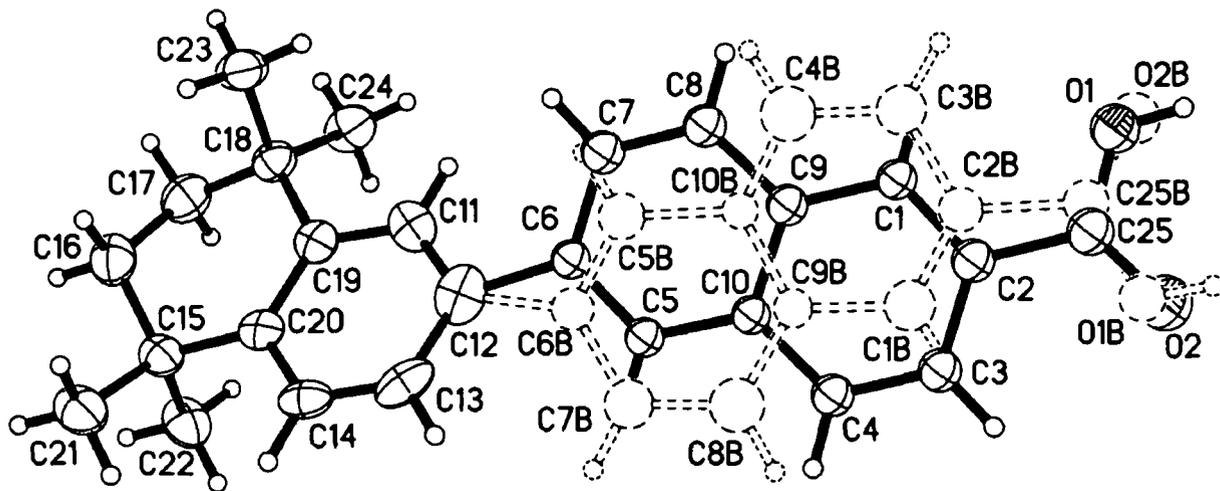


Figure 2-6: 6-(1,1',4,4'-tetramethyl-1,2,3,4-tetrahydro-6-naphthyl)-2-naphthalene carboxylic acid (CEB18, 5) also crystallises with two crystallographically disordered independent positions. Triclinic crystals (space group P-1) were grown from a concentrated solution of 5 in acetone at low temperature. The two disordered molecules are almost co-planar (2.4°), the dihedral angle between the 1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene group and the naphthoic acid groups is 31.1° for the major molecule and 28.8° for the minor, which have occupancies of 63% and 37% respectively. This compound also dimerises through intermolecular hydrogen bonding.

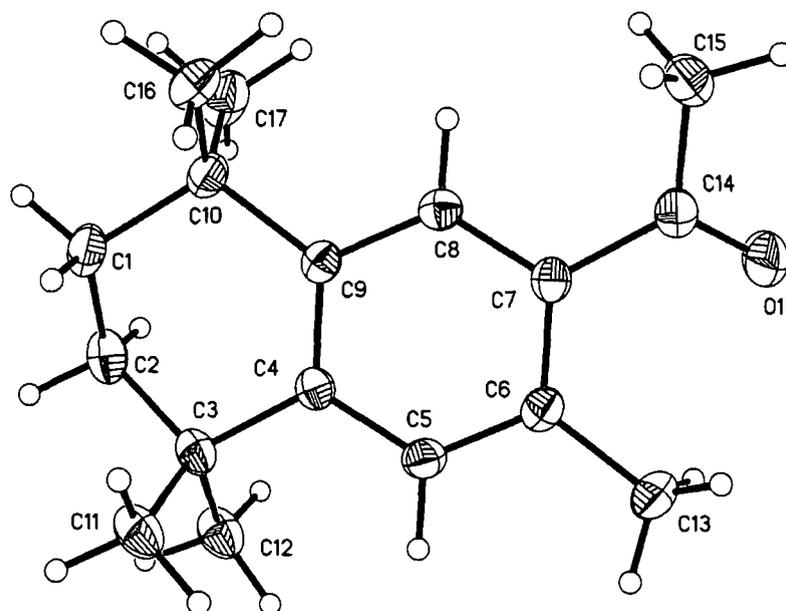


Figure 2-7: Single molecule of (5,6,7,8-tetrahydro-3,5,5',8,8'-pentamethyl-2-naphthyl)ethan-1-one (**14**). Orthorhombic crystals (space group $P2_12_12_1$) were grown by slow evaporation of solvent from a concentrated solution of **14** in ethyl acetate.

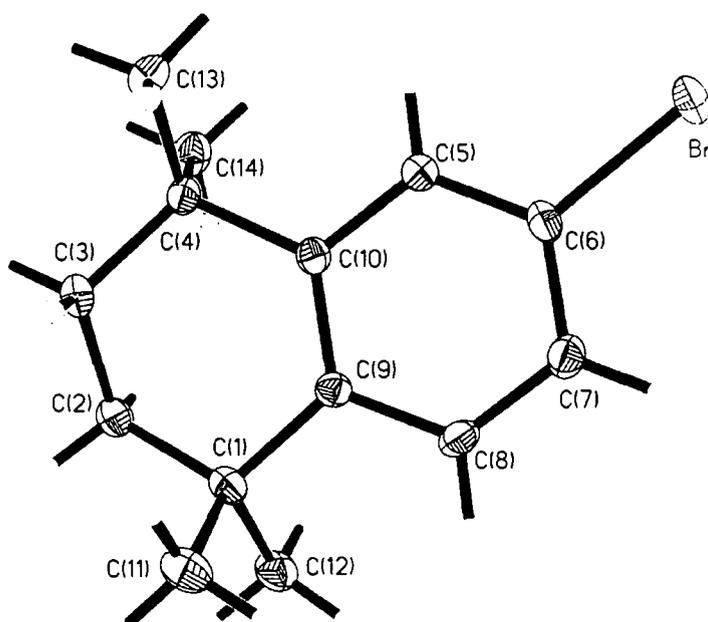


Figure 2-8: Single molecule of 6-bromo-1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene (**17**). Monoclinic crystals (space group $P2(1)/c$) were grown from a concentrated methanolic solution of **17** at low temperature.

2.7 Conclusions

These experiments illustrate the successful application of emerging C-H activation methodologies to the preparation of biologically active retinoids. Three compounds (**3**, **4** and **5**) were prepared, rigorously purified and have been screened for biological activity (Chapter 3). Significant progress was also made towards the synthesis of two groups of styrene arotinoids (**1** and **2**), investigating and optimising the reactions. However, due to low yields after rigorous purification of the intermediates, insufficient material was available to complete the synthesis. The *meta* and *para* isomers of the methyl esters of both TTNPB and 3-Me TTNPB series have subsequently been prepared by other researchers.³⁹⁷ TTNPB is a strong pan-RAR agonist and the archetypal arotinoid which served as a lead for a large number of analogues.^{214,362,395,398-400} Introducing the sterically demanding methyl group at the 3 position produces eclipsed torsional interactions that force TTNPB from its near co-planar conformation such that the benzoic acid and pentamethyltetrahydronaphthalene groups lie orthogonal to each other. In this conformation, 3-Me TTNPB has a 50-fold selectivity for RXR over RAR.³⁹⁹

Crude purification of intermediates should be performed throughout the synthesis, but ultimately the final product must be completely pure to be useful for biological testing. Any impurities may themselves effect cell differentiation, disrupt the normal behaviour of the cells or interact with the retinoid. If the identity of impurities is known they can be tested alongside the target molecules with the aim of subtracting any observed effect. The only incidental advantage to this would be the preliminary assessment of cytotoxicity of impurities, which may help to assess the tolerance of impurities. Fortunately less than 10 mg of material is sufficient to perform initial biological screening. The retinoids CEB16 (**3**), CEB17 (**4**) and CEB18 (**5**) were problematic to purify. It should be possible to improve the yield and simplify the purification of these compounds by reducing the proportion of bromo carboxylic acid starting materials. It would also be advantageous to the synthesis of retinoids to develop HPLC methods to analyse and purify the products. Such methods could also be used to determine the stability of synthetic compounds and perform simple metabolic studies *in vitro*.

All-*trans*-retinoic acid (ATRA) is notoriously unstable and is readily isomerised when exposed to light and heat.⁴⁰¹ Over the past thirty years thousands of synthetic retinoids have been designed by modifying the structure of the natural retinoids with numerous permutations of hydrophobic rings, linker groups and bioisosteres for carboxylic acid.⁴⁷ Some of the resulting compounds have been found to be agonists or antagonists of individual or multiple isoforms of the RAR and RXR receptors. Replacing the labile polyene chain of ATRA and modifying other elements of its structure is likely to increase the stability of the compound and improve selectivity for one or more of the receptor binding domains. Compounds CEB16 (**3**), CEB17 (**4**) and CEB18 (**5**) proved to be stable during synthesis and lengthy purification and a preliminary stability study confirmed that these compounds were more stable than the natural retinoids under daylight and after heating although CEB16 (**3**) and CEB17 (**4**) decomposed slightly on exposure to UV light.⁴⁰²

**CHAPTER 3: BIOLOGICAL SCREENING
AND CHARACTERISATION OF NATURAL
& SYNTHETIC RETINOIDS IN HUMAN
EC STEM CELLS**

3.1 *In vitro* retinoid screening

3.1.1 Introduction

All-*trans*-retinoic acid (ATRA), the parent compound of a large family of natural and synthetic retinoids, possesses a wide range of fundamental endogenous activities that are believed to present considerable opportunity for clinical therapy (see Section 1.6). In order to study the biological effects of retinoids, and thus explore their therapeutic potential, a suitable model system of an appropriate endogenous effect must be identified. Classical retinoids bind and activate one or more of the retinoic acid receptors (RARs) or retinoid X receptors (RXRs) to control the expression of their target genes and realise their biological effects.^{355,356} Many researchers have therefore used retinoid receptor binding and transactivation assays to compare the effects of novel retinoids using various cell types, as appropriate to the investigators' area of interest.⁴⁰³ Although well suited to high-throughput screening,²³² not only does binding affinity not correlate directly with ability to activate receptors, but the data from different assays may give inconsistent results.^{213,214} For example, the synthetic retinoid Am580 (Table 3-1) is described as an RAR- α selective agonist in its binding affinity, but actually transactivates all three RAR isotypes.^{349,404-406}

It is therefore preferable to use functional assays to investigate characteristic gene expression. Early structure-activity relationship (SAR) studies utilised the standard hamster tracheal organ culture (TOC) assay, which measures the ability of biologically active retinoids to reverse keratinisation.^{341,407} More recently, stem cells have been used extensively to investigate the ability of retinoids to control cellular differentiation in an *in vitro* model system that closely resembles *in vivo* development.^{408,409} The characteristic ability of classical retinoids to induce differentiation is of particular interest as retinoids have great potential to act as chemo-preventative and chemo-therapeutic agents.^{12,301}

3.1.2 Stem cells

Stem cells are characterised by two fundamental properties: their capacity to self-renew by generating identical progeny and the ability to differentiate into multiple yet distinct specialised cell types. At each cell division the alternative outcomes of self-renewal and differentiation are decided by the interplay between intrinsic factors and extrinsic instructive or selective signals.⁴¹⁰⁻⁴¹³ During the course of development, totipotent stem cells of the zygote that have the potential to form all types of cells in the foetus or adult are succeeded by those with a more restricted potential. These multipotent stem cells differentiate into a smaller range of cell types as exemplified by embryonic neural stem cells that differentiate into neurons, astrocytes, and oligodendrocytes.^{414,415} All tissues of the embryo are derived from the three germ layers of the inner cell mass (Figure 3-1). Stem cells that are able to differentiate into cell types of the mesoderm, ectoderm and endoderm, either in response to exogenous stimuli or when xenografted, are described as pluripotent and offer the most representative model of embryonic development.

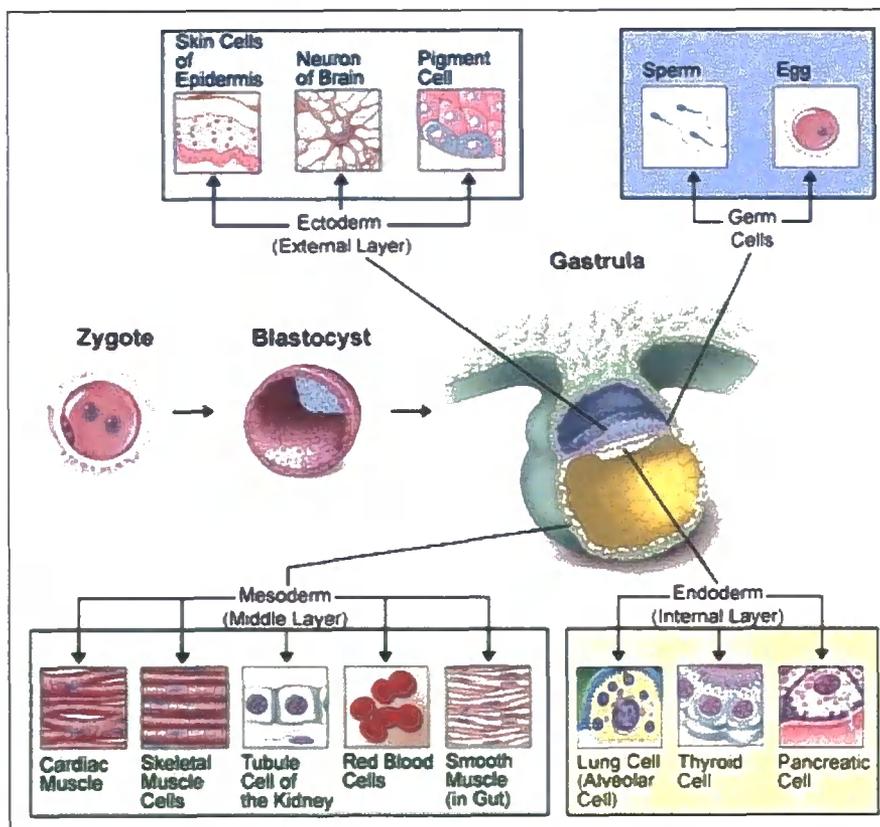


Figure 3-1: Human development begins when a sperm fertilises an egg and creates a single cell that has the potential to form an entire organism, called the zygote. In the first hours after fertilisation, this cell divides into identical cells. These cells then begin to specialise, forming a hollow sphere of cells, called a blastocyst. The blastocyst has an outer layer of cells (yellow), and inside this hollow sphere, there is a cluster of cells called the inner cell mass (light blue). The inner cell mass can give rise to germ cells, as well as cells derived from all three germ layers (ectoderm, light blue; mesoderm, light green; and endoderm, light yellow), including nerve cells, muscle cells, skin cells, blood cells, bone cells, and cartilage. Figure from National Institutes of Health website.⁴¹⁶

Embryonal carcinoma (EC) cells are the stem cells of teratocarcinomas, *i.e.* germ cell tumours, which contain an array of haphazardly arranged differentiated cell types and are thought to reflect the process of embryonic development.⁴¹⁷⁻⁴¹⁹ *In vitro* cultures of EC cells were initially obtained from murine xenografts and subsequently isolated directly from tumours.^{419,420} Although early teratocarcinoma lines showed little capacity for differentiation, EC lines were eventually obtained that were pluripotent and able to differentiate.⁴²¹ F9 and P19 are two well characterised murine EC cell lines that have been used extensively as models of early embryonic differentiation.⁴²²⁻⁴²⁴ Several human EC lineages have also been developed including TERA2.cl.SP12, which was clonally derived from TERA2 huEC cells.⁴²⁵ TERA2.cl.SP12 cells were isolated from the more heterogeneous parent line to provide an EC model of human embryonic development that is highly homogeneous and capable of differentiating into both neurons and glia.^{425,426} Although EC cells cannot yield any kind of therapy directly due to their cancerous nature, such clones are useful models and aid the understanding of molecular mechanisms that control cell differentiation.⁴²⁶⁻⁴²⁸

Embryonic stem (ES) cells are derived from the inner cell mass of mammalian blastocysts. ES cells proliferate rapidly and infinitely while maintaining pluripotency and offer a greater differentiation potential than EC cells.⁴²⁹ Murine ES (muES) cells were first described in 1981,^{430,431} and have been used extensively *in vitro* to study the molecular control of lineage commitment and differentiation. Human ES (huES) cells were finally isolated in 1998⁴³² and are believed to hold great potential in the fields of cell therapy, tissue engineering, drug discovery, *in vitro* toxicology and the chemical testing required under REACH.[‡] ^{409,433-435} It is also hoped that huES cells could be used to treat a host of degenerative diseases such as Alzheimer's, Parkinson's disease and diabetes.⁴³⁶⁻⁴³⁸ Unfortunately, the majority of existing human ES cell lineages have been found to be contaminated with animal antigens, as they were established and maintained on animal-derived feeder layers and in media supplemented with foetal calf serum.⁴³⁹ Though it is proposed that this xeno-antigen contamination is largely reversible,⁴⁴⁰ all stem cell lines derived using animal components are now considered unsuitable for human transplants. Human ES cells have subsequently been successfully derived under defined, animal-free conditions.^{441,442}

Contentious ethical and supply issues have resulted in legislation governing the use of human embryos to produce ES cells.^{411,443} Although there are many similar characteristics between mouse and human ES cells, there are clear differences between the gene expression profiles, growth requirements, expression of surface markers, and response to various developmental signals.^{444,445} It is therefore preferable to use human stem cells as an *in vitro* system in which to study embryonic development, since species differences may exist, making huEC cells a less controversial alternative for initial

[‡] Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is an EU regulation that came into force in June 2007 to control the production and use of chemical substances. Chemicals must be tested to establish the effects on human health and the environment.

studies. Furthermore, EC lines have several advantages as standards for comparison between laboratories: they grow without feeder cells, are relatively simple to passage, resist spontaneous differentiation, and are a rich source of the proteins and mRNAs used to characterize huES cells.⁴⁴⁶ It is therefore convenient for the researcher to use a more robust model, such as EC cells, that replicates the behaviour of ES cells.^{426,446,447}

Stem cell pluripotency is maintained by the precise control of a complex transcriptional regulatory network.^{448,449} The forced expression of four of these master regulators, Oct4, Sox2, Klf4, and c-Myc has recently enabled the reprogramming of somatic cells into induced pluripotent stem cells (iPS).⁴⁵⁰⁻⁴⁵³ This exciting discovery circumnavigates the ethical issues which hamper the derivation of ES cells and could enable patient specific stem cells to be generated for stem cell therapy.⁴⁵⁴

ES and EC cells can be propagated indefinitely in an undifferentiated state by maintaining them under optimal culture conditions. However, they also undergo differentiation to more specialised cell types spontaneously in response to exogenous stimuli and under sub-optimal culture conditions.⁴⁵⁵ A key step in controlling differentiation is defining the pathways that function in undifferentiated cells and the ways in which culture conditions and extrinsic factors can activate or repress them.⁴⁵⁶ A wide range of chemicals have been shown to potentiate the differentiation of stem cells into numerous different cell types. Given its role as a putative morphogen, ATRA has been used extensively to induce the differentiation of EC cells and ES cells in an attempt to delineate its *in vivo* roles.^{408,424,457} Other agents including HMBA (hexamethylene bisacetamide)^{426,458,459} and DMSO (dimethyl sulfoxide)^{460,461} also induce differentiation in EC cells, but result in distinctly different terminal cell types. A major barrier to the use of ES cell-derived tissues for treating human disease is the inability to control, with high efficiency, the differentiation of ES cells into transplantable precursors of a specific lineage. Researchers therefore aim to develop a greater understanding of the molecular mechanisms underlying pluripotency and differentiation to harness the potential of ES cells.

Despite the convenient nature of cell culture, it is an inherently artificial environment for cell growth. Although efforts have been made to mimic the complexity and natural stimuli found in living tissue, for example through co-culture, three-dimensional scaffolds and surface functionalisation,⁴⁶²⁻⁴⁶⁴ the behaviour of cells *in vitro* cannot be taken as fully representative of developmental processes in animal or clinical studies.

3.1.3 Retinoid induced cell differentiation

ATRA-induced differentiation of stem cells has been widely reported and extensively characterised.^{408,465,466} Retinoid treatment typically induces a heterogeneous range of cell types and its effects vary depending on the concentration and cell line, making it difficult to compare data.²¹³ In P19 muEC cells ATRA induces endodermal and neural differentiation,^{467,468} whilst F9 muEC cells can be induced to differentiate into cells of the endodermal lineage.^{457,469} The huEC cell line NTERA2/D1 cells yield a variety of cell types including functional, post-mitotic neurons,^{427,470-472} and similarly treating adherent monolayers of TERA2.cl.SP12 cells with ATRA induces the formation of approximately 10-15% of terminally differentiated neurons, whilst the remainder of the culture differentiates into non-neural cell types.⁴⁷³ Electrophysiological experiments have determined that electrical impulses are communicated between perikarya, just like their *in vivo* counterparts.⁴⁷⁴

It is believed that this heterogeneous behaviour is due to the activation of multiple receptors and genes, mirroring the *in vivo* pleiotropic abilities of endogenous ATRA.¹⁴⁹ The inability to generate specific cell types “on demand” currently precludes the potential to use such derivatives for cell replacement. A range of culture strategies have consequently been developed to expedite and increase the neuronal differentiation efficiency.^{428,433,475} Terminally differentiated functional neurons, for example, are a valuable resource for screening drugs, testing the toxicity of new compounds, and as a potential source of transplantable material to correct neurological deficits.^{426,476}

Differentiation is characterised by marked changes in gene expression that can be monitored to investigate the nature and maturity of resulting cell types. In excess of 500 genes have been identified as targets of ATRA;¹⁷⁸ these genes or their protein products can also be used to characterise and quantify differentiation products. For example, a number of cell surface antigens have been used extensively to investigate ATRA-induced differentiation of EC and ES cells.^{421,445,477} EC stem cell markers including SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49 and TRA-2-54 are suppressed after cells commit to differentiate.⁴⁷⁸⁻⁴⁸¹ In a reciprocal fashion, markers of differentiated cells that were absent in the stem cell population are up-regulated, for example VIN-IS-53, VIN-IS-56, VIN-2B-22, A2B5 and ME311.^{459,482}

A wide range of other retinoids have also been tested in similar systems, initially comparing to them to the activity of natural retinoids,⁴⁶⁸ and subsequently screening the biological effects of a large number of novel synthetic retinoids that have been prepared in the search for therapeutic entities that are safer and more efficacious than ATRA.²¹³

3.1.4 Retinoid (in)stability

It is well known that ATRA and its geometric isomers 9-*cis*-retinoic acid (9-*cis*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA), by virtue of their common labile polyene chain, are highly unstable in the presence of oxidants, heat and light, which cause oxidative degradation or isomerisation.^{280,402,483-485} It is therefore routine laboratory practice to handle these retinoids in subdued light and to store them at low temperature, ideally in an inert atmosphere.^{225,401} The degradation of retinoids during culture will reduce cellular exposure and may lead to concentration dependent effects. The isomers, metabolites and other degradants produced may also affect cell development, leading to a heterogeneous and poorly reproducible response.

However, relatively little research has been published into the nature and magnitude of retinoid degradation and its effects on *in vitro* experiments. This is particularly surprising given the large number of papers published in this field that apparently ignore the consequences of retinoid degradation on their data. Under standard cell culture conditions several factors will contribute to the degradation of ATRA including: incubation at 37 °C, exposure to oxygen, dissolution in aqueous media and interaction with media supplements, in addition to absorption and metabolism by cells.

A limited number of studies have analysed the degradation of retinoids in culture. The initial concentration of applied retinoid is observed to decline rapidly, in some instances to less than 5% of the original concentration within 24 hours,²⁸⁰ though the rate of degradation is significantly affected by the cultured cell type and cell density,^{486,487} media and presence of supplements such as serum.^{488,489}

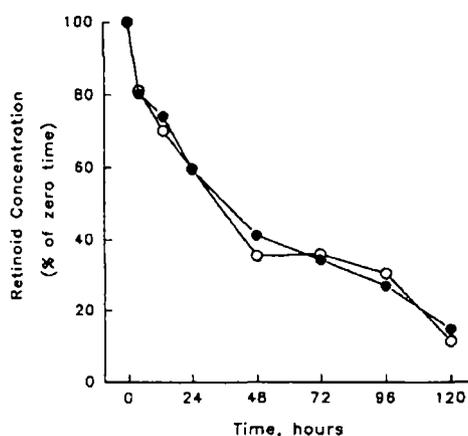


Figure 3-2: Decline of retinoid levels in medium of cultured LA-N-5 cells. Cells were treated with 1 μ M of either ATRA (O) or 9-*cis*-RA (●) under similar conditions. From Han *et al.*, 1995.²⁸⁵

Two independent groups have presented kinetics of ATRA-degradation that are in very good agreement. After 24 hours 75% of the original concentration of ATRA remains, declining to 40-50% after 48 hours and 35-40% after 72 hours (Figure 3-2).^{285,488} It is unclear whether this significant

decrease in retinoid concentration will affect cell behaviour under normal culture conditions. Although a number of synthetic retinoids are more stable than ATRA outside of the cell culture environment,^{352,402} their stability *in vitro* has yet to be tested.

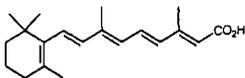
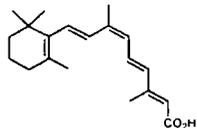
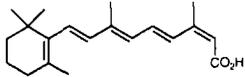
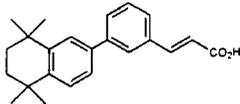
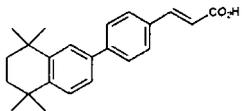
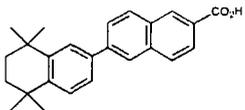
3.1.5 Chapter aims

TERA2.cl.SP12 human embryonal carcinoma stem cells will be used to investigate the biological activity of the stable retinoic acid analogues prepared in Chapter 2. All-*trans*-retinoic acid has previously been demonstrated to induce differentiation of this cell line, although it has not been established if this activation is achieved *via* the classical retinoid molecular pathway (Section 1.5). The activity of the synthetic retinoids will be thoroughly characterised and compared to the differentiation induced by ATRA and its geometric isomers.

3.2 Methodology and compounds investigated

The retinoids listed in Table 3-1 were screened for the ability to induce differentiation in the TERA2.cl.SP12 human embryonal carcinoma cell line. The compounds were dissolved in solvent and diluted in culture media to a final concentration of 10 μ M and applied to the cells for up to 28 days. During this time, morphological changes were observed and flow cytometry was used to analyse the expression of antigens specific to pluripotent stem cells and their differentiated derivatives. A number of other compounds were also screened at 10 μ M for 14 days using the above methods but were not comprehensively investigated by the author (Table 3-2). The compounds presented in Table 3-2 were tested because their structures bear a superficial similarity to known retinoids.

Table 3-1: Retinoid compounds.

Compound	Synonym(s)	Comment
all-trans-retinoic acid 	ATRA tretinoin	Natural metabolite. Strong pan-RAR agonist. Extensively studied <i>in vivo</i> and <i>in vitro</i> . ¹⁶³
9-cis-retinoic acid 	9-cis-RA alitretinoin	Putative endogenous ATRA isomer. Pan-RAR & RXR agonist. ^{161,162}
13-cis-retinoic acid 	13-cis-RA isotretinoin	Putative endogenous ATRA isomer. Pan-RAR & RXR agonist. ^{163,490,491}
CEB16 3 	CD2908	Synthesis and dermal applications patented. No biological data published. ^{492,493}
CEB17 4 	—	Examined in QSAR study. RAR- β & RAR- γ specific agonist. ^{494,495}
CEB18 5 	TTNN AGN 191650 SR3957	RAR- β & RAR- γ specific agonist. ^{496,497}

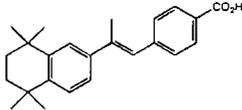
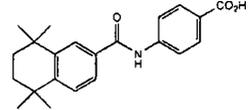
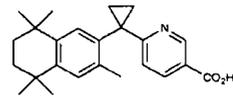
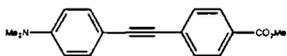
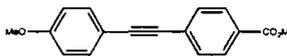
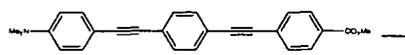
Compound	Synonym(s)	Comment
TTNPB 	TTNPB Ro 13-7410	Purchased from Sigma. Highly teratogenic. Pan-RAR agonist, no RXR activity. Strong inducer of apoptosis. >500-fold greater potency than ATRA. ^{214,362,398,399,498-501}
Am580 	CD366	Purchased from Sigma. Binds selectively to RAR- α , but actually transactivates all three RAR subtypes and with greater potency at RAR- α . No RXR activity. This specificity causes lower topical toxicity, but has higher teratogenic potency than RAR- β & RAR- γ specificity. Improved therapeutic effects compared to ATRA. ^{349,404-406,502}
LG100268 	—	RXR- β specific agonist. ^{201,217}

Table 3-2: Other compounds screened for differentiation activity.

Compound	Synonym(s)	Comment
NMe₂ tolan 	—	Prepared by Dr. Tolu Fasina. Donor-acceptor tolan (diphenylacetylene).
OMe tolan 	—	Prepared by Dr. Tolu Fasina. Donor-acceptor tolan (diphenylacetylene).
BPEB 	—	Prepared by Dr. Tolu Fasina. Donor-acceptor BPEB (1,4-bis(phenylethynyl) benzene).

The BPEB compound was dissolved in DMF and all other compounds were dissolved in DMSO before dilution in culture media to a final concentration of 10 μ M. These vehicles were also tested in the same manner to confirm that the solvents had no effect on the cells. Ethanol (an alternative vehicle) and an acetone solution equivalent to 5 mol% contamination, a potential synthetic impurity, were also tested.

Compounds found to be of interest in the initial screen were investigated further to confirm these data and rigorously characterise the observed differentiation. Analyses included: repeat flow cytometric analysis to confirm the reproducibility of differentiation, immunocytochemical localisation and western blot analysis of antigens associated with neural and non-neural differentiation and, in some cases, proliferation studies.

3.3 Observations of changes to cell phenotype

Proliferative TERA2.cl.SP12 EC stem cells are morphologically homogeneous, consisting of rounded cells with a high nuclear to cytosol ratio. Below approximately 30 passages, cell growth can be maintained in a reproducible and robust manner with a doubling time of approximately one day. Under certain conditions, for example the addition of an exogenous compound such as all-*trans*-retinoic acid (ATRA), reduced serum content of media or other non-ideal growth conditions, and sometimes spontaneously, these self-renewing stem cells may be induced to differentiate. Initially committed progenitor cells are produced, which may continue to proliferate. If the culture conditions are appropriate, this progenitor may develop into one of a restricted number of terminally differentiated cell types. The simplest method of monitoring the effects of test compounds is to regularly observe the morphology of the culture by phase microscopy, in comparison to a negative control (cells in normal proliferative media) and a positive control (cells grown in media supplemented with ATRA). Identification of cell types by morphology alone is unreliable; assumptions should be confirmed using immunocytochemical localisation (ICC) of phenotypic antibodies. Further images are presented in Section 3.6.

3.3.1 Results

Control cultures, LG100268, tolans and BPEB do not induce differentiation of EC cells

The control cultures, LG100268 culture and all cultures grown with the tolans and BPEB shown in Figure 3-3 failed to differentiate; representative phase images are shown in Figure 3-3. No discernable differentiation or other morphological changes were observed during typical culture periods. Elevated levels of cell debris were observed in cultures grown with LG100268 (but not control cultures), possibly due to apoptosis.

On one occasion, cells from a 28 day old DMSO culture were re-plated after harvesting for flow cytometric analysis and maintained for a further four weeks. After this time, a number of neuron-like cell types were observed (Figure 3-4). These conditions are sub-optimal for EC cell growth, but representative of the results that might be expected in an experiment with a compound that fails to induce differentiation.

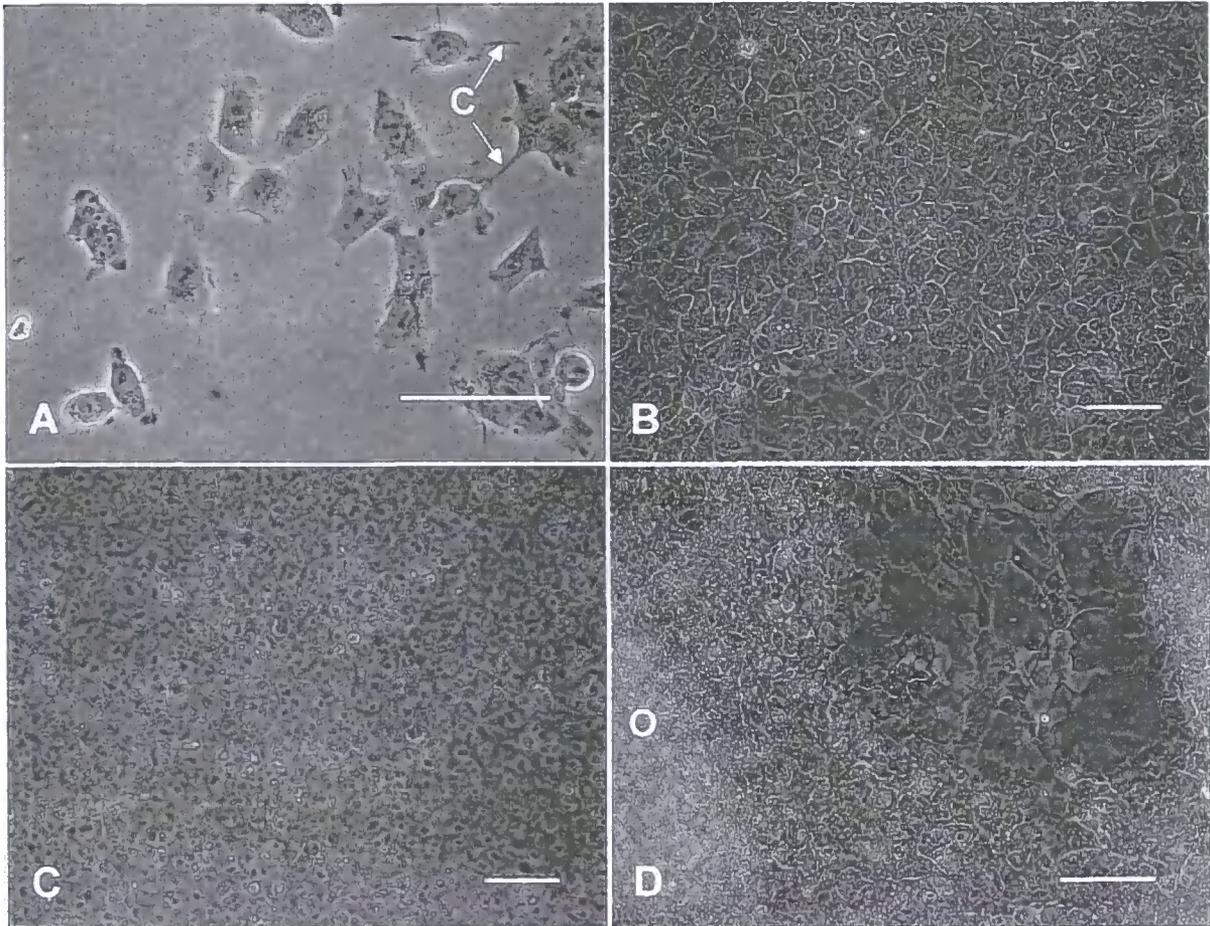


Figure 3-3: Control cultures: all of the control cultures failed to differentiate, no discernable morphological changes were observed during typical culture periods. All scale bars represent 50 µm.

A: TERA2.cl.SP12 control on day 1. Freshly plated cells divide rapidly and extend cytoplasmic extensions (C) towards adjacent cells.

B: TERA2.cl.SP12 control on day 5. Culture is confluent, cells exhibit typical high nuclear to cytosol ratio.

C: DMSO culture after 9 days. Cells are tightly packed, at this density cells are stressed and become sub-optimal - they may undergo spontaneous differentiation.

D: DMSO culture after 28 days. Dense culture, with some heterogeneity of cell types and lots of overgrowing cells (O). The top cell layers are lost into the media.

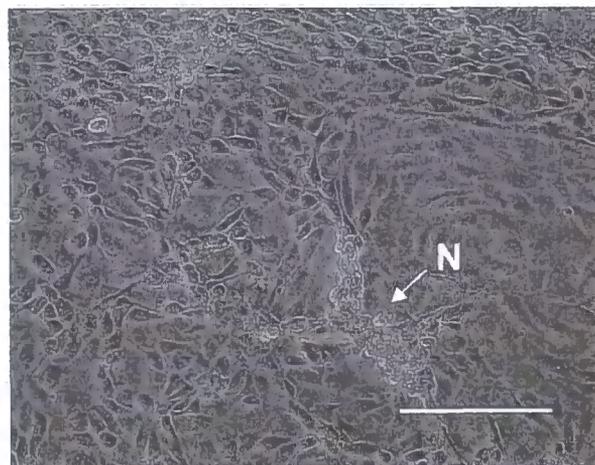


Figure 3-4: DMSO culture after 57 days showing putative neuronal colony (N). Culture was harvested for flow cytometric analysis at 28 days, and then replated and maintained for further 29 days. Scale bar = 50 µm.

ATRA, 9-cis-retinoic acid and 13-cis-retinoic acid cultures

Cultures grown in the presence of 10 μ M ATRA, 9-*cis*-RA or 13-*cis*-RA, the three natural retinoids, were highly comparable (Figure 3-5). As previously observed in TERA2.cl.SP12 cells induced to differentiate with ATRA, heterogeneous cultures develop, rosette-like aggregations of cells are visible (Figure 3-5A), and at about 2 weeks' maturity young neurons can be identified by their dense rounded cell bodies and extending processes (Figure 3-5B).⁴²⁶ These neurons migrate and aggregate to form perikarya connected by extensive neurites that coalesce to form nerve bundles, reaching a maximal length of 150 μ m in length after 28 days. These neuronal cells grow over a background of non-neural cell morphologies and some epithelial colonies are also observed. Cultures were less confluent and significantly more mature than those observed in response to CEB compounds (Figure 3-6).

CEB16, CEB17 and CEB18 cultures

The cultures grown with 10 μ M CEB16, CEB17 and CEB18 initially developed similarly to each other, becoming heterogeneous within a few days (Figure 3-6). The cultures reached confluency in about 6 days, were reproducibly composed of multiple cell layers, and had high media demands relative to ATRA and its isomers. The cultures matured differentially, with CEB18 effecting the development of highly neural cultures, whilst CEB17 and CEB16 in particular, possessed less morphologically identifiable neural cell types. All these synthetic retinoids, but especially CEB16, caused the TERA2.cl.SP12 cells to develop into significant numbers of large flat epithelial colonies (Figure 3-6B). Undifferentiated EC cell colonies were also observed in CEB16, which appear to be resistant to the retinoid (Figure 3-6B).

Am580 and TTNPB

These two commercially available synthetic retinoids are also highly effective at inducing phenotypic neural differentiation at the screening concentration of 10 μ M. The rate of apparent neurogenesis appears comparable to that of ATRA and its isomers, but both synthetic retinoids seem to enable a slightly greater level of maturity in the neural networks observed after 28 days' culture. (Figure 3-7)

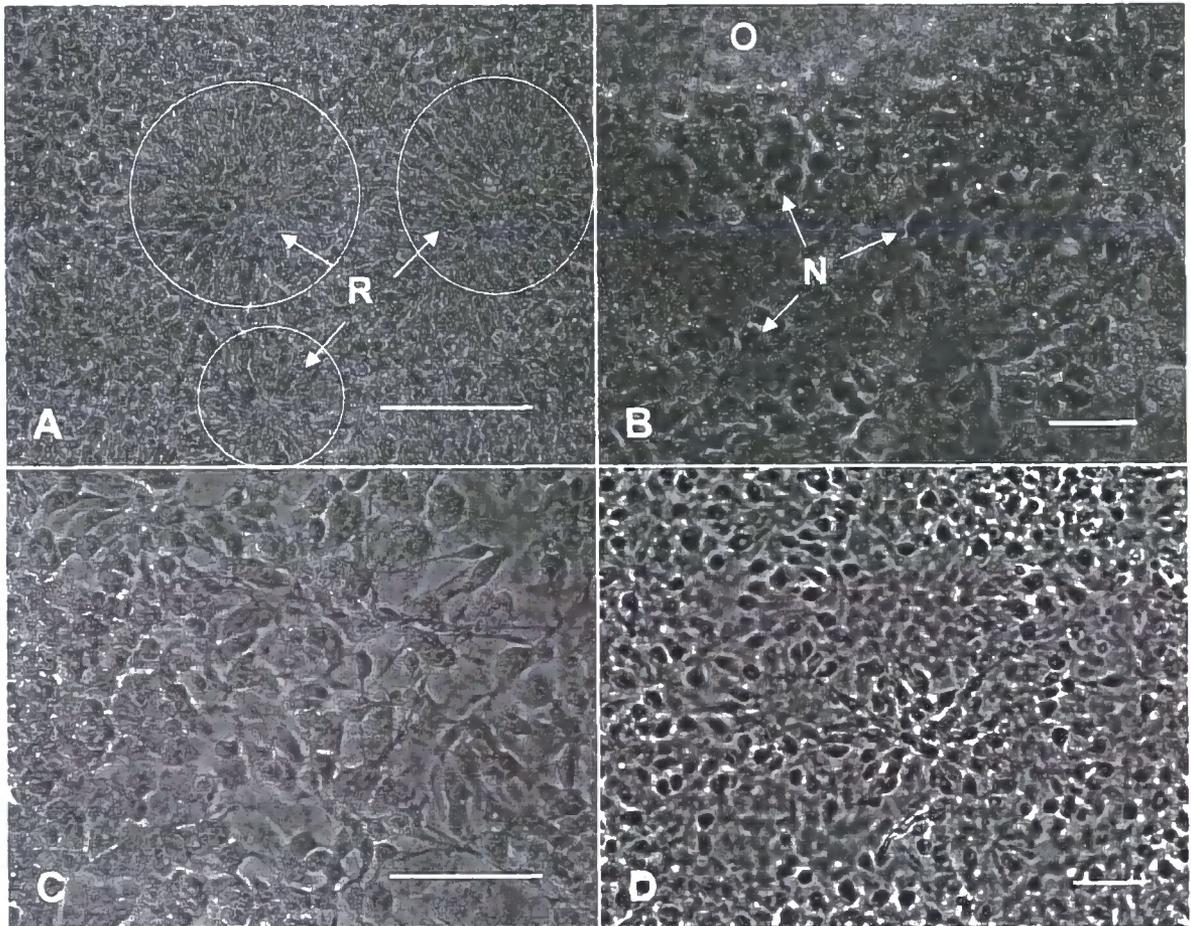


Figure 3-5: TERA2.cl.SP12 cells differentiated with 10 μ M ATRA, 9-*cis*-RA or 13-*cis*-RA for 28 days. Scale bars represent 50 μ m.

A: 9-*cis*-RA RA culture after 13 days. Large numbers of rosettes (**R**) are present. These features are rich in nestin (see immunocytochemical studies), a marker of neural progenitors.

B: 20 day-old 13-*cis*-RA culture. Significant numbers of neurons present (**N**), which are starting to migrate together. Overgrowing area of apparently un-response cells (**O**) also visible.

C: 13-*cis*-RA culture after 28 days' culture showing large numbers of interconnecting cells with neuronal phenotype.

D: The morphology of the ATRA-treated cells after 28 days is highly neural and mature.

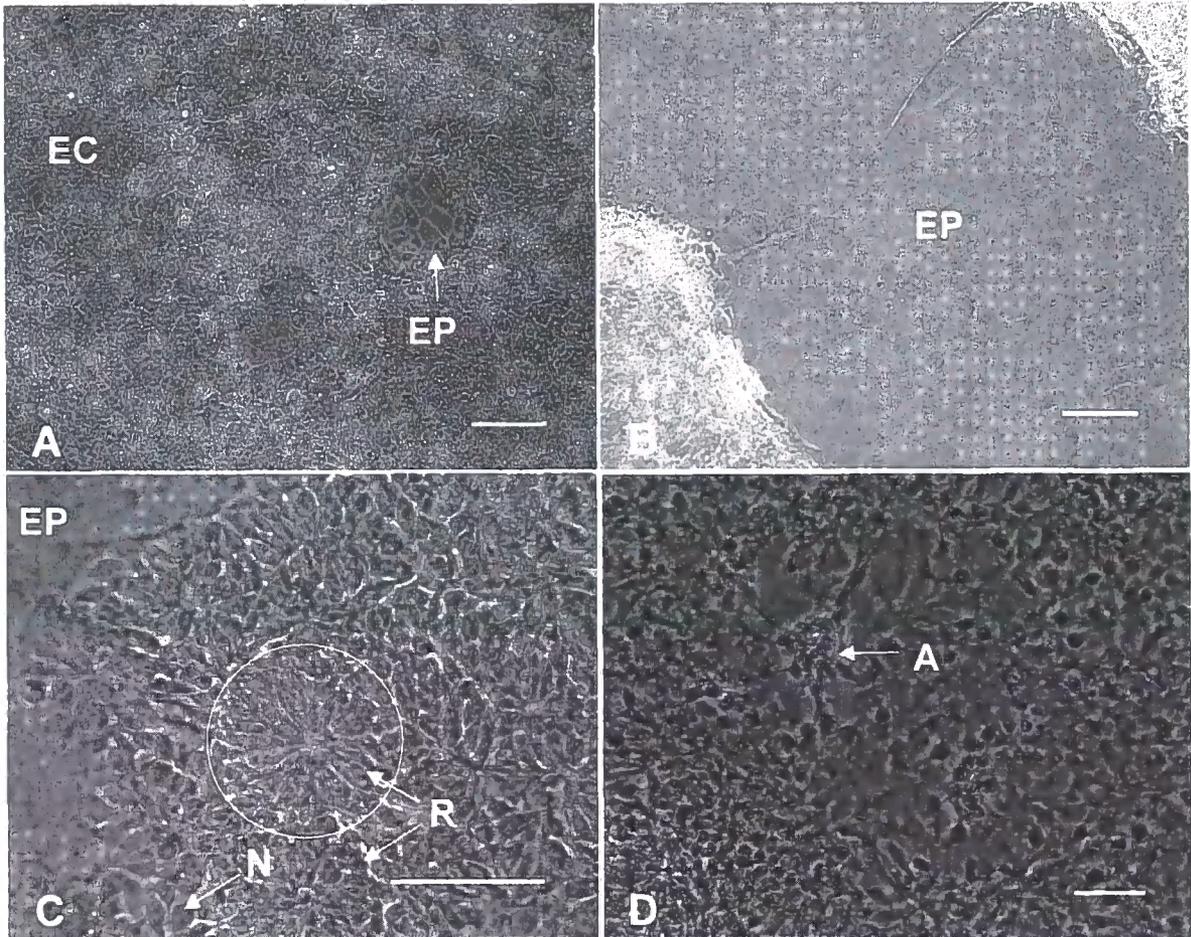


Figure 3-6: TERA2.cl.SP12 cells differentiated with 10 μ M CEB16, CEB17 or CEB18 for 28 days. Scale bars represent 50 μ m.

A: CEB17 after 9 days' culture. Heterogenous, multi-layered culture. Majority of cells retain EC morphology (EC), a small epithelial colony is also visible (EP).

B: CEB16 after 28 days' culture. A large epithelial colony (EP) composed of a single layer of large flat cells surrounded by areas of overgrowing cells.

C: CEB16 on day 28. Neural rosettes (R), a few individual neurons (N) and an epithelial colony (EP, out of focus due to different focal depth) are visible: culture appears to be at an earlier stage of neurogenesis than observed with other retinoids at this time point.

D: CEB18 after 28 days (fixed with MAA for ICC and stained with TUJ-1, a neuron-specific antibody, see Section 3.6). TUJ-1 staining emphasised a large neuronal aggregate (A) amongst numerous individual neurons.

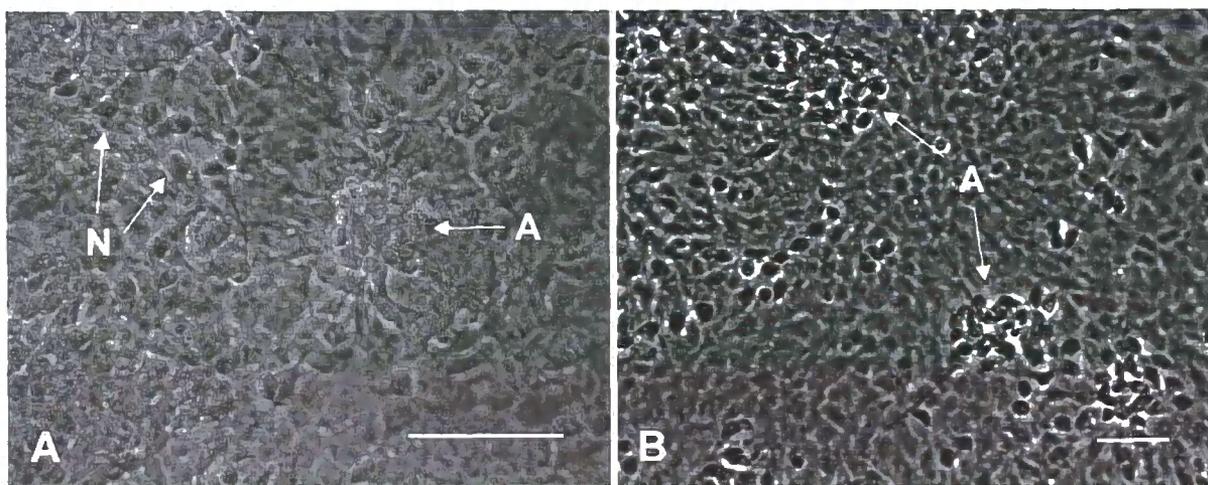


Figure 3-7: TERA2.cl.SP12 cells treated with 10 μM Am580 and TTNPB for 28 days. Scale bars represent 50 μm .

A: TTNPB culture after 28 days. Apparent large neuronal aggregate (A) with extensive neurites and large numbers of individual neuron-like cells (N) are visible.

B: Am580 after 28 days' culture. This culture had been stained with TUJ-1, highlighting two large neuronal aggregates (A) connected to an extensive network of nerve bundles (Section 3.6). Neuronal soma are tight, and extensions are normally bipolar indicating well formed neurons.

3.4 Flow cytometry: quantitative determination of cell fate

Flow cytometry was chosen as the technique to confirm morphological observations that showed TERA2.cl.SP12 cells either undergoing differentiation or not responding to test compounds. This method also provided the first characterisation of differentiation by using phenotype specific antibodies to provide a quantitative comparison of the effects induced by the different treatments. Primary monoclonal antibodies specific to the antigens associated with globoseries glycolipids and glycoproteins of pluripotent stem cells (SSEA-3 and TRA-1-60)^{478,480} and neural cell types (VIN-IS-53 and A2B5)^{459,482} were used in indirect fluorescence. TERA2.cl.SP12 cells were analysed before treatment and after 7, 14 and 28 days' treatment with 10 μ M retinoids.

Cells were incubated with one of the primary antibodies and then with a fluorophore-conjugated secondary antibody. The percentage of single, live and fluorescent cells was measured by flow cytometry. A minimum of three independent batches of TERA2.cl.SP12 cells were treated with 10 μ M CEB16, CEB17, CEB18, ATRA, 9-*cis*-RA, 13-*cis*-RA and LG100268 for up to 28 days. DMSO (0.1% v/v, vehicle), 5mol% acetone (a potential synthetic impurity) and normal media were also used to maintain cells for this period. Fresh retinoids were added at every media change, which was performed initially after three/four days and then every one/two days as required. The percentages of live cells positive for each antigen were averaged and standard deviations calculated. Statistical analysis was performed on arcsin transformed data. Analysis of variance (ANOVA) was used to compare the effects of the different retinoid treatments on antigen expression, with Bonferroni correction post-hoc analysis. Significance levels were set at: * P -value < 0.05, ** P -value < 0.01 and *** P -value < 0.001.

One batch of cells was tested with 10 μ M Am580 or TTNPB and tested after 7 and 14 days' culture, to confirm morphological observations. A further batch of cells was tested with the BPEB, two tolan, DMF and ethanol after 7 and 14 days' growth. The two tolan compounds were soluble in DMSO and were applied to the cells at a final concentration of 10 μ M. The BPEB compound was poorly soluble in both DMSO and DMF, and insoluble in a number of other common vehicles (ethanol, glycerol, PBS + 0.1% BSA and 4 mM HCl + 0.1% BSA). DMF and DMSO solutions of BPEB were tested, at \sim 3 μ M and \sim 5 μ M, respectively.

3.4.1 Results

Retinoid compounds from Table 3-1

Seven day flow cytometric data of cultures grown with retinoids confirm the initiation of differentiation (Figure 3-8 and Figure 3-9). The pluripotency markers SSEA-3 and TRA-1-60 are down-regulated relative to undifferentiated EC cells (day 0), whilst markers of neural phenotypes are concomitantly up-regulated (VIN-IS-53 and A2B5). The control cultures and LG100268 culture show no significant differences in any of the antigens from the seven day control culture (2nd group of bars, TERA2.cl.SP12 cells grown in normal media). These cells have grown beyond confluency, resulting in sub-optimal culture conditions as revealed in the down-regulation of SSEA-3 ($P < 0.001$ for all 7 day cultures), the most sensitive stem cell marker tested. This may be due to spontaneous differentiation.

The different efficacies of the retinoid treatments are highlighted by comparing the results for TRA-1-60 (Figure 3-9, **B** and **D**). Significant down-regulation is observed in all retinoid conditions compared to the DMSO control. ATRA, 9-*cis*-RA, 13-*cis*-RA and CEB18 show the greatest down-regulation ($P < 0.001$), and CEB16 and CEB17 show a significant, but smaller difference ($P < 0.05$). The TRA-1-60 results for CEB16 and CEB17 are also significantly different to the expression of TRA-1-60 in the ATRA culture ($P < 0.01$), confirming the slower rate of differentiation observed morphologically. A significant difference between ATRA and CEB16 is also observed in the A2B5 results ($P < 0.01$, Figure 3-9 **C**) consistent with a slower exit of the cell cycle.⁴²⁷ No statistically significant differences in the expression of VIN-IS-53 were found between any of the 7 day old cultures.

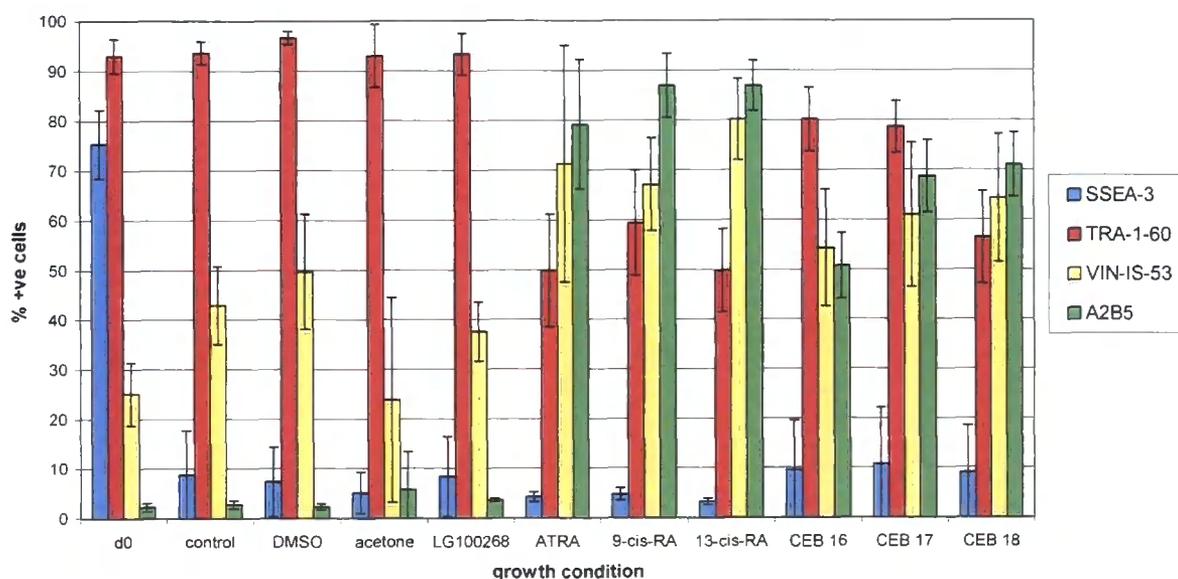


Figure 3-8: Expression of cell surface antigens in TERA2.cl.SP12 huEC cells after treatment with 10 μ M retinoid or control conditions for 7 days (mean percent positive cells of ≥ 3 batches ± 1 SD).

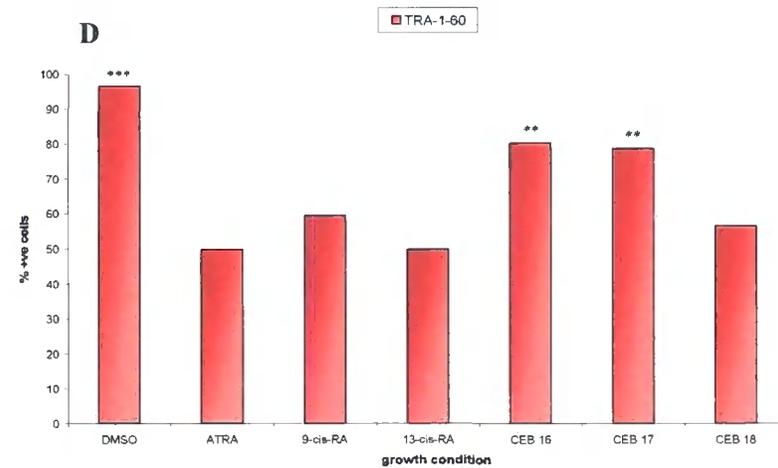
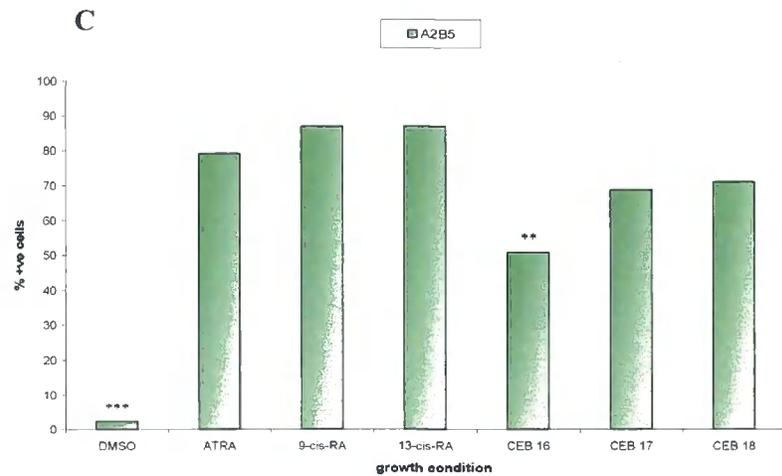
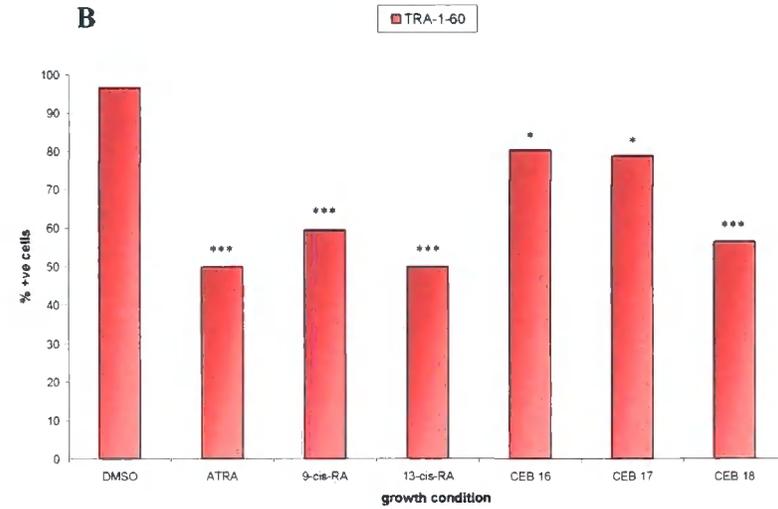
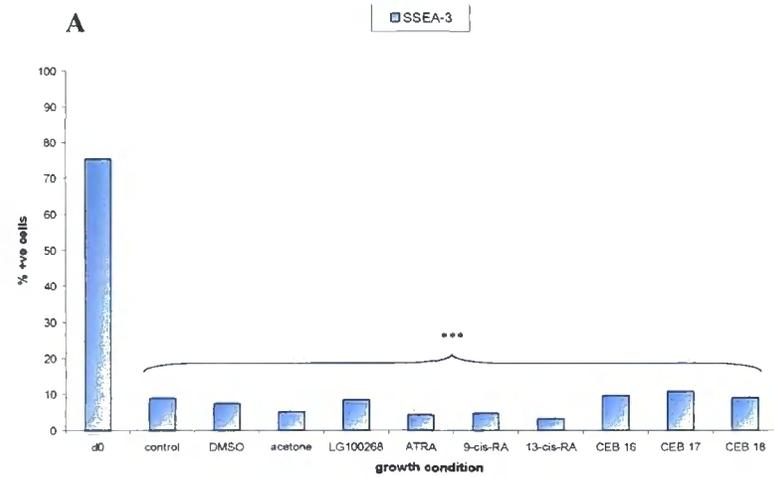


Figure 3-9: Mean percent positive cell expression of SSEA-3 (A), TRA-1-60 (B and D) and A2B5 (C) in TERA2.cl.SP12 huEC cells after treatment with 10 μ M retinoid or control conditions for 7 days ($n \geq 3$). Significant differences from d0 (A, SSEA-3), DMSO (B, TRA-1-60) and ATRA (C, A2B5 and D, TRA-1-60) are presented. Statistical significance between the growth conditions is denoted by * (P -value < 0.05), ** (P -value < 0.01) and *** (P -value < 0.001).

14 day data show the continued commitment towards neural cell types (Figure 3-10). All retinoid cultures (excluding LG100268) have suppressed both stem cell markers completely, and strongly express VIN-IS-53 and A2B5. The distinction between differentiating cultures and non-differentiating cultures is very clear. There are no significant differences in antigen expression between the differentiating cultures. There are significant differences between the control/LG100268 cultures and the other retinoid cultures in the expression of TRA-1-60 ($P < 0.001$), VIN-IS-53 ($P < 0.05$) and A2B5 ($P < 0.05$).

After 28 days' culture, the antigen expression pattern has changed again (Figure 3-11). The control, DMSO, acetone and LG100268 data show some evidence of differentiation. In differentiated cultures, the levels of VIN-IS-53 are maintained, but A2B5 is starting to be down-regulated. A2B5 is characteristic of neural progenitor cells and is not acquired permanently.⁵⁰³

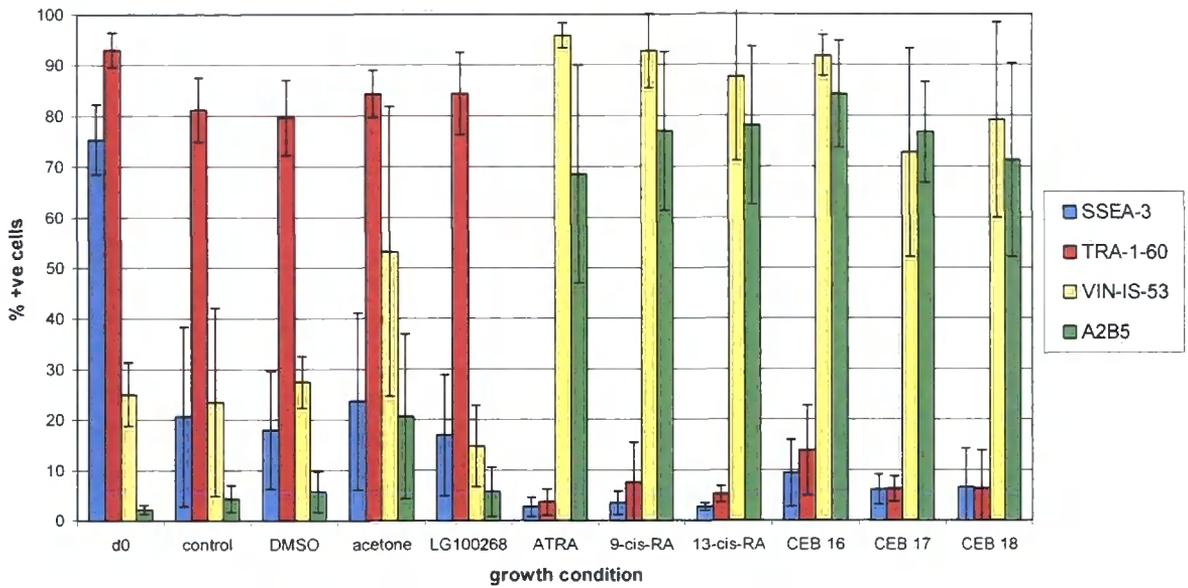


Figure 3-10: Expression of cell surface antigens in TERA2.cl.SP12 huEC cells after treatment with 10 μ M retinoid or control conditions for 14 days (mean percent positive cells of ≥ 3 batches ± 1 SD).

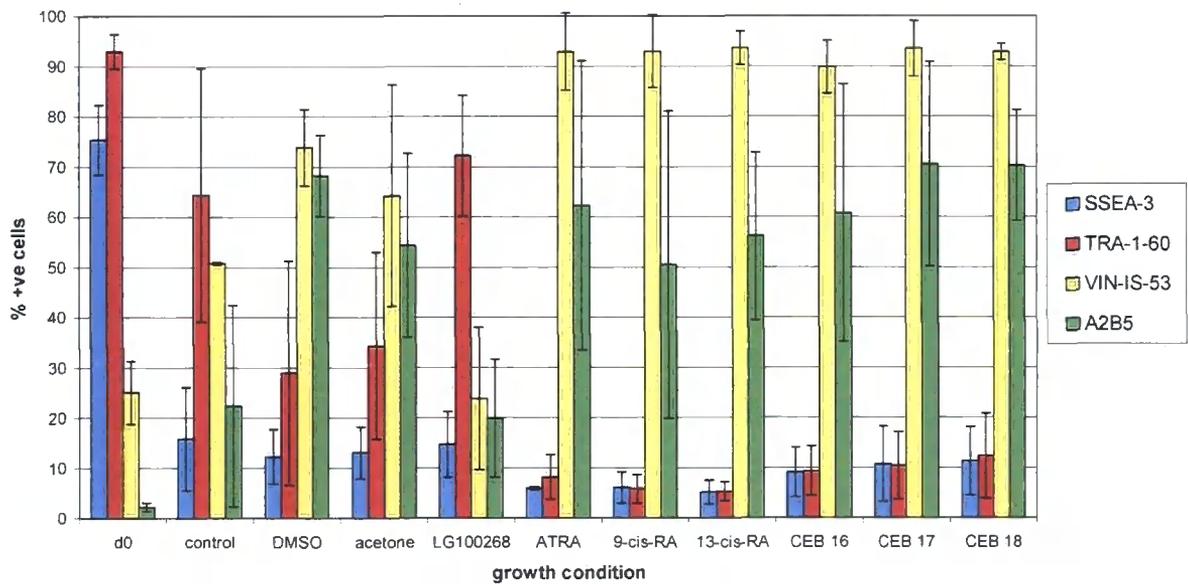


Figure 3-11: Expression of cell surface antigens in TERA2.cl.SP12 huEC cells after treatment with 10 μ M retinoid or control conditions for 28 days (mean percent positive cells of 3 batches (2 for “control”) ± 1 SD).

Compounds from Table 3-2

Flow cytometric analysis clearly confirms that the BPEB and tolans do not induce differentiation and that DMF and ethanol induce only the same phenotypic changes seen in similarly maintained control cultures. By contrast, 10 μ M Am580 and TTNPB appear to be as effective as ATRA at inducing neural differentiation (Figure 3-12 and Figure 3-13).

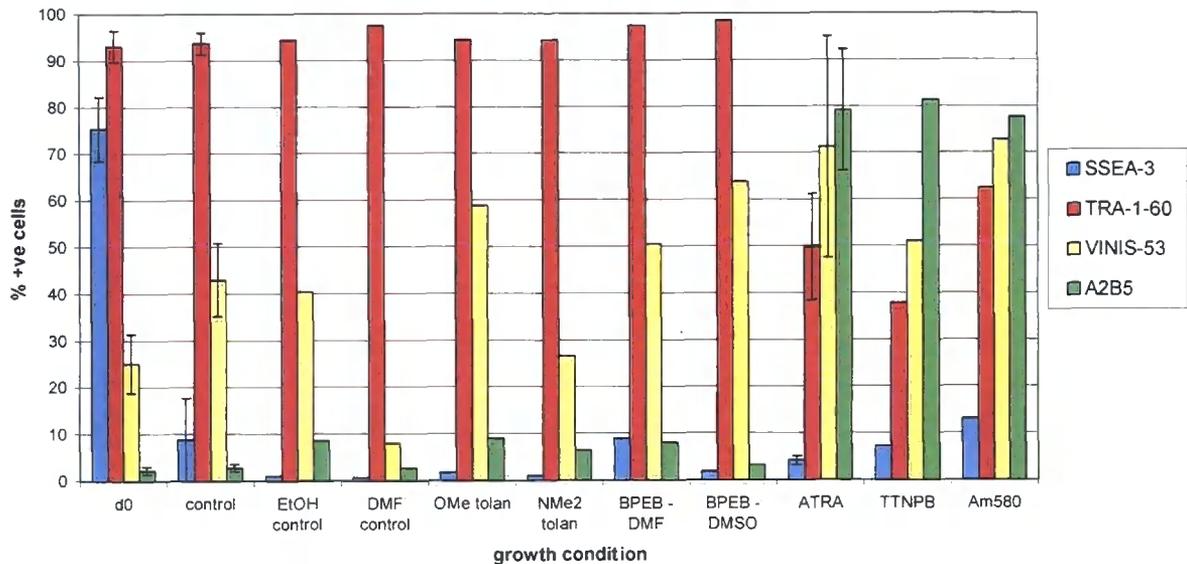


Figure 3-12: Expression of cell surface antigens in TERA2.cl.SP12 huEC cells after treatment with 10 μ M test compound or control conditions for 7 days (n=1, except where error bars are shown when n=3, \pm 1SD).

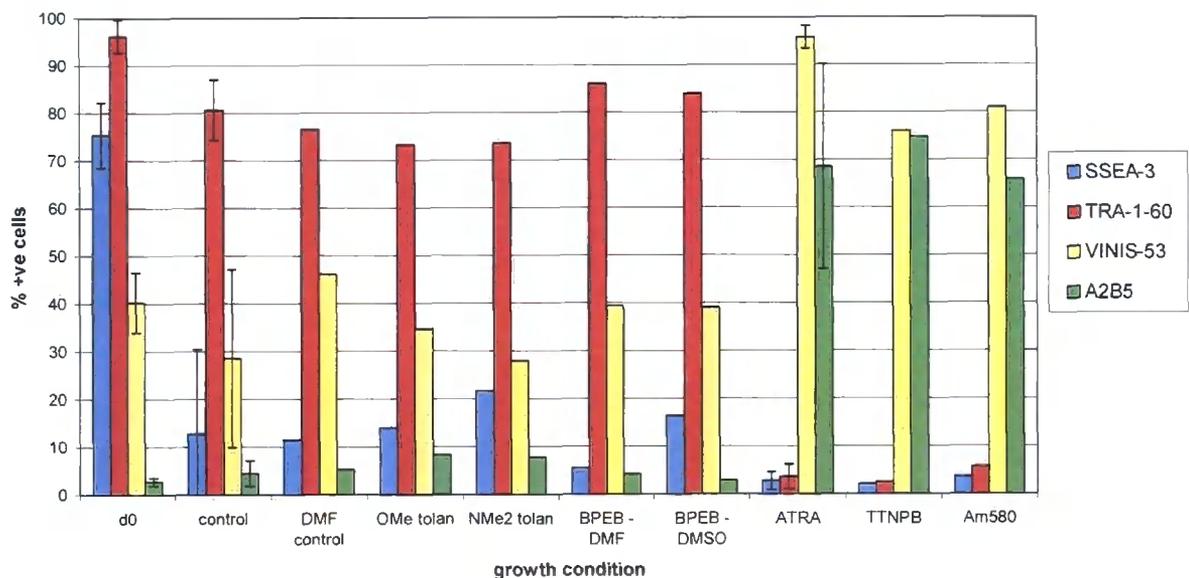


Figure 3-13: Expression of cell surface antigens in TERA2.cl.SP12 huEC cells after treatment with 10 μ M test compound or control conditions for 14 days (n=1, except where error bars are shown when n=3, \pm 1SD).

3.5 Proliferation

Flow cytometric analysis and morphological observations of the differentiating cultures show that the different retinoids initiate differentiation at varying rates. ATRA and its isomers induce a faster response than CEB18, and CEB16/CEB17 were the least efficacious. The initial response of TERA2.cl.SP12 cells (first seven days) to retinoid exposure is observed as a reduction in cell proliferation and the appearance of non-EC cell morphologies in characteristically heterogeneous cultures. This apparent delayed exit of the cell cycle results in significantly more confluent cultures and higher media demands for the CEB compounds.

In order to quantify this observation, a tetrazolium-based proliferation assay was used to measure the number of viable cells in cultures exposed to 10 μ M of each retinoid over 14 days. Metabolism in proliferating cells produces reducing equivalents such as NADH or NADPH that reduce MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) from a yellow salt to a blue formazan product. The amount of formazan produced during 4 hours' incubation with cultures was measured by reading the UV absorbance of the solution at 490 nm, and is proportional to the number of viable cells in the culture.

3.5.1 Results

A total of four independent batches of cells were analysed independently after 0, 3, 7, 10 and 14 days' culture. The UV absorbencies of each growth condition were averaged and used to generate growth curves (Figure 3-14). The number of viable cells present in the control (normal media) and DMSO (vehicle) cultures were similar and at time points after three days were substantially greater than any of the cultures treated with retinoid. The retinoid cultures can be clearly divided into two different groups with appreciably different numbers of cells: the natural retinoid cultures were less proliferative than those grown with synthetic retinoids. There were an average of 18%, 60% and 39% more cells in the synthetic cultures, compared to those treated with natural retinoids, after 3, 7 and 10 days' culture respectively. All batches showed the same trend in results, but differences in proliferation between batches (biological variation) reduced the average magnitude of difference between compounds and produced large standard deviations (Figure 3-15). Consequently, no statistically significant differences were observed between the retinoid compounds at any time point.

Comparing the relative number of cells in the retinoid cultures to the control cultures further highlighted differences between the two groups of compounds (Figure 3-15). After three days, CEB16, CEB17 and CEB18 cultures all contained more cells than the controls, while 9-*cis*-RA, 13-*cis*-RA and ATRA cultures had slightly suppressed proliferative capacity. Later time points

demonstrate a large reduction in cell number of all retinoid cultures. The 7 and 10 day data provide the best comparison between retinoid groups.

At the 10 and 14 day time points, large number of overgrowing cells were present in the cultures that were easily dislodged during media changes and in the PBS wash prior to addition of MTS reagent. The data for these time points consequently show greater variation, and are more difficult to compare. However, the growth curves of all cultures appear to be in a stationary phase of cell growth by the end of the experiment (Figure 3-14).

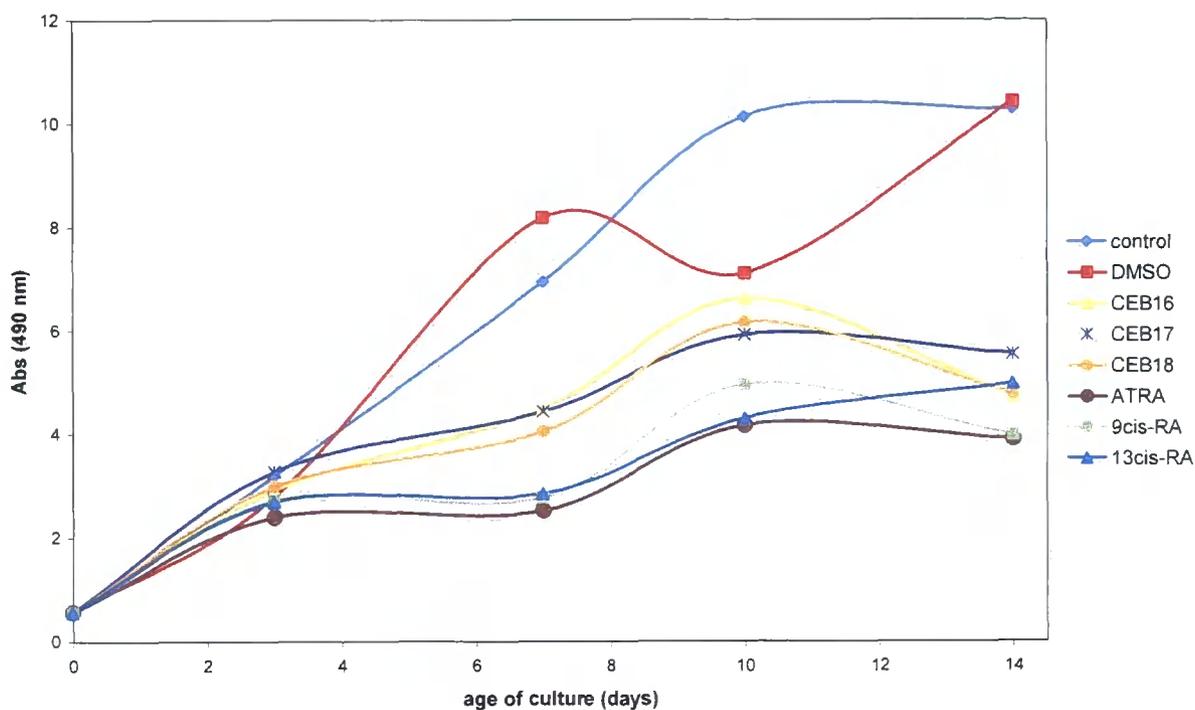


Figure 3-14: TERA2.cl.SP12 huEC growth curves, derived from MTS viability assay data, after continuous treatment with 10 μ M retinoid or control conditions ($n \geq 3$). Error bars have been omitted from this figure for clarity; the magnitude of the standard deviations can be observed in Figure 3-15.

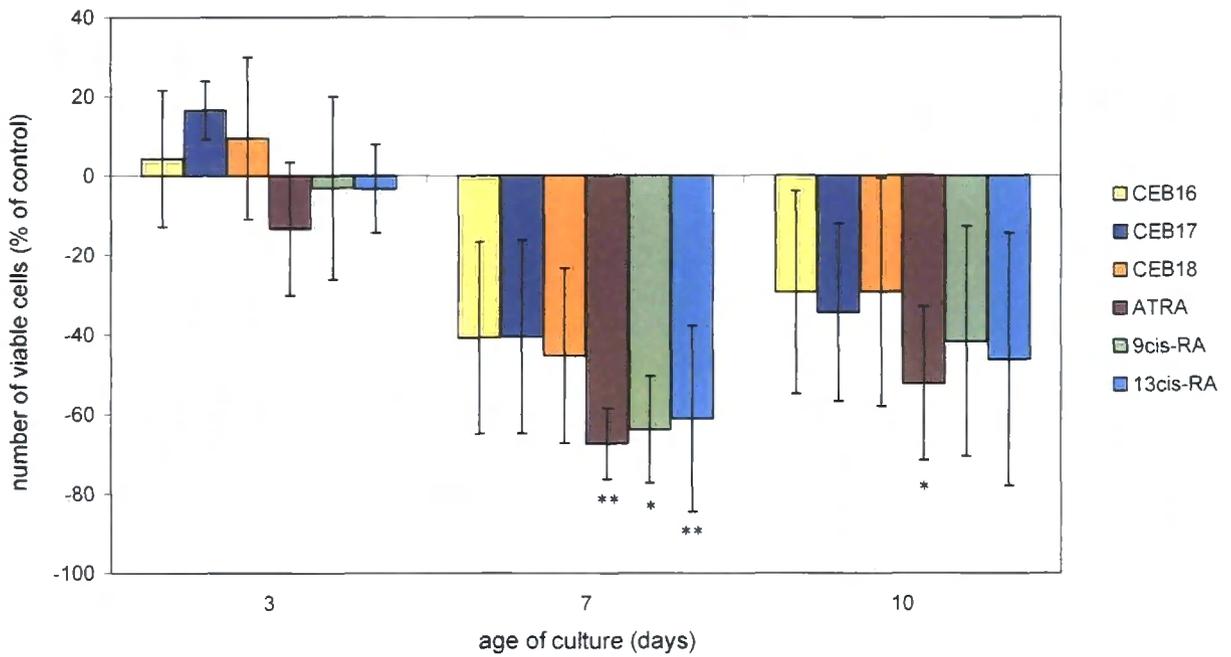


Figure 3-15: Relative number of viable TERA2.cl.SP12 huEC cells produced after treatment with 10 μ M retinoid for 3, 7 and 10 days. Data are presented as \pm percent difference of the retinoid culture from DMSO control (3 and 7 days) or control culture (10 day data), using the mean absorbance values for ≥ 3 batches of independent cells, ± 1 SD. Statistical significance between the relevant control and the growth conditions indicated is denoted by * (P -value < 0.05), ** (P -value < 0.01) and *** (P -value < 0.001).

3.6 Immunocytochemical (ICC) localisation of antibodies

TERA2.cl.SP12 cells were differentiated on cover-slips using the retinoids listed in Table 3-1. After 14 and 28 days, the resulting cultures were fixed and a range of phenotypically defined antibodies was used to localise cellular protein markers (Table 3-3). Phase images were compared with the fluorescent image(s) in same field of view; stained features were visualised through an appropriately coloured filter on a fluorescence microscope. Co-staining with different antibodies raised in different species permitted the use of species-specific secondary antibodies conjugated to different fluorophores. Fluorescent images from co-stained cultures were overlaid where appropriate to examine for interactions between different cell types. All cover-slips were mounted in a medium containing Hoechst to indiscriminately counterstain all nuclei blue.

The presence and patterns of expression of each antibody were examined in each retinoid culture, and compared to the other conditions.

Table 3-3: Primary antibodies used in ICC staining.

Antibody: anti-	Information
nestin	Visualises putative neural stem/progenitor cells.
β -III-tubulin (clone TUJ-1)	Neuron-specific tubulin, stains the nuclei and neurites of developing neurons.
cytokeratin 8 (C8)	Marker of epithelial cytoplasm.
fibronectin	This antibody recognises an extracellular matrix protein associated with epidermal layer development.
neurofilament-68 (NF68)	A low MW intermediate filament expressed in young neurons.
neurofilament-200 (NF200)	A high MW intermediate filament expressed in mature neurons.
neuron specific enolase (NSE)	Detected only in mature neurons.
microtubule associated protein-2 (MAP-2)	Reacts with MAP-2a, MAP-2b and MAP-2c. Present in mature neuronal cytoskeleton.
glial fibrillary associated protein (GFAP)	An intermediate filament uniquely expressed in astrocytes.

3.6.1 Results

A total of nine antibodies for a range of neural and non-neural cell types were used on cultures grown with eight different retinoids. Representative fluorescent and corresponding phase micrographs were recorded; a selection of images is presented in Figures 3-16 to 3-30, and notable features are described in the figure legends. TERA2.cl.SP12 cells were also grown in normal media to confluency and stained to provide basal expression information (Figure 3-17). Antibody controls were also prepared alongside ordinary staining to confirm the absence of non-specific binding (Figure 3-17).

Four antibodies were used to stain all retinoid cultures: β -III-tubulin (clone TUJ-1), nestin, C8 and NSE, and six of the 14 days old retinoid cultures were stained with fibronectin. The cover-slips were examined carefully and a qualitative assessment of the relative intensity and degree of phenotype maturity was made, these data are summarised in Table 3-4.

Nestin expression was present in all cultures at all time points, although at varying levels. The most intense expression was seen on the 14 day cover-slips, distributed throughout the cultures, but especially radiating out from rosettes (for example Figure 3-18, 9-*cis*-RA and Figure 3-19, CEB18). By 28 days, the amount and intensity of nestin staining was significantly decreased and it was typically isolated to proliferative centres (for example 13-*cis*-RA in Figure 3-24 and TTNPB in Figure 3-26). The exception to this observation was the CEB16 cover-slip: nestin expression was still intense, and distributed throughout the culture indicating an earlier stage of neurogenesis (Figure 3-25). Nestin has also been identified in a range of non-neural cells and has more recently been proposed as a common marker of multi-lineage progenitor cells capable of forming derivatives of all three germ layers.⁵⁰⁴ Where nestin expression is co-localised with distinctive features such as rosettes, which have previously been demonstrated to develop into neural tubes,⁵⁰⁵ these cells can be confidently described as neural progenitors.

After fourteen days, the TUJ-1 antibody stained the cell bodies of young neurons and the short processes extending towards other neurons; TUJ-1 positive cells were often found in islands, but were also present throughout the culture. Less TUJ-1 staining was present in CEB16 and CEB17 compared to the other retinoid-treated cultures, consistent with the less mature morphology observed. At the 28 day time-point all cultures were highly stained with TUJ-1. The neurons had migrated and formed numerous aggregates in the upper layers of the culture over a background of non-neural cells (for example, Figure 3-25 CEB17). Long neurites form networks of connections between aggregates, coalescing into nerve bundles of up to 160 μ m in length (ATRA in Figure 3-24 and Am580 image in Figure 3-26). Again, ICC staining reveals the CEB16 culture to be significantly less mature: less neurons were present, which were distributed throughout the culture with little organisation and their

neurites are shorter and often not bi-polar (Figure 3-25). The TTNPB and Am580 cultures show some of the most advanced neuronal networks (Figure 3-26).

Table 3-4: Qualitative analysis of ICC staining.

Time-point	Retinoid	Antibody				
		nestin	TUJ-1	C8	fibronectin	NSE
14 days	ATRA	+++	++	++	++	+
	9- <i>cis</i> -RA	+++	++	+	+	+
	13- <i>cis</i> -RA	+++	++	++	++	+
	CEB16	++	+	+++	N	-
	CEB17	++	+	++	N	-
	CEB18	++	++	++	++	+
	Am580	+++	++	+++	++	+
	TTNPB	+++	++	++	++	+
	28 days	ATRA	+	+++	++	N
9- <i>cis</i> -RA		+	+++	++	N	+++
13- <i>cis</i> -RA		+	+++	++	N	+++
CEB16		++	++	+++	N	++
CEB17		+	++	+++	N	+++
CEB18		+	+++	++	N	+++
Am580		+	+++	++	N	+++
TTNPB		+	+++	++	N	+++

KEY: + positive – small amount present
 ++ significant amounts of staining
 +++ antigen expression is extensive and well-developed
 - absent
 N antibody not used

TUJ-1 cell body staining and nestin staining were absent in the colonies of flat grey cells that were highly stained with cytokeratin 8 (C8) (Figure 3-18, 9-*cis*-RA and Figure 3-25, CEB17). As cultures matured, processes extended between these epithelial cells into the colony, as seen in the ATRA culture in Figure 3-24. C8 staining was observed in all cultures at all time-points. This cytoplasmic epithelial marker was localised in several different patterns distinguishing between different cell types (illustrated in Figure 3-16, (C) and (D), and observed in Figure 3-22, CEB17). The largest epithelial

colonies were observed in cultures grown with 10 μ M CEB16 and CEB17, as previously noted (Figure 3-22 CEB16, CEB17 and CEB18 in Figure 3-28).

Fibronectin was present at a low level in all the cultures to which the antibody applied. This extra-cellular protein marker showed little apparent correlation to features observed by phase contrast microscopy. Low levels were also observed in undifferentiated TERA2.cl.SP12 cells (Figure 3-17). Undifferentiated cells also showed low levels of TUJ-1, cytokeratin 8 and nestin expression. The TUJ-1 staining was cytoplasmic and diffuse, unlike neuronal staining observed in the retinoid cultures where it is intensely expressed in only the neuronal cell body and processes. Staining was not observed in the secondary control. Low levels of nestin and C8 are present uniformly throughout the undifferentiated cultures.

An antibody against neuron specific enolase (NSE) was used to stain all cover-slips. CEB16 and CEB17 treated cells did not express this protein after 14 days' culture, but low levels of staining were observed in all other cultures at this time point (Figure 3-21, Figure 3-22 and Figure 3-23, data for CEB16 and CEB17 cultures is not shown). NSE is a marker of mature neurons, so it is to be expected that it will be expressed at a later stage in neurogenesis than TUJ-1. After 28 days' culture, NSE levels are highly up-regulated, demarcating mature perikarya and neurons (compare the staining of the natural retinoid cultures, Figure 3-24, with CEB16 in Figure 3-25). The low molecular weight neurofilament NF68 is normally expressed in young neurons, as demonstrated by its expression in CEB16 and CEB17 cultures after 14 days (Figure 3-22).

Three further antibodies were also used to stain the 28 day old cultures grown with ATRA and CEB18. The results of this staining are summarised in Table 3-5. Expression of GFAP was not detected in either of the mature cultures, consistent with previous analysis of TERA2.cl.SP12 cells grown in monolayer.⁴²⁸ NF200 and MAP2 were expressed in both cultures (Figure 3-30) further demonstrating the level of neuronal development and maturity induced by both synthetic and natural retinoids.

Table 3-5: Qualitative assessment of the results of staining ATRA and CEB18 28 day cultures with additional antibodies (key as in Table 3-4).

Time point	Retinoid	Antibody		
		GFAP	NF200	MAP-2
28 days	ATRA	-	+++	++
	CEB18	-	++	+

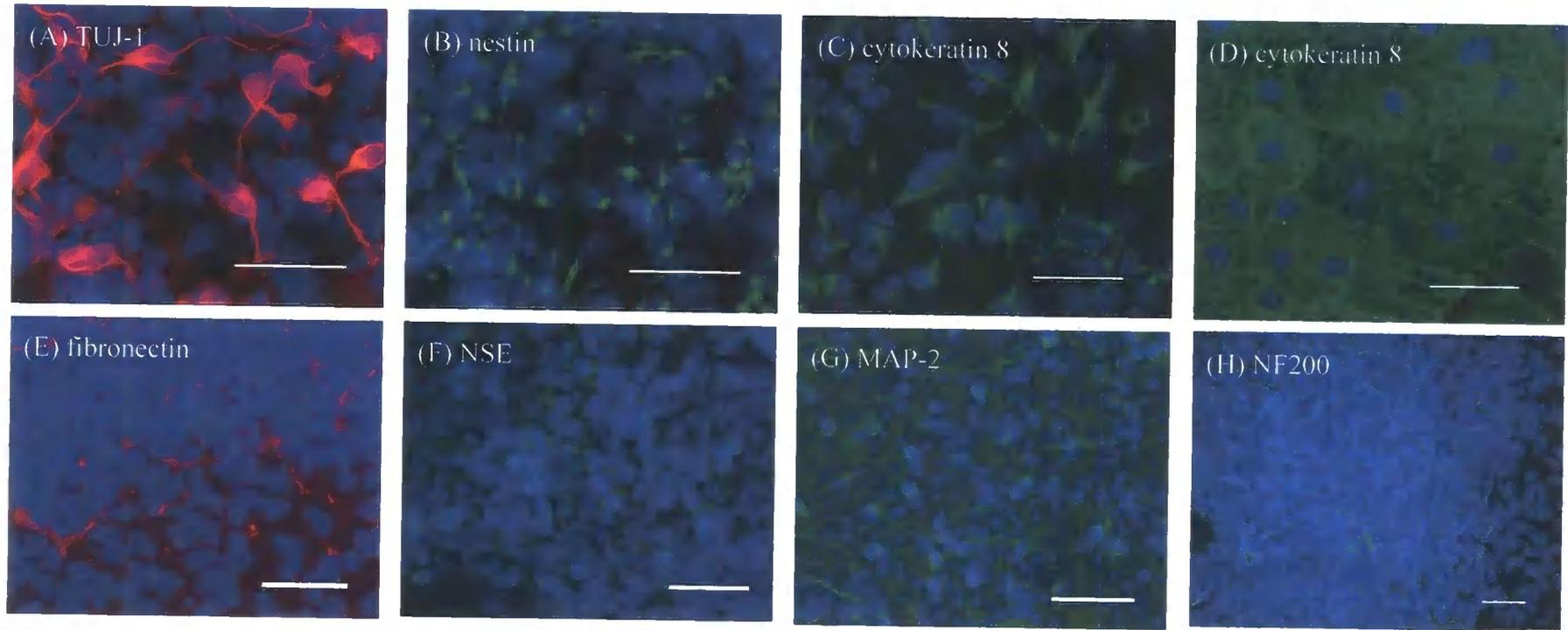


Figure 3-16: Example images of antibody staining localisation in differentiated cultures, overlaid with corresponding Hoechst image. A: 10 μ M 13-*cis*-RA, 14d; B: 10 μ M 13-*cis*-RA, 14d; C: 10 μ M 9-*cis*-RA, 28d; D: 10 μ M CEB16, 28d; E: 10 μ M 13-*cis*-RA, 14d; F: 10 μ M 13-*cis*-RA, 14d; G: 10 μ M ATRA, 28d; H: 10 μ M 9-*cis*-RA, 28d. Note the two different cell types stained by C8: C is not epithelial and D is typical of an epithelial colony. Scale bars represent 50 μ m.



Figure 3-17: Antibody control images (top row) and undifferentiated TERA2.cl.SP12 huEC phase and ICC staining. Corresponding Hoechst images are shown inset. Scale bars represent 50 μ m.

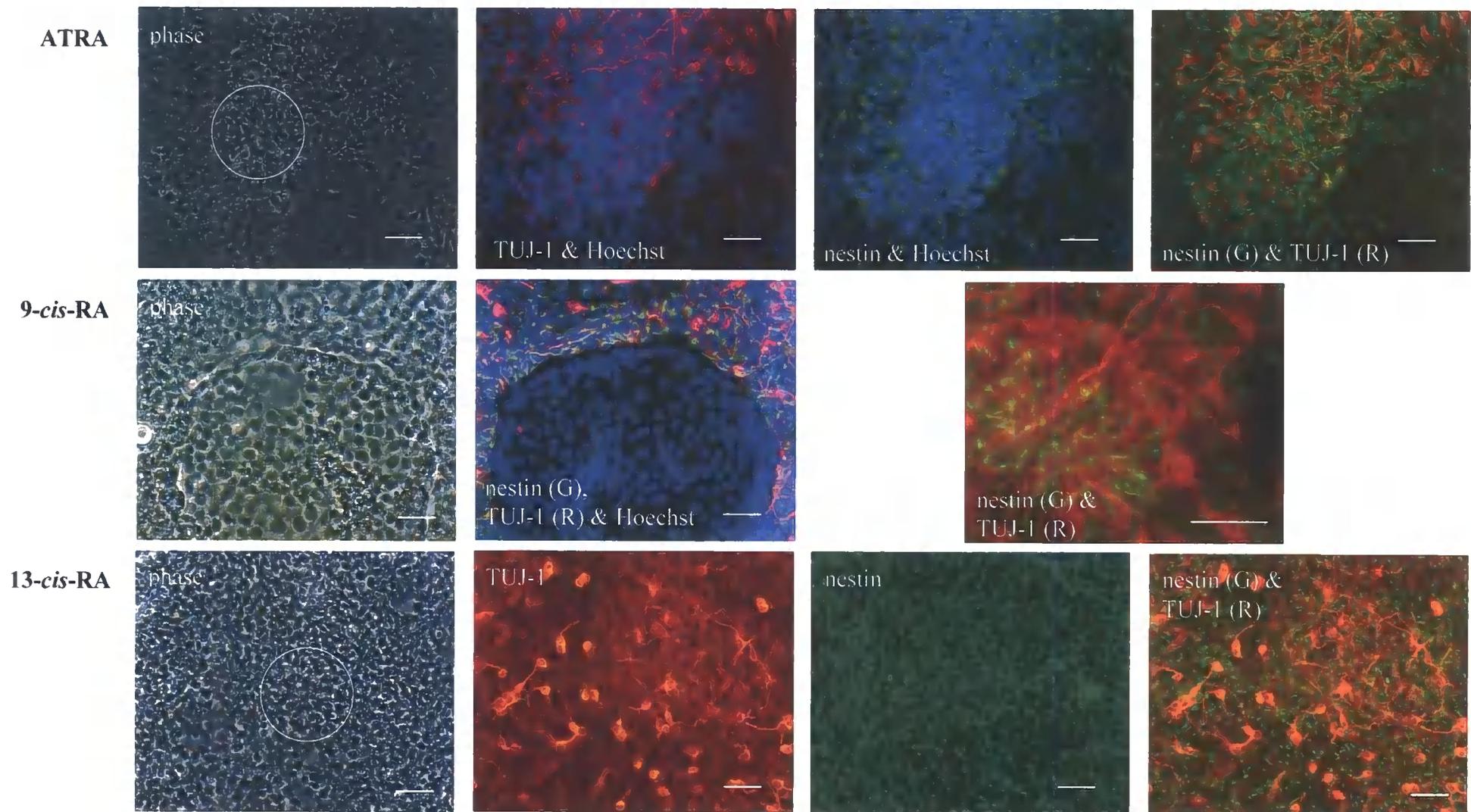


Figure 3-18: Phase and corresponding ICC images of TUJ-1 (stained red, R) and nestin (stained green, G) staining of cultures grown with 10 μ M ATRA, 9-*cis*-RA or 13-*cis*-RA for 14 days. In the ATRA images, nestin and TUJ-1 co-localise in a hyper-proliferative area, where rosettes are starting to form. An epithelial colony in 9-*cis*-RA images expresses neither TUJ-1 nor nestin. Scale bars represent 50 μ m.

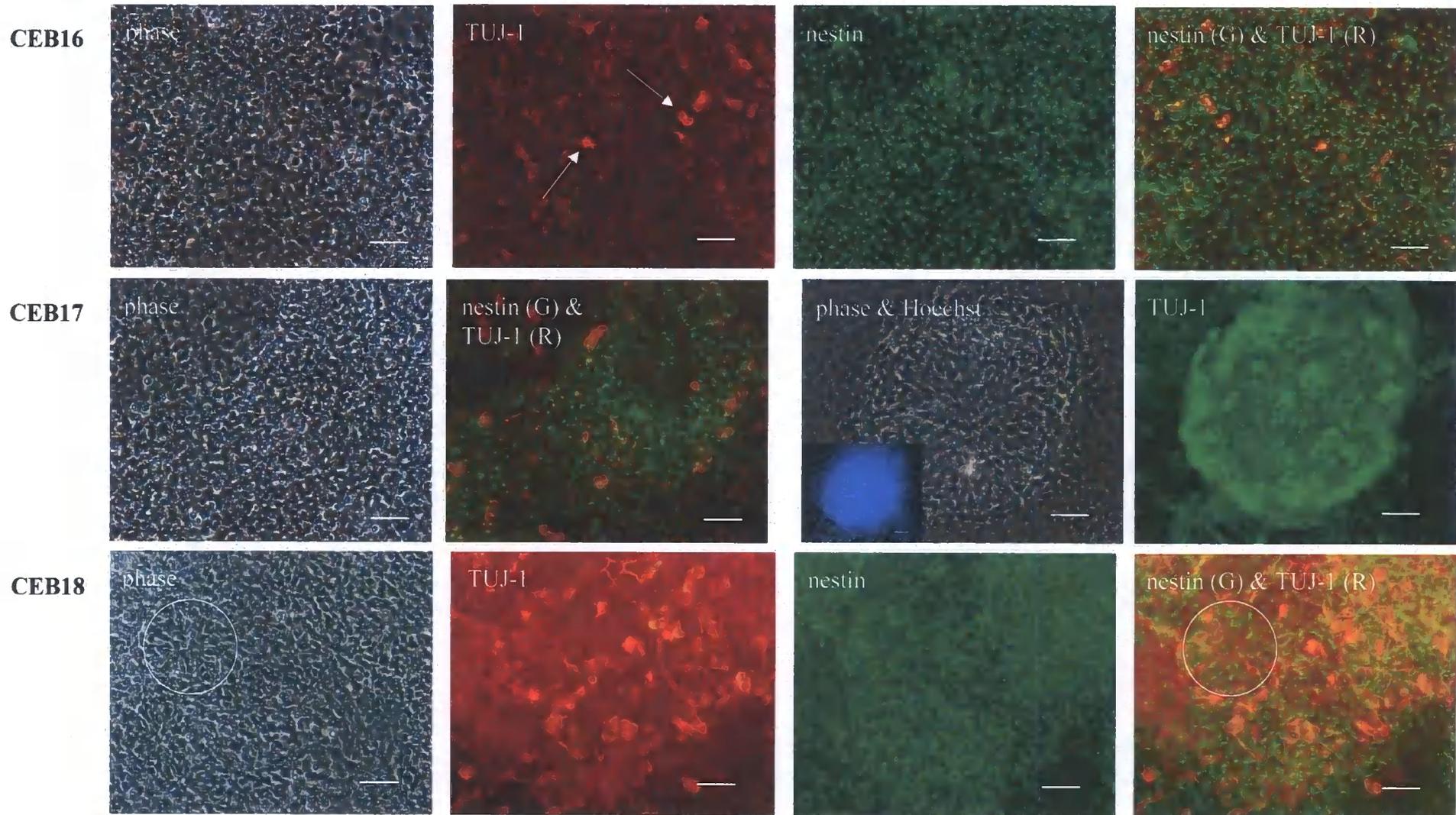


Figure 3-19: Phase and corresponding ICC images of TUJ-1 (red, R) and nestin (green, G) staining of cultures grown with 10 μ M CEB16, CEB17 or CEB18 for 14 days. Note the small number of individual, immature neurons in the CEB16 culture. A hyper-proliferative neuronal colony is shown in the second pair of CEB17 images. A neural rosette is clearly visible in the CEB18 images. Scale bars represent 50 μ m.

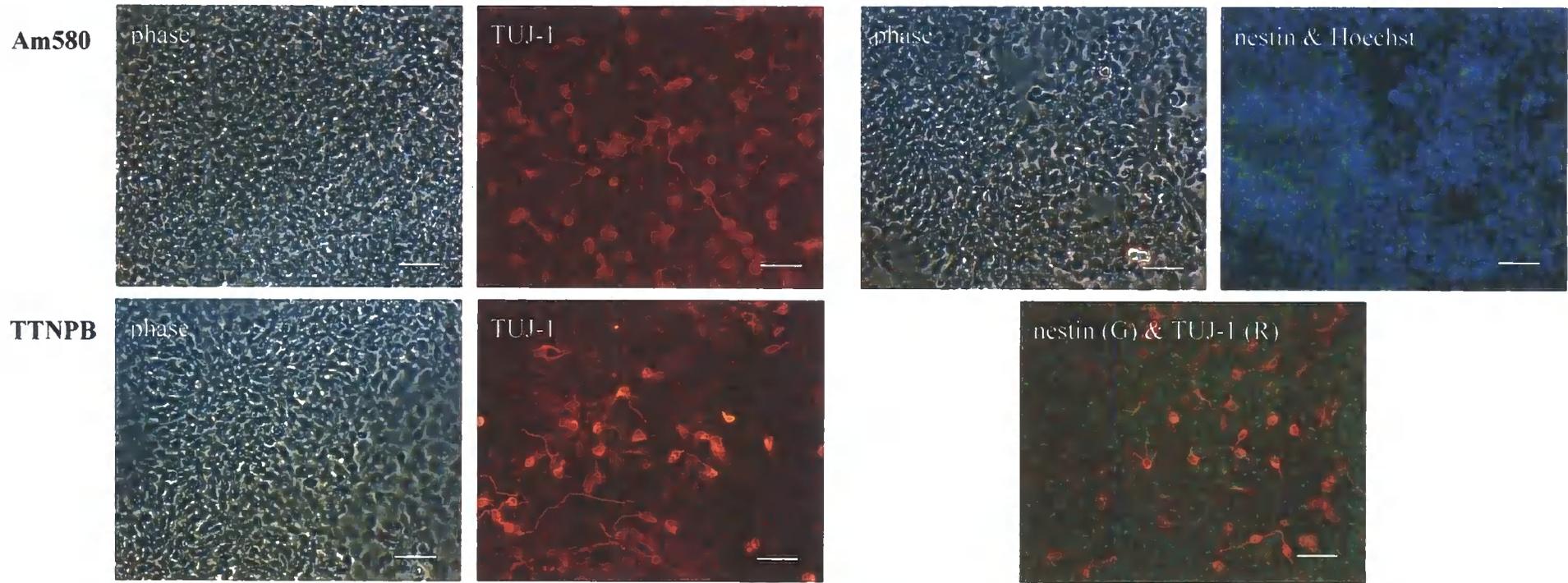


Figure 3-20: Phase and corresponding ICC images of TUJ-1 (red, R) and nestin (green G) staining of cultures grown with 10 μ M Am580 or TTNPB for 14 days. Large numbers of neurons are observed in both cultures, some neurons are extending long neurites. Scale bars represent 50 μ m.



Figure 3-21: Phase and corresponding ICC images of cultures co-stained with cytokeratin 8 (C8, green G)) and fibronectin (red, R), or NSE after 14 days' exposure to 10 μ M ATRA, 9-*cis*-RA or 13-*cis*-RA. Scale bars represent 50 μ m.

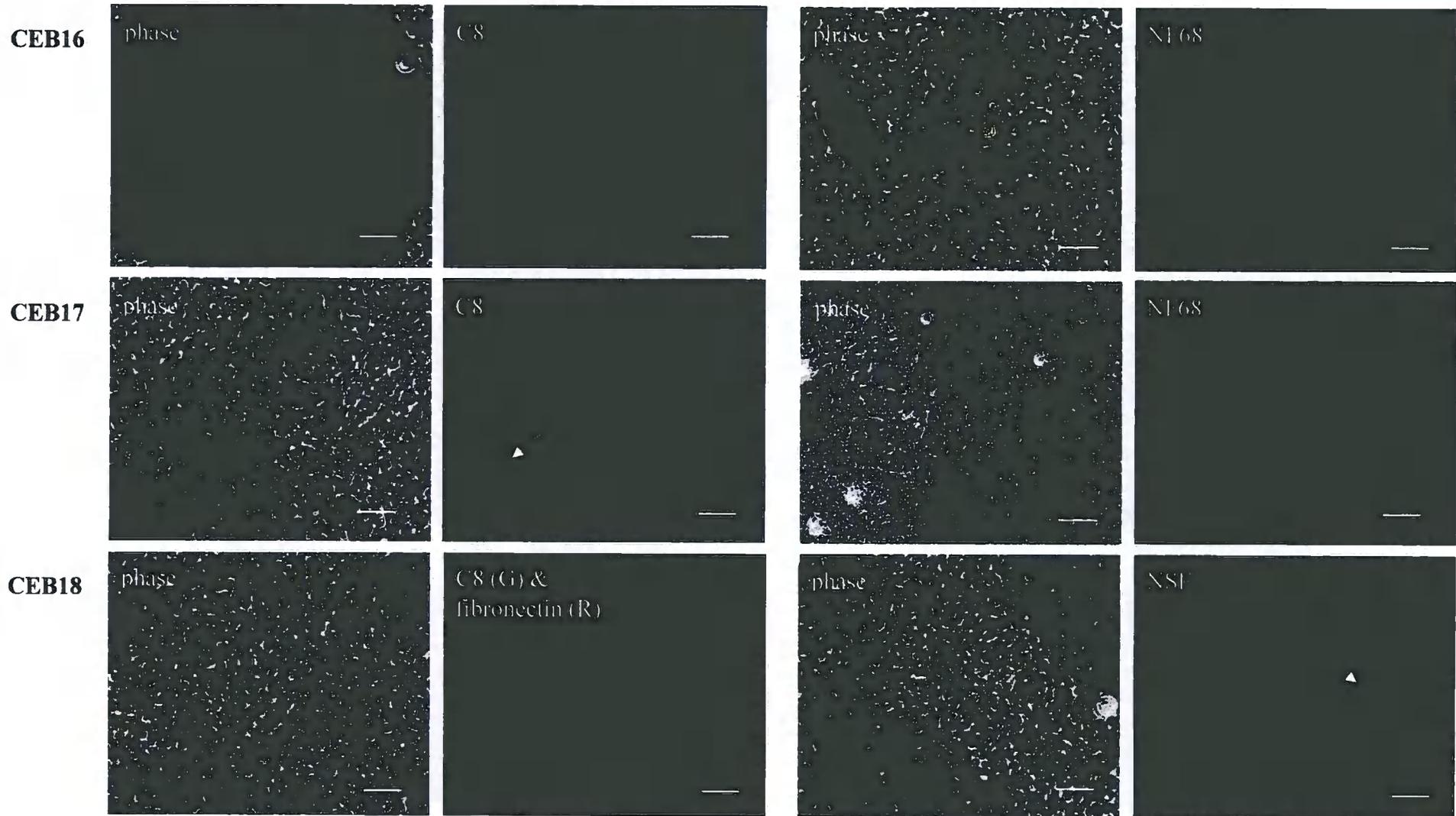


Figure 3-22: Phase and corresponding ICC images of cultures co-stained with cytokeratin 8 (C8, green G), fibronectin (red R), NSE and NF68 after 14 days' exposure to 10 μ M CEB16, CEB17 or CEB18. No NSE staining was observed in the CEB16 and CEB17 cultures (data not shown) and only a few neurons expressed NSE in the CEB18 culture. CEB16 and CEB17 cultures were not stained with fibronectin, but were positive for NF68. A large C8 +ve epithelial colony is shown in the CEB16 images; the CEB17 C8 images show different patterns of C8 staining in different phenotypes. Scale bars represent 50 μ m.

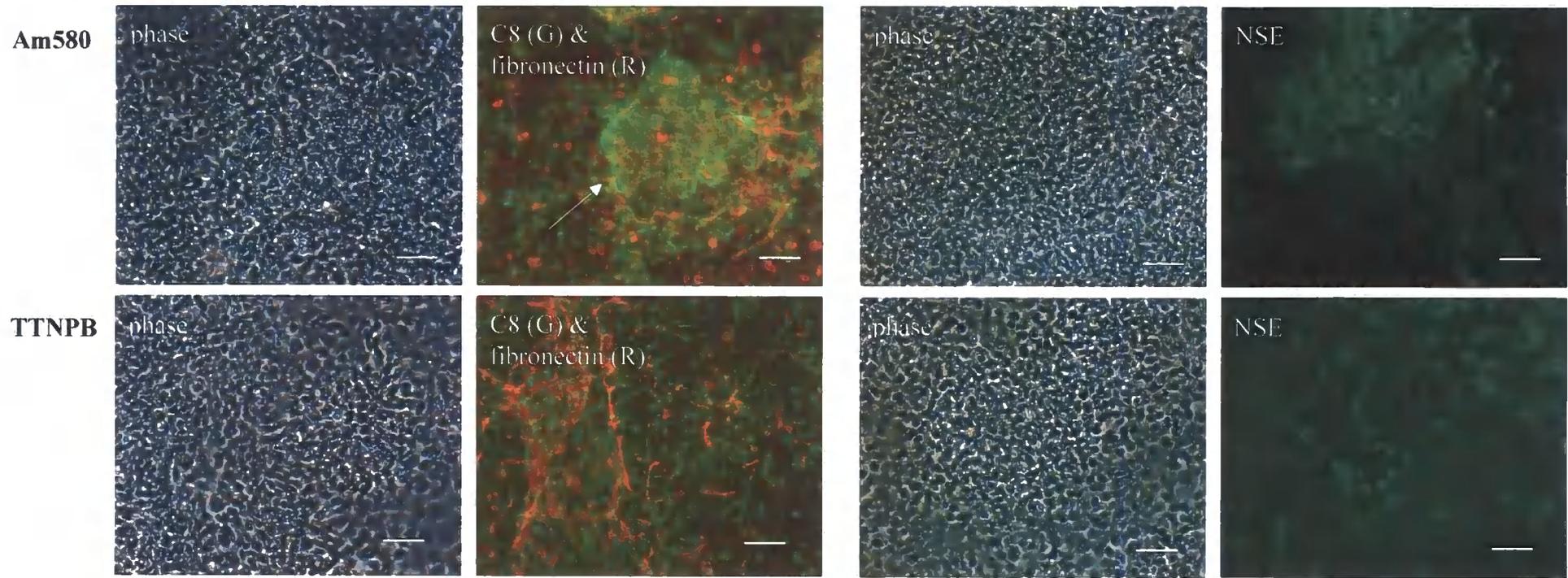


Figure 3-23: Phase and corresponding ICC images of cultures co-stained with cytokeratin 8 (C8, green G) and fibronectin (red R), or NSE after 14 days' exposure to 10 μ M Am580 or TTNPB. A dense colony of non-neuronal cells is highlighted by C8 in the Am580 culture. Scale bars represent 50 μ m.

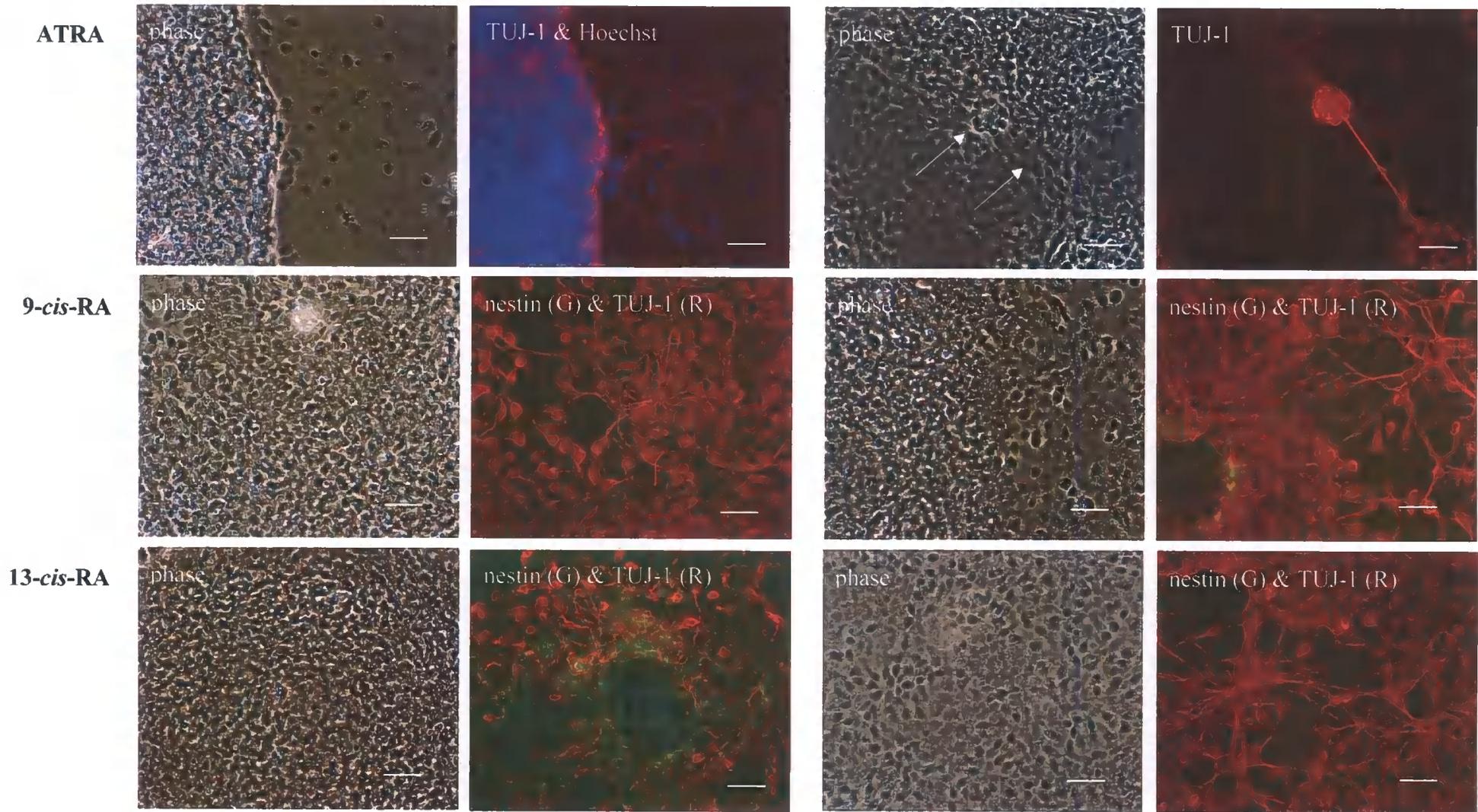


Figure 3-24: Phase and corresponding ICC images of TUJ-1 (red R) and nestin (green G) staining of cultures grown with 10 μ M ATRA, 9-*cis*-RA or 13-*cis*-RA for 28 days. Large numbers of TUJ-1 stained neurons are present. Some neurons have aggregated with long nerve fibres formed from coalesced neurites (ATRA, nerve fibre \sim 160 μ m long). Neurites extend into epithelial colonies (ATRA). Nestin expression is restricted to weak staining in proliferative areas. Scale bars represent 50 μ m.

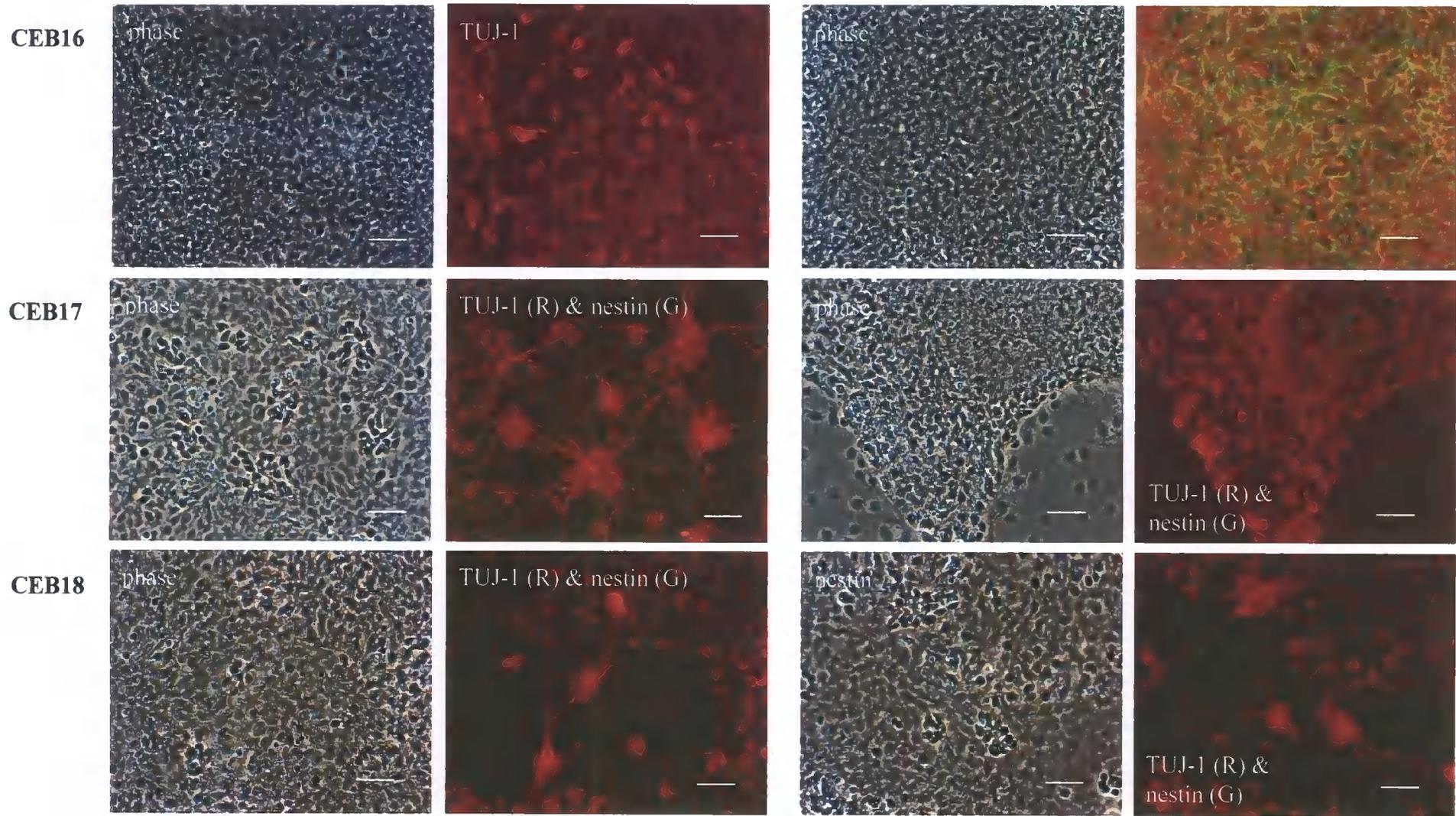


Figure 3-25: Phase and corresponding ICC images of TUJ-1 (red R) and nestin (green G) staining of cultures grown with 10 μ M CEB16, CEB17 or CEB18 for 28 days. Neurons appear immature in some areas of CEB16 cultures, whereas neuronal aggregates and other mature features are common in CEB17 and CEB18 cultures. Nestin expression remains high in CEB16, but is barely visible in CEB17 and CEB18 cultures. Scale bars represent 50 μ m.

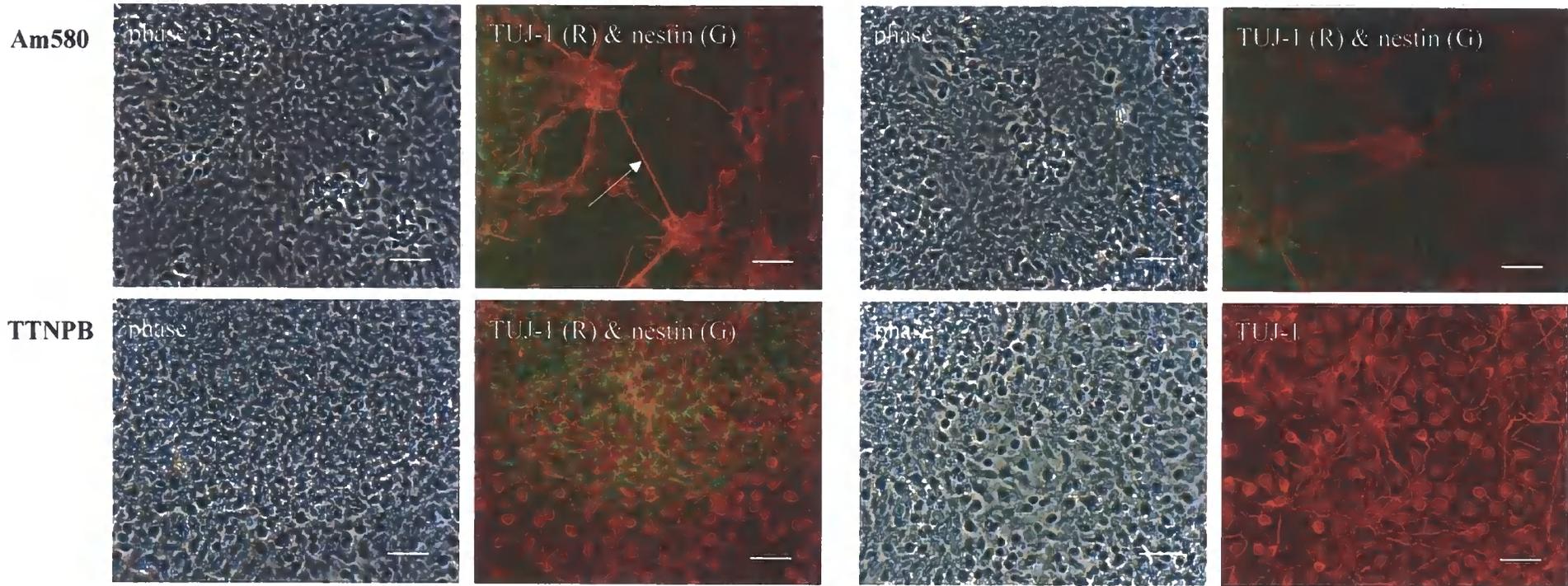
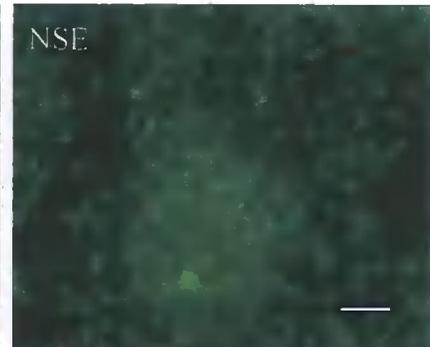
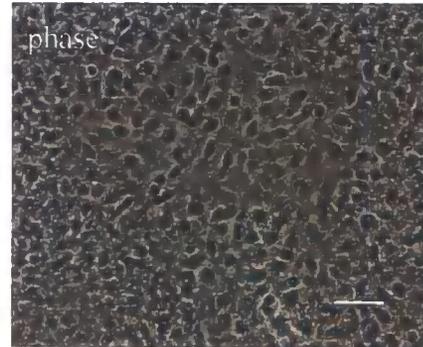
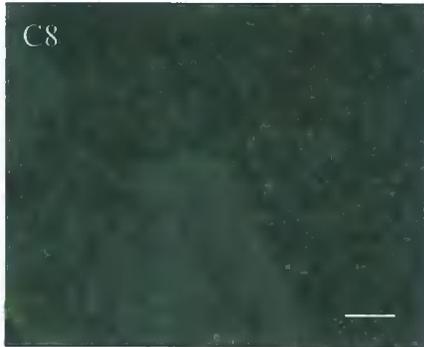
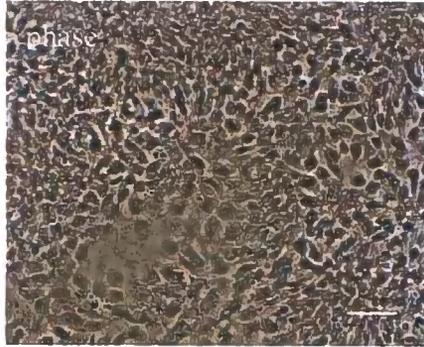
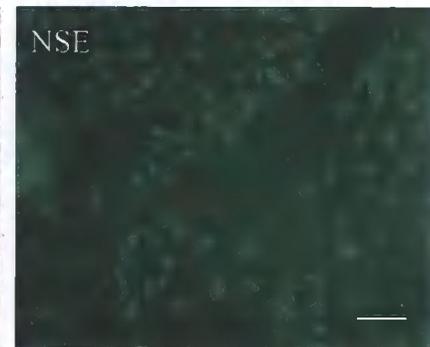
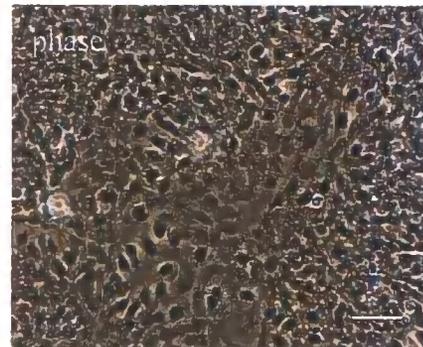
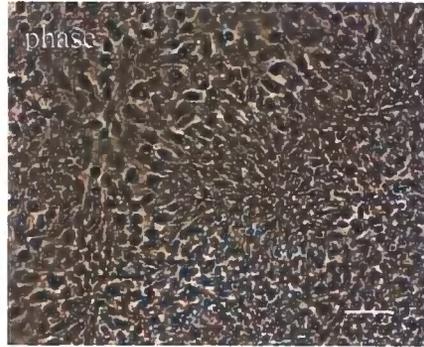


Figure 3-26: Phase and corresponding ICC images of TUJ-1 (red R) and nestin (green G) staining of cultures grown with 10 μ M Am580 or TTNPB for 28 days. These are mature neuronal cultures. Nerve bundle in first Am580 view is \sim 150 μ m long. Scale bars represent 50 μ m.

ATRA



9-*cis*-RA



13-*cis*-RA

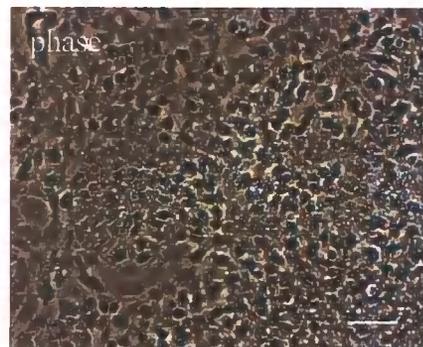
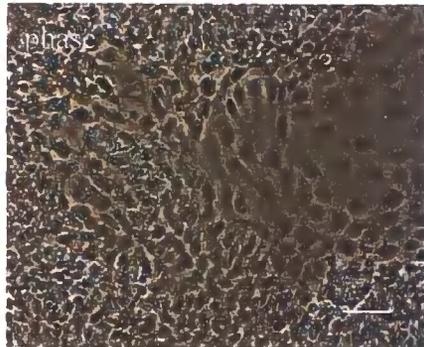


Figure 3-27: Phase and corresponding ICC images of cultures stained with cytokeratin 8 (C8) or NSE after 28 days' exposure to 10 μ M ATRA, 9-*cis*-RA or 13-*cis*-RA. The heterogeneous nature of these differentiated cultures is highlighted by the different patterns of C8 staining seen in these cultures. Large numbers of mature neurons are visualised by NSE. Scale bars represent 50 μ m.

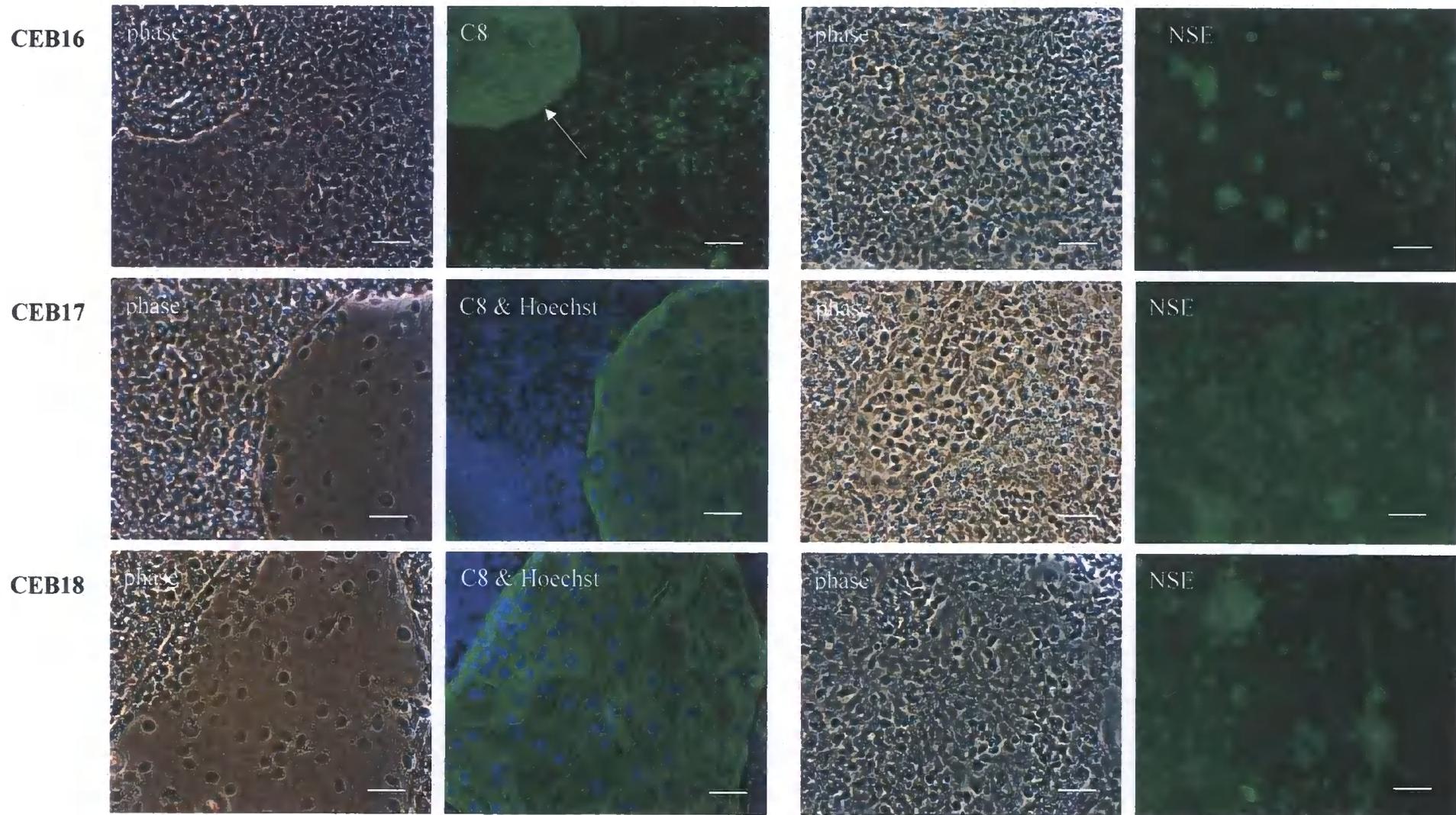


Figure 3-28: Phase and corresponding ICC images of cultures stained with cytokeratin 8 (C8) or NSE after 28 days' exposure to 10 μ M CEB16, CEB17 or CEB18. Numerous large epithelial colonies are present, especially in the CEB16 culture, which are highlighted by C8 staining (see also Figure 3-16). A proliferative colony, possibly resistant to retinoid, is depicted in the CEB16 C8 images in this figure. Significant levels of NSE staining are observed. Scale bars represent 50 μ m.

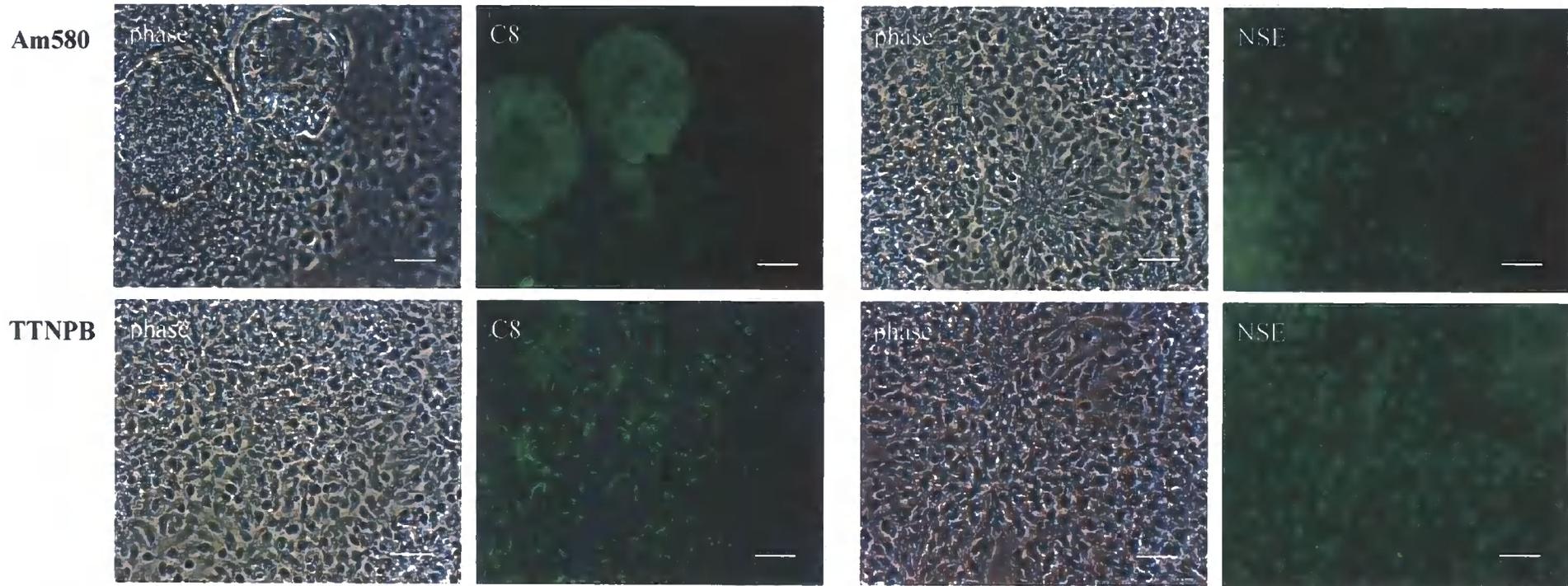


Figure 3-29: Phase and corresponding ICC images of cultures stained with cytokeratin 8 (C8) or NSE after 28 days' exposure to 10 μ M Am580 or TTNPB. Small numbers of C8-positive epithelial colonies are present and there is strong NSE expression. Scale bars represent 50 μ m.

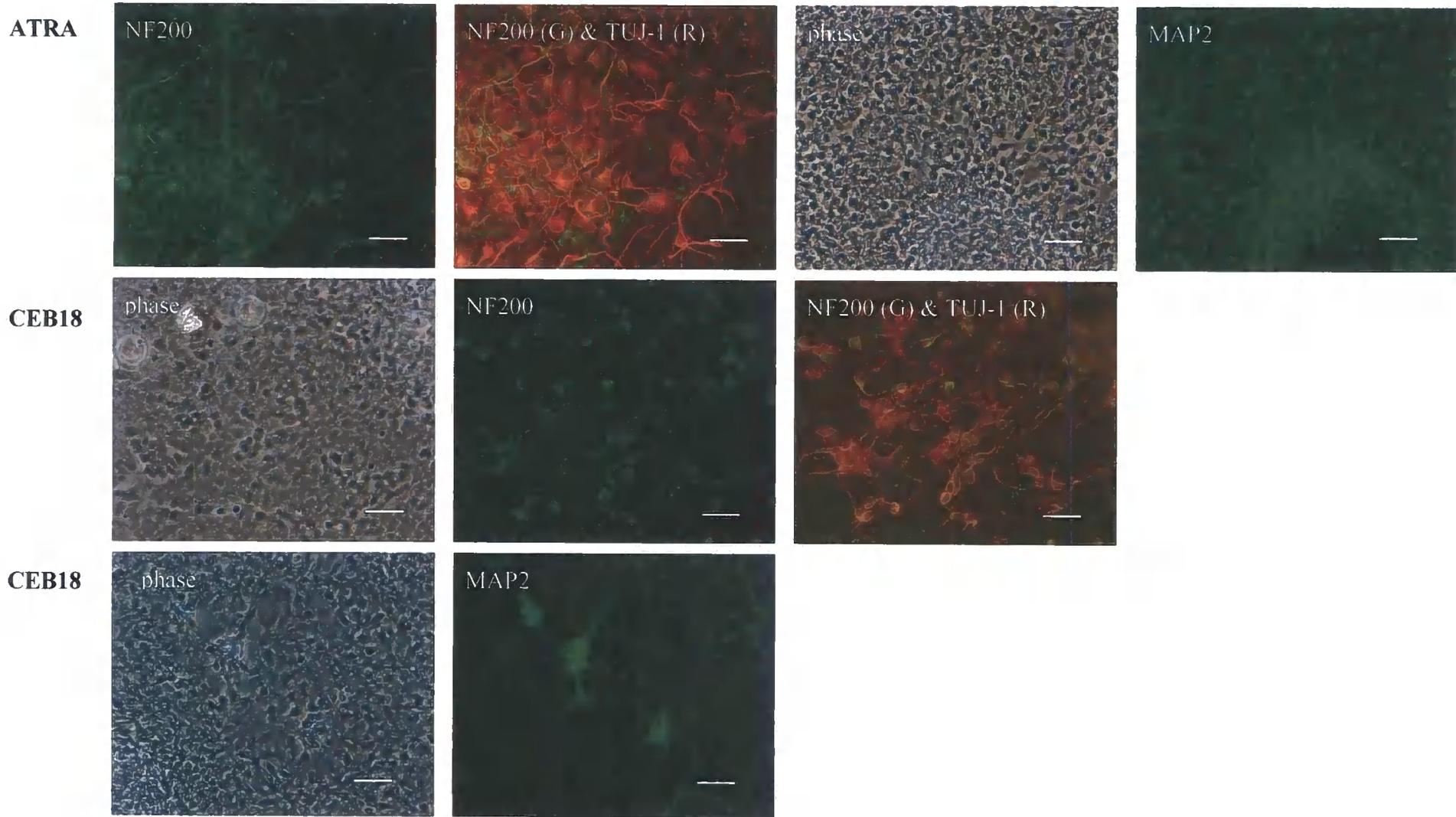


Figure 3-30: Phase and corresponding ICC images of cultures stained with NF200 (green G) and TUJ-1 (red R) or MAP2 after 28 days' exposure to 10 μ M ATRA or CEB18. NF200 and MAP2 are markers of mature neuronal phenotypes. These staining results confirm that the ATRA cultures are more developed than those differentiated with CEB18. Both cultures express high levels of the high molecular weight neurofilament emphasising the maturity of the cultures and long neurites. Scale bars represent 50 μ m.

3.7 Protein expression profiling by western blot

The retinoid-differentiated derivatives of TERA2.cl.SP12 huEC cells were also analysed by western blot. Protein samples were collected from cells grown with 10 μ M of the retinoids in Table 3-1 or maintained under control conditions (DMSO, acetone and normal media) after 7, 14 and 28 days' culture. Thirty three samples were analysed for the expression of thirteen antigens associated with neural and non-neural differentiation and the three retinoic acid receptors (Table 3-6). 12 μ g of each protein sample was loaded onto the gels and β -actin expression was used to confirm equal loading.

Table 3-6: Antibodies used in western blot analysis.

Antibody anti-	Predicted MW (kDa)	Information
β -actin	42	Ubiquitous in TERA2.cl.SP12 cells. Used as loading control.
OCT 4	43	A transcription factor expressed by undifferentiated EC, ES and embryonic germ cells. "Gatekeeper of pluripotency."
NSE	45	Detected only in mature neurons.
β -III-tubulin (clone TUJ-1)	50	Detects neuronal microtubulin.
cytokeratin 8 (C8)	52.5	Marker of simple and complex epithelia.
RAR- α	50 / 55	Retinoic acid receptor, subtypes α_1 and α_2 .
RAR- β	51	Retinoic acid receptor, subtypes β_1 and β_2 .
RAR- γ	60	Retinoic acid receptor, subtype γ_1 .
NF68	68	A low molecular weight (MW) intermediate filament expressed in young and mature neurons.
NF160	160	Medium MW intermediate filament expressed in maturing neurons.
NF200	200	High MW intermediate filament expressed in mature neurons.
fibronectin	220-240	An extracellular matrix protein associated with epidermal layer development.
nestin	220-240	Marker of putative neural stem/progenitor cells.
Microtubule associated protein-2 (MAP-2)	2 x 280 & 70	Antibody detects MAP-2a, MAP-2b and MAP-2c. Present in mature neuronal cytoskeleton.

3.7.1 Results

Images of the western blot data are presented in Figure 3-31. The pluripotency marker OCT4 is expressed at high levels in the day 0 sample (d0, undifferentiated TERA2.cl.SP12 cells) and remains highly expressed in all control cultures up to 14 days. These cells continued to proliferate beyond confluency, and were maintained in sub-optimal conditions for EC cells, yet retained expression of OCT4. The 7 day sample from the LG100268 culture also expressed high levels of OCT4. Although no expression was seen after 14 days' culture, the first time point confirms that this retinoid is not inducing differentiation. OCT4 was not detected in any of the cultures grown with ATRA, 9-*cis*-RA, 13-*cis*-RA, Am580 or TTNPB demonstrating that these cultures had undergone differentiation. By comparison, OCT4 was observed in some of the CEB retinoid cultures establishing that these compounds are less efficient than the natural retinoids at inducing differentiation. The low level of OCT4 present in CEB17 and CEB18 7 day cultures demonstrates that these cultures had begun to differentiate, but complete loss of pluripotency was only observed after 14 days' culture. Expression of this marker is maintained at a low level at all time points in the CEB16 cultures highlighting its low efficacy.

Expression of the two neuronal markers TUJ-1 and NSE was up-regulated in the retinoid treated cultures. TUJ-1 was detected in all undifferentiated cultures but expression increased significantly during differentiation, which is seen most clearly in the CEB compounds, Am580 and TTNPB blots. Low levels of NSE were also present in the EC sample and its expression increased slightly in the control cultures. The retinoid-treated cultures all show temporal increases in NSE expression, beyond that observed in the control cultures. Levels of expression are consistent with the previously observed order of efficacy.

Expression of the three retinoic acid receptors, RAR- α , RAR- β and RAR- γ was also investigated. RAR- β was not detected in any of the samples (data not shown), although a positive control was not analysed so this cannot be interpreted as absence of expression in TERA2.cl.SP12 cells and their retinoid-differentiated derivatives. RAR- α expression was observed at very low levels in undifferentiated TERA2.cl.SP12 cells and cells maintained under control conditions. Moderately elevated levels of RAR- α protein were detected in all retinoid cultures and also the day 7 LG100268 culture. RAR- γ is also expressed in TERA2.cl.SP12 cells. The control cultures contain the most protein although the day 0 result appears anomalous and further samples should be analysed to confirm the large difference. Expression of RAR- γ is decreased in the retinoid differentiated cultures. The levels of both RAR- α and RAR- γ appear to fluctuate between time points, which may suggest multi-phasic expression of the receptors during differentiation, or be indicative of high levels of biological variation.

Cytokeratin 8 (C8) and fibronectin protein expression were analysed as indicators of non neural differentiation. The delicate C8 protein underwent significant fragmentation prior to analysis which resulted in the visualisation of multiple bands of decreasing molecular weight. C8 expression at day 0 is very low; it appears to increase slightly in the control cultures and more significantly in the retinoid cultures during the course of the experiment. The highest levels of C8 appear to be present in some of the CEB cultures but fragmentation makes a thorough analysis of expression impossible with these data. A trace amount of fibronectin protein was observed in the undifferentiated sample, which increases in all other cultures in a time-dependent manner. The highest expression of this protein was detected in cultures grown with the natural retinoids and CEB16.

NF68, NF160, NF200, RAR- β , nestin and MAP-2 were not detected by this procedure; however, positive controls were not attempted.

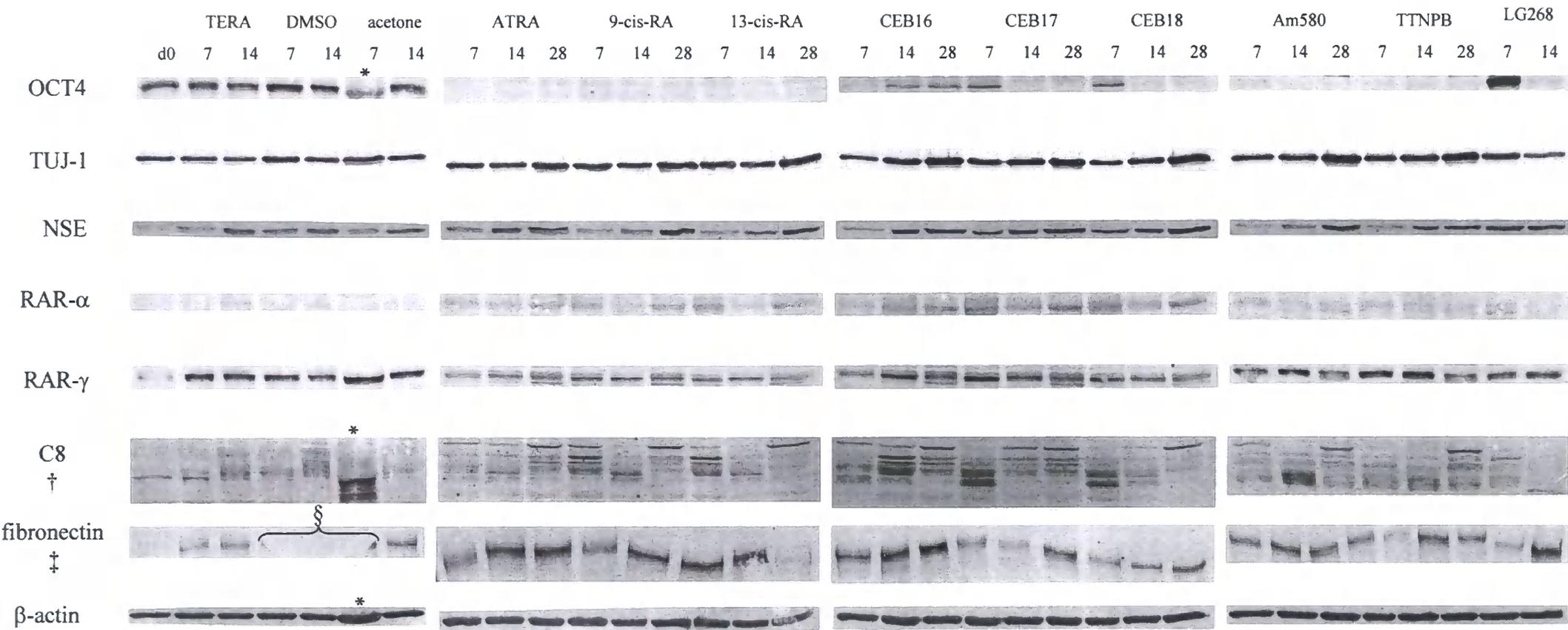


Figure 3-31: Western blot analysis of selected neural and non-neural antigens during retinoid-induced differentiation of TERA2.ci.SP12 huEC cells. Cells were continuously treated with 10 μ M of the specified retinoid or maintained under control conditions for 7, 14 or 28 days. Expression of β -actin was used as a loading control.

Notes: * protein sample was unusually viscous and difficult to pipette accurately. Some of the resulting protein bands are broader or ran faster than expected (OCT4, C8, β -actin). † Protein has degraded to varying degrees during storage/processing. ‡ Protein samples were separated on a 5% polyacrylamide-SDS gel to enable detection of large (220-240 kDa) protein. Resulting elution was slower than a 10% gel and lead to large variations in elution rates between lanes. § Transfer from gel to membrane failed for these three samples.

3.8 Synergism with a retinoid X selective agonist

Retinoid X agonists (rexinoids) are unable to induce differentiation by themselves, as demonstrated by morphological and flow cytometric analysis of the activity of LG100268 on TERA2.cl.SP12 huEC cells. However, rexinoid agonists are able to synergistically increase the differentiation potential of RAR agonists,²⁰⁵ as has previously been demonstrated in EC cells.¹⁹⁹ The synergistic potential of LG100268 was therefore investigated in combination with five of the other retinoids in Table 3-1, applying a combination of 5 μ M LG100268 and 5 μ M of RAR agonist simultaneously for a continuous period of 14 days. TERA2.cl.SP12 cells were grown in either a vehicle control (0.1% v/v DMSO), or culture media supplemented with 5 μ M LG100268, ATRA, 9-*cis*-RA, CEB16, CEB17 or CEB18, or a combination of 5 μ M LG100268 and 5 μ M ATRA, 9-*cis*-RA, CEB16, CEB17 or CEB18. Phenotypic observations were made as the cultures matured, and flow cytometric analysis of the stem cell marker TRA-1-60 and two markers of neural phenotypes, VIN-IS-53 and A2B5, was performed after 7 and 14 days' culture.

3.8.1 Results

Phenotypic observations

Representative phase micrographs of the developing cultures were recorded after 7 and 14 days' culture (Figure 3-32-Figure 3-35). As observed previously, treatment with LG100268 (LG268) alone did not induce morphological differentiation: the cultures developed identically to the DMSO control cultures (Figure 3-32 and Figure 3-34). Cultures treated with 5 μ M ATRA, 9-*cis*-RA, CEB16, CEB17 or CEB18 all underwent phenotypic changes consistent with differentiation into neural and non-neural cell types, with the same order of efficacy observed earlier, but to a lesser degree than the corresponding 10 μ M cultures. Addition of 5 μ M LG100268 to the retinoid cultures induced small changes in some of the cultures consistent with enhanced differentiation, when compared to the corresponding 5 μ M culture without LG100268. At the 7 day time point, this was only noted in the CEB18 + LG100268 culture (Figure 3-33), but by 14 days' growth small differences were observed in the 9-*cis*-RA + LG100268 culture (Figure 3-34) and significantly greater neural development was present in the CEB16+, CEB17+ and CEB18+ LG100268 cultures (Figure 3-35).

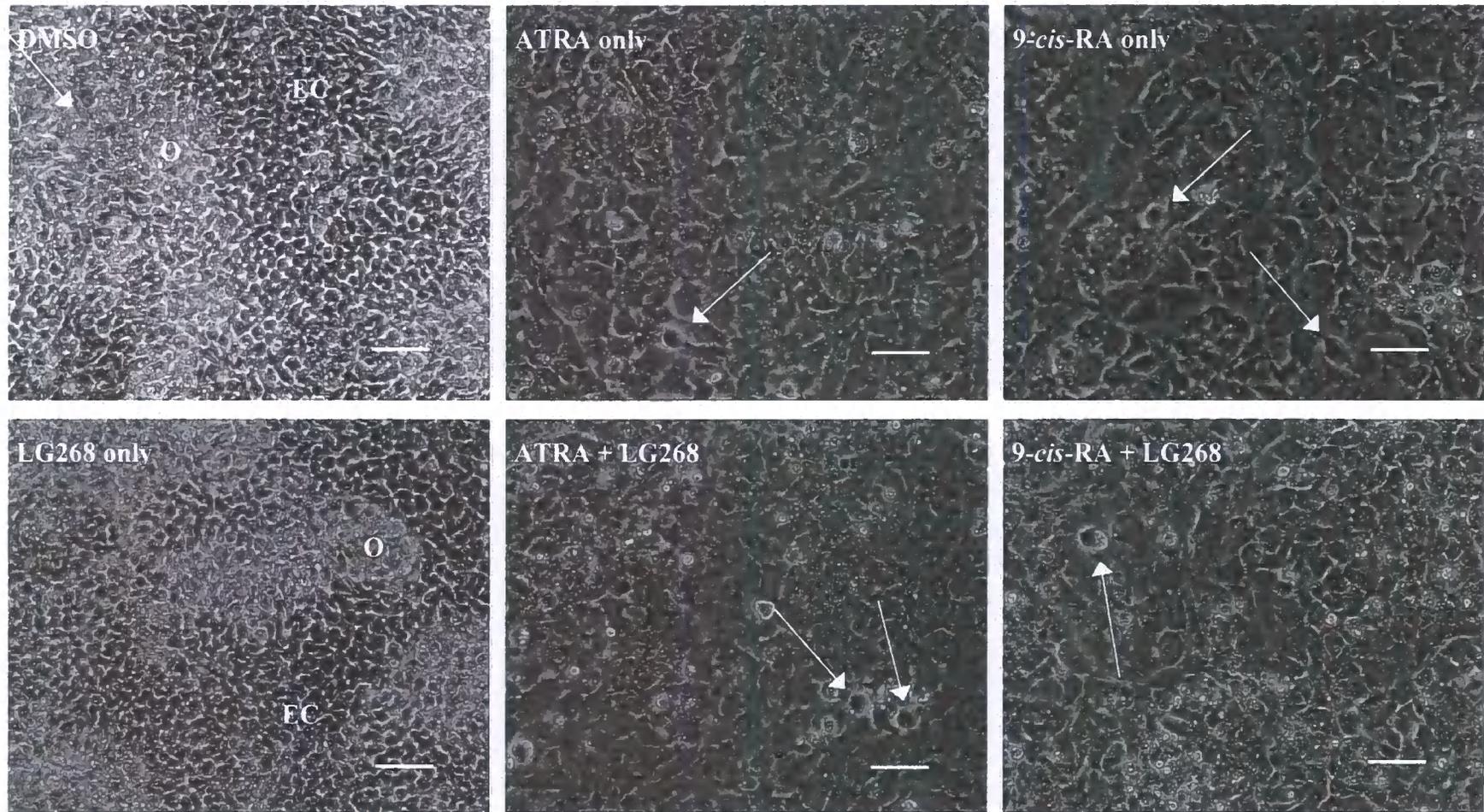


Figure 3-32: Phase images of 7 day old TERA2.cl.SP12 huEC cultures in LG100268 synergism study. Cultures grown with DMSO only and only 5 μ M LG268 possess only EC cell morphologies (EC), cells are tightly packed and starting to overgrow (O). The ATRA-only culture is low density and slightly heterogeneous, a few putative neuronal cell bodies are visible (arrow). The 5 μ M ATRA + 5 μ M LG268 culture has a slightly higher cell density than the culture treated with only 5 μ M ATRA, and has a similar number of individual neuron-like cells. Both 5 μ M 9-*cis*-RA cultures appear to be at a similar stage of differentiation as the ATRA-only culture, though the 9-*cis*-RA-only culture has a slightly higher cell density. Scale bars represent 50 μ m.

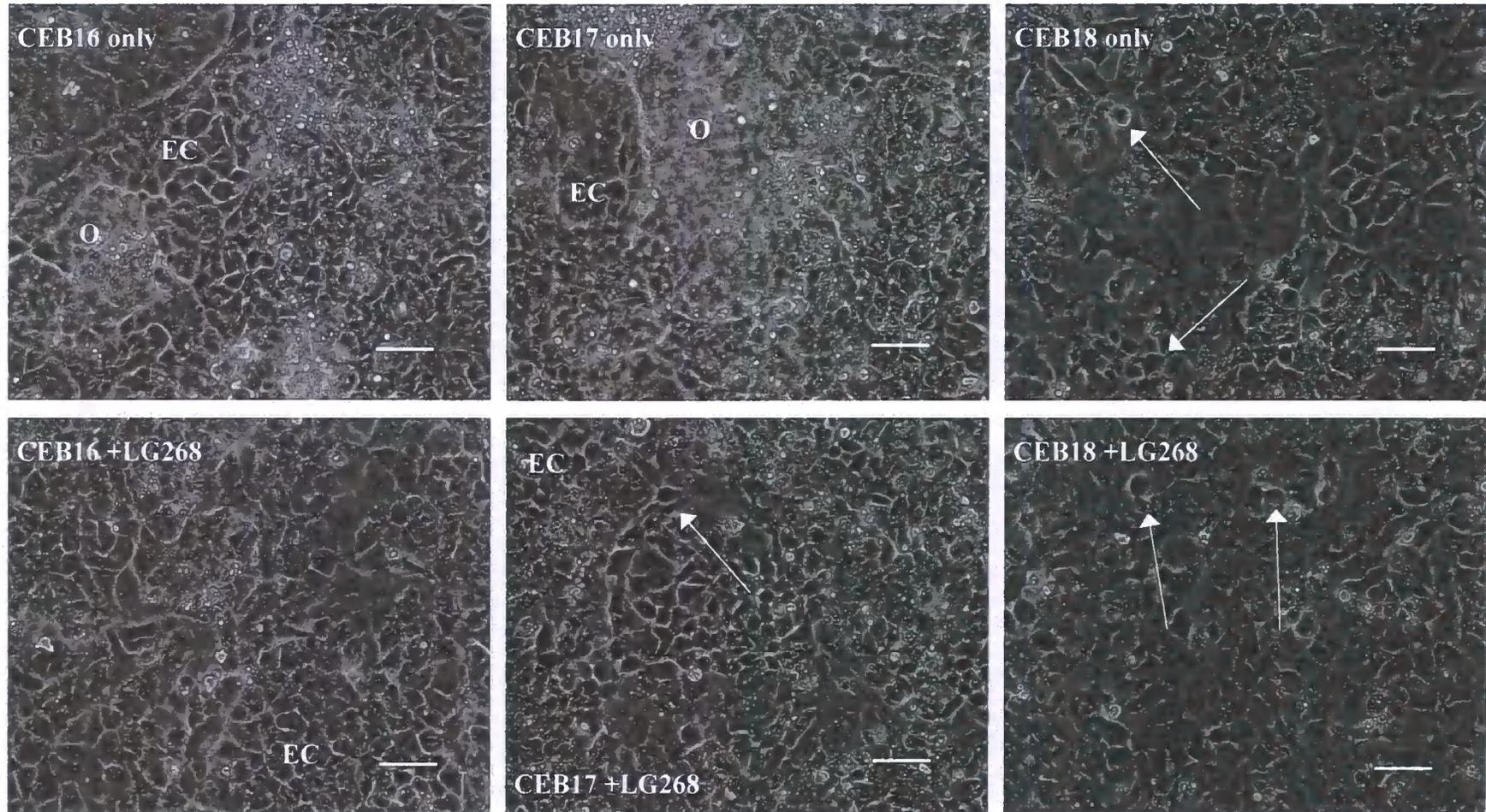


Figure 3-33: Phase images of 7 day old TERA2.cl.SP12 huEC cultures in LG100268 synergism study. Both 5 μ M CEB16 and 5 μ M CEB16 + 5 μ M LG268 cultures appear to be at a very early stage of differentiation. Cell densities are lower than control cultures, but only a few areas show heterogeneity – the majority of cells present a typical EC morphology. Addition of 5 μ M LG268 to 5 μ M CEB17 and 5 μ M CEB18 has enhanced phenotypic changes characteristic of differentiating cultures. Without LG268, the cultures are mostly EC like (CEB18 culture is slightly more mature). More putative neuronal cell bodies develop upon addition of the rexinoid, and cell densities are lower. Scale bars represent 50 μ m.

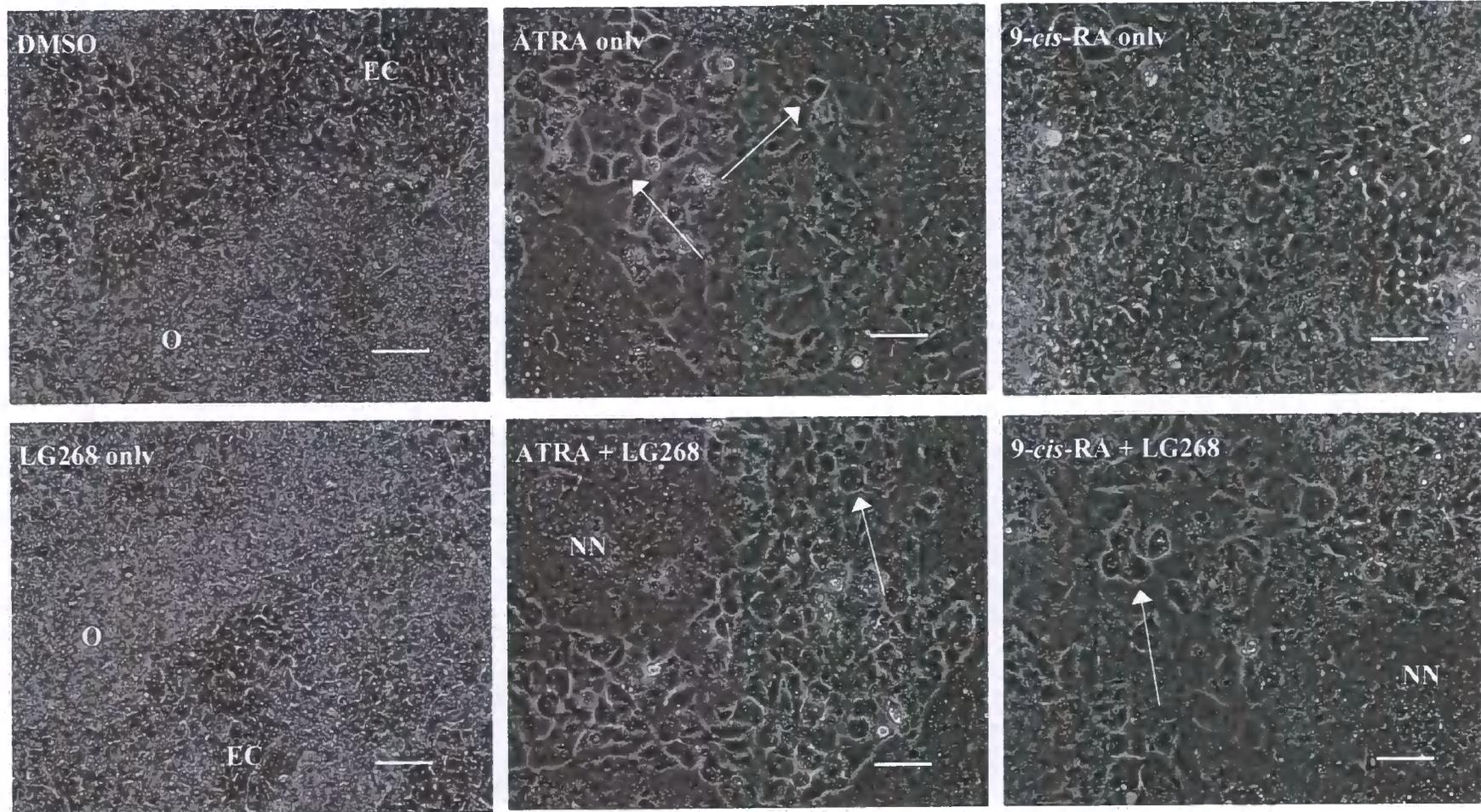


Figure 3-34: Phase images of 14 day old TERA2.cl.SP12 huEC cultures in LG100268 synergism study. The DMSO and 5 μ M LG268 cultures remain undifferentiated; EC-like cells are visible amongst dense and overgrowing (O) cultures. Both ATRA cultures are low density and composed of a range of cell phenotypes. Cultures are predominantly non neural, with a few small colonies of putative neurons and a few rosettes. The 5 μ M ATRA + 5 μ M LG268 image shows a putative group of immature neurons, amongst large numbers of non neural (NN) phenotypes. The 9-*cis*-RA cultures are more heterogeneous than the ATRA cultures, containing large areas of non neural cells and lots of apparent neurons. The 5 μ M 9-*cis*-RA + 5 μ M LG268 culture contained more neuron-like cells. Scale bars represent 50 μ m.

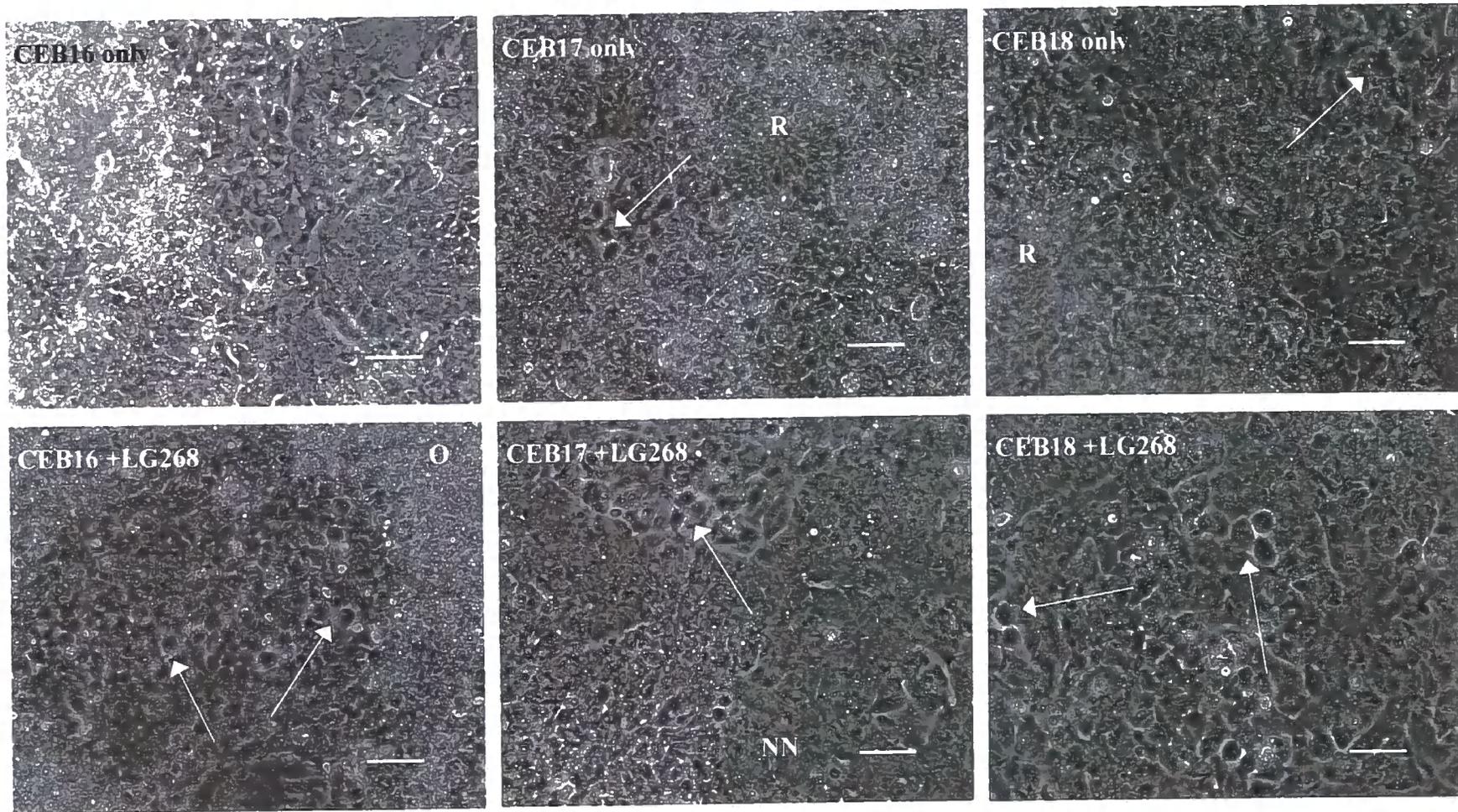


Figure 3-35: Phase images of 14 day old TERA2.cl.SP12 huEC cultures in LG100268 synergism study. Addition of 5 μ M LG268 to 5 μ M CEB16, CEB17 and CEB18 cultures appears to potentiate enhanced neuronal differentiation. All + LG268 cultures are lower density, with larger numbers of putative neurons and rosettes (R) than the corresponding 5 μ M retinoid alone cultures. As seen previously (section 3.3), at this concentration CEB18 is the most efficacious inducer of neural differentiation and CEB16 the least. Scale bars represent 50 μ m.

Flow cytometric analysis

The expression of three cell surface antigens in the cultures we measured by indirect flow cytometry after 7 and 14 days' continuous exposure to retinoids. The geometric mean fluorescence intensity (MFI) of stem cell marker TRA-1-60 and neural markers VIN-IS-53 and A2B5 is presented in Figure 3-36 and Figure 3-37. On both graphs the MFI of one antigen is shown on a secondary axis for clarity.

At both time points, the results of the 5 μ M LG100268 culture are highly comparable to the DMSO control. As before (Section 3.4), LG100268 did not up-regulate the neural antigens and expression of the stem cell marker VIN-IS-53 remained high, confirming the morphological observation that no differentiation had occurred. All 5 μ M retinoid and 5 μ M retinoid + 5 μ M LG100268 cultures underwent antigen changes consistent with the induction of neural differentiation. In all conditions, TRA-1-60 was down regulated after 7 days' culture, with varying efficiency, and was further suppressed by 14 days. The levels of VIN-IS-53 and A2B5 were both up-regulated relative to the control cultures in all conditions.

Data for the 5 μ M retinoids highlight large differences in differentiation-inducing efficiency. Cells from the ATRA and 9-*cis*-RA cultures had similar MFIs for all antigens at both time points. All three synthetic retinoids induced a lower level of expression of neural antigens, and reduced suppression of TRA-1-60 compared to either of the natural retinoids. In particular, the CEB16 culture contained high levels of TRA-1-60 after 7 days' culture (Figure 3-36). The results for 5 μ M CEB17 and 5 μ M CEB18 are similar at 7 days, but CEB17 appeared to induce neural differentiation more efficiently at the 14 day time point. However, as the data are only drawn from one batch, this may be insignificant.

Combining 5 μ M LG100268 with 5 μ M retinoid appears to potentiate differentiation in all of the cultures after 14 days. After seven days, the MFI of both neural antigens was markedly higher in the CEB17 + LG100268 and CEB18 + LG100268 cultures than in cultures grown in the presence of only CEB17 or CEB18 respectively. No differences were observed in any of the other cultures at this time point. After 14 days' culture, small differences are observed upon addition of LG100268 to both ATRA and 9-*cis*-RA, although repeat analyses of further batches would be required to confirm the trend. A large difference was observed when CEB18 was supplemented with LG100268, continuing the trend observed after 7 days. Small differences in MFI were also visible in the CEB16 and CEB17 + LG100268 combinations.

The synergistic improvement of differentiation upon addition of the retinoid LG100268 is identical for all retinoids tested in this experiment, even 9-*cis*-RA which has both RXR and RAR binding affinity. It therefore seems likely that repeat analyses would confirm this relationship.

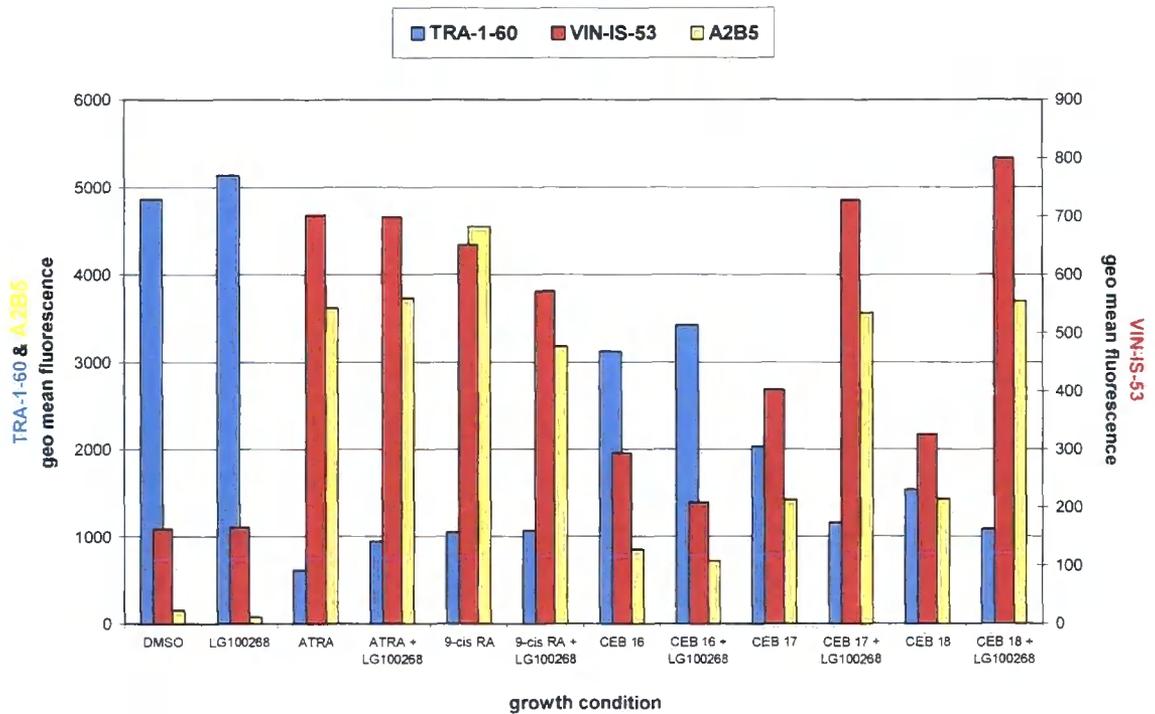


Figure 3-36: Flow cytometric analysis of the ability of retinoid LG100268 to synergistically potentiate retinoid-induced differentiation in TERA2.cl.SP12 huEC cells. A combination of 5 μ M LG100268 and 5 μ M retinoid were applied continuously. Data presented are the geometric mean fluorescent intensity of three antigens after 7 days' culture. (n=1)

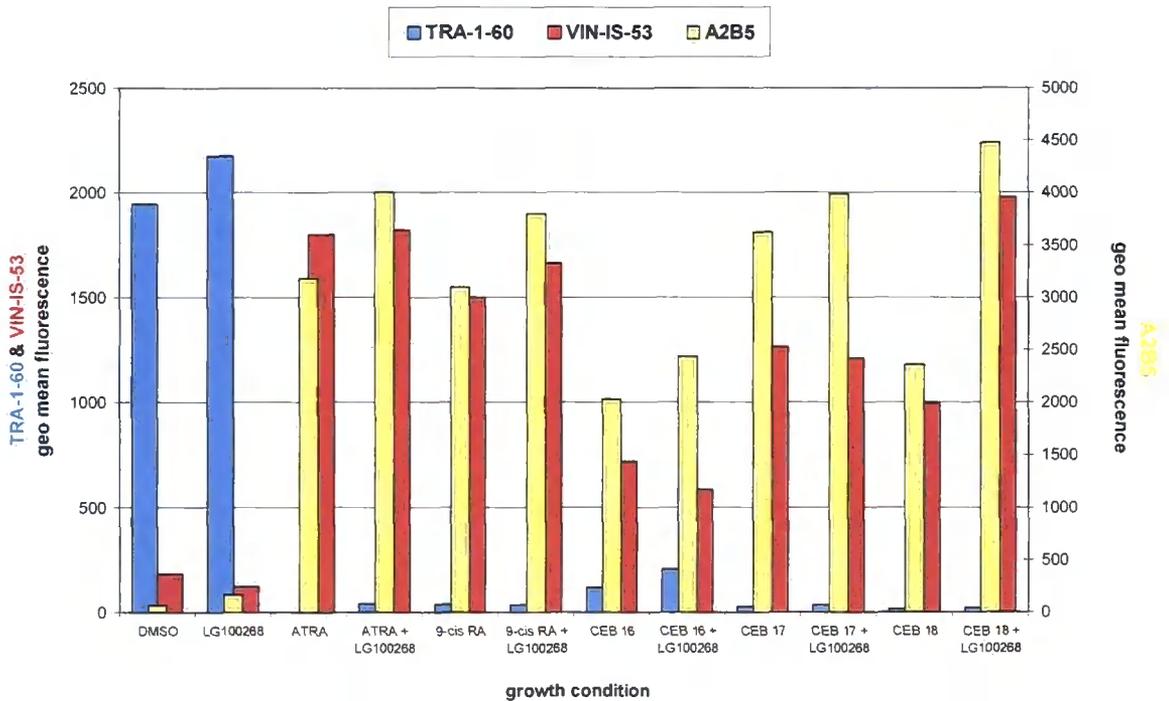


Figure 3-37: Flow cytometric analysis of the ability of retinoid LG100268 to synergistically potentiate retinoid-induced differentiation in TERA2.cl.SP12 huEC cells. A combination of 5 μ M LG100268 and 5 μ M retinoid were applied continuously. Data presented are the geometric mean fluorescent intensity of three antigens after 14 days' culture. (n=1)

3.9 Retinoid effects on SH-SY5Y neuroblastoma cells

Neuroblastoma is the most common extracranial solid tumour of childhood and is responsible for approximately 15% of all childhood cancer deaths.⁵⁰⁶ It is an embryonal cancer of the sympathetic nervous system, and presents both a heterogeneous malignancy and prognosis.²⁷⁹ ATRA, 13-*cis*-RA and fenretinide are three retinoids that have been used with some success as chemotherapeutic agents in the treatment of neuroblastoma.²⁵⁶ However, the search continues to find a treatment with improved pharmacokinetic properties and improved clinical results.

The third generation human neuroblastoma cell line SH-SY5Y shows morphological and biochemical properties of cells similar to those derived from sympathetic neurons of the embryonic neural crest.⁵⁰⁷ These cells can be used to further investigate the ability of synthetic retinoids to induce differentiation in a clinically relevant model. Working in collaboration with Dr. Chris Redfern's research group at the Northern Institute for Cancer Research, Newcastle University, the effects of CEB16, CEB17 and CEB18 compounds on SH-SY5Y cells were investigated. Data pertaining to the morphological changes induced in response to the compounds, and the ability of these compounds to protect the cells against fenretinide-induced cell death are presented.

3.9.1 Neurite outgrowth

When cultured in the presence of ATRA and a number of other retinoids, SH-SY5Y cells morphologically differentiate towards mature ganglion-like cells.⁵⁰⁸ Changes are observed within a few days such that the ability of compounds to induce neuronal differentiation can be assessed quickly by comparing neurite outgrowth. This observation provides a simple and fast method of screening the efficacy of retinoids in the SH-SY5Y neuroblastoma cell line.

Control cells treated with only the vehicle (ethanol, 0.1% v/v) exhibited the same morphological appearance as before treatment. Cell bodies were rounded or slightly elongated, with short, stunted processes emanating from the cell body. After treatment, cells typically appear more neuronal with more rounded cell bodies and long thin processes, or neurites.⁵⁰⁸ In this experiment the differentiating cell bodies have yet to adopt the dense, rounded morphology, but there are clear differences between the different treatments in neurite outgrowth. ATRA was the most effective, then CEB16, CEB17 and finally CEB18 (Figure 3-38).

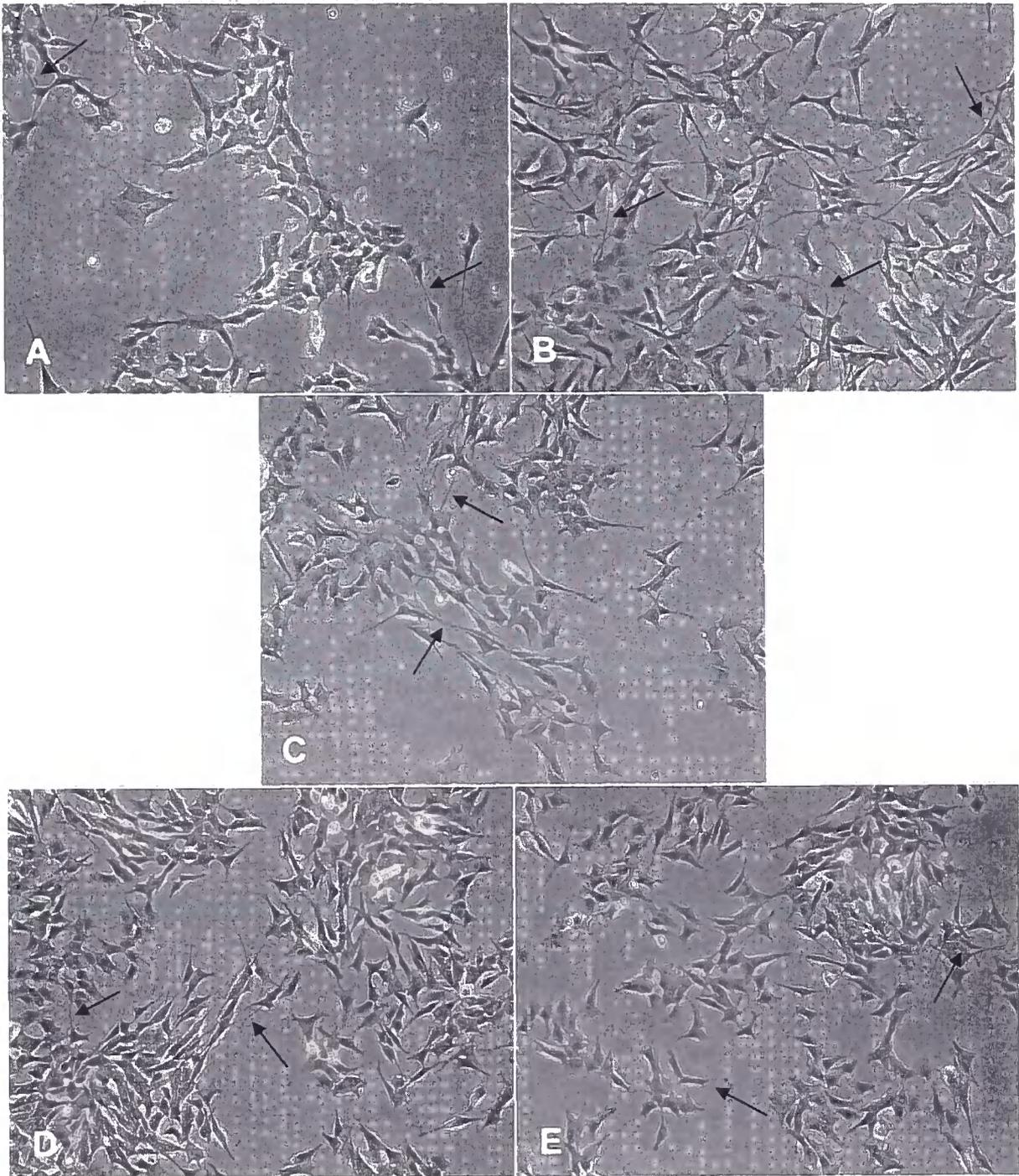


Figure 3-38: SH-SY5Y morphological observations.

A: Control (0.1% v/v ethanol): cell bodies are generally rounded, with short processes (arrows).

B: Positive control (10 μ M ATRA + ethanol: cell bodies have become elongated, extending long neurites.

C: 10 μ M CEB16 + ethanol: more processes are visible than in the control, but less than observed in response to ATRA. The most effective of the three synthetic retinoids tested.

D: 10 μ M CEB17 + ethanol: some neurite extension visible.

E: 10 μ M CEB18 + ethanol: least evidence of neuronal differentiation.

3.9.2 Fenretinide induced apoptosis

Fenretinide (all-*trans*-*N*-(4-hydroxyphenyl)-retinamide, 4-HPR, structure **J** in Appendix 1) is an extremely active synthetic retinoid that inhibits proliferation in cancer cells and induces apoptosis in certain cancer cells. It also shows minimal toxicity during long-term administration to patients in clinical trials. These features have led to large scale clinical trials in a range of cancer studies.²⁵³ It is a highly selective activator of RAR- γ ,²⁴⁷ which may explain its favourable pharmacological properties, but also exerts activity *via* non-retinoid pathways.^{237,253}

As a second experiment to measure the activity of the synthetic retinoids on SH-SY5Y neuroblastoma cells compared to ATRA, the ability of the retinoids to inhibit fenretinide-induced apoptosis was investigated. Cultures were treated with 10 μ M of ATRA or test retinoid for 24 hours, then with 5 μ M fenretinide for a further 24 hours to induce apoptosis. Apoptosis was quantified using flow cytometry to measure the percentage of propidium iodide-stained cells. The activity of CEB16, CEB17 and CEB18 was then expressed as a percentage of the observed ATRA inhibition of fenretinide-induced apoptosis (relative to non-retinoid-treated cells). As shown in Figure 3-39, all the synthetic retinoids were less effective at preventing apoptosis than ATRA at this concentration. CEB16 had the strongest effect, then CEB17 (with a large standard error of mean (SEM)), and finally CEB18.

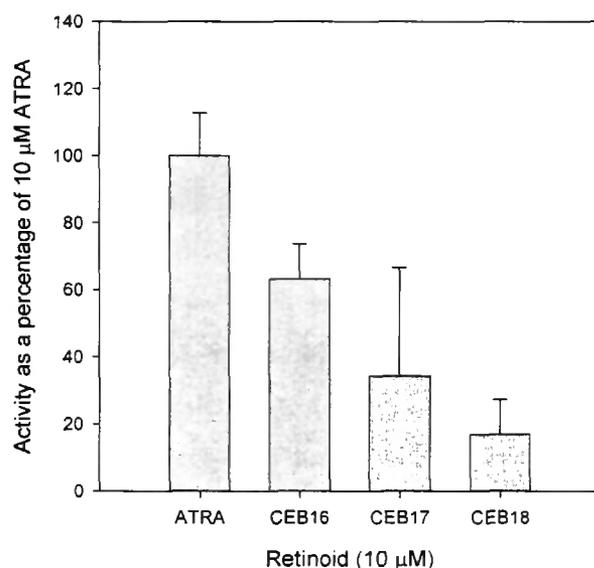


Figure 3-39: Apoptosis-inhibitory activity of three synthetic retinoids relative to 10 μ M ATRA (n=3, error bars +1 SEM).

3.10 Discussion

All-*trans*-retinoic acid has been used to modulate embryonal carcinoma (EC) stem cell fate for over thirty years and induces a range of well defined morphological and immunological changes.⁴⁵⁷ The differentiation propensity of different EC lines varies, for example F9 murine (mu) EC cells differentiate into endodermal cell types,⁴²² the P19 muEC cell line undergoes neuro-ectodermal differentiation,⁴⁶⁰ whereas NTERA-2 human EC (huEC) cells and their derivatives predominately differentiate in an ectodermal direction.⁴⁵⁹ However, ATRA alone is not sufficient to recapitulate the neural specification observed *in vivo*.^{475,509} Embryonic stem (ES) cell culture requires the addition of further trophic factors, including sonic hedgehog, to induce development and specification beyond caudally fated neuroepithelial cells.⁵¹⁰ Furthermore, it has been suggested that neuro-epithelial differentiation may be the default differentiation pathway of ES cells *in vitro*⁵¹¹ and that induction of this phenotype may not require addition of exogenous ATRA.⁵¹²

In common with many human EC cell lines, TERA2.cl.SP12 retains the capacity of its germ cell origins to differentiate *in vitro*.^{421,513} Spontaneous differentiation occurs in sub-optimal culture conditions, which can be limited by consistent handling. Treatment with compounds such as ATRA and hexamethylene bisacetamide (HMBA) also induces differentiation, enabling the convenient use of huEC cells as a model of embryonic development.⁴²⁶ In response to exogenous ATRA TERA2.cl.SP12 cells differentiate into heterogeneous cultures; approximately 10-15% of cells commit toward becoming mature neurons, the remainder of cells produce non-neuronal cell types including epithelial colonies.^{426,428} This pattern of differentiation was observed in this work in response to natural⁵¹⁴ and synthetic retinoids, although the proportions of cell types observed and the speed of exit from the cell cycle varied significantly between compounds.

In the screening and characterisation experiments presented in this chapter, except for the retinoid synergism study, the retinoids were all applied at a final concentration of 10 μ M. ATRA has previously been used to successfully differentiate TERA2.cl.SP12 huEC cells at this concentration.⁴²⁶ 10 μ M ATRA is at the upper end of the range of retinoid concentrations that have been described in many published *in vitro* experiments,⁵¹⁵ which may be partly explained by the reduced sensitivity of EC cells and the ratio of cell number to number of moles of retinoid in culture medium.⁴⁸⁶ Significant differences in uptake of retinoids has previously been noted between different cell lines.⁴⁸⁷ If the cells were to metabolise the retinoid only slowly, then the exposure of each cell to the retinoids would be constant throughout the experiment. However, it is known that ATRA is rapidly metabolised by cells and it is also degraded by the culture environment.^{285,486-488} Furthermore, cell culture density increases rapidly during the experiment, as the cells continue to proliferate, such that the concentration of

retinoid that the cells experience will decrease significantly during the course of the experiment. Anecdotal observations, based upon a unique experiment, indicate that the frequency of media change appears to be a significant variable in differentiation studies (data not shown). When retinoid-supplemented culture media was replaced daily, differentiation was induced more quickly compared to the standard frequency of every three days. Whereas changing the media less frequently than normal (every five or six days) resulted in even slower phenotypic changes. Regular supply of fresh media may give the cells an advantage over cells grown predominantly in old media containing cell debris and other degradants, but it also seems that the depletion of ATRA between media changes has an effect on cell growth and development. In high density cultures the frequency of media changes and therefore presumably fluctuations in retinoid concentration are an important factor in differentiation efficacy. Synthetic retinoids that are less labile than ATRA may provide a more consistent source of differentiation induction.

During all differentiation experiments the cultures were continuously exposed to the test retinoid. At every media change the appropriate concentration of retinoid was mixed thoroughly into the replacement media. In studies that used concentrations of less than 10 μM , the retinoids were added to media immediately before use, as stability studies have demonstrated degradation of the natural retinoids to be first order²²⁴ and, consequently, lower concentrations of retinoid are expected to show elevated degradation kinetics. It was not investigated whether continuous exposure was necessary for optimal or terminal differentiation, though this has been demonstrated in other cancer cell lines.⁵¹⁶

Human EC and ES cells tend to differentiate and/or grow poorly at low cell densities due to the loss of cell-cell contacts,⁴⁷⁸ mediated through the NOTCH pathway.^{517,518} In accordance with these observations, TERA2.cl.SP12 EC cells are also maintained at high density.⁴²⁵ To limit and control for the effects of spontaneous differentiation during experiments, TERA2.cl.SP12 cells were seeded at a density of 20 000 cells/cm³ in every experiment for which data are presented. Cultures seeded at lower densities were observed to respond differently to treatment (data not shown), which is consistent with other studies.⁵¹⁹ Unfortunately, high density seeding coupled with the relatively fast doubling time and slow initiation of differentiation (and consequent late exit of cell cycle) led to cultures quickly attaining and surpassing confluency. High culture densities made morphological and immunocytochemical features difficult to record photographically.

Phenotypic observations

The vehicles (0.1% v/v DMSO, DMF and ethanol), 5 mol% acetone, LG100268 and the three “conjugated rigid rod” compounds (Table 3-2) induced no changes in culture appearance compared to a TERA2.cl.SP12 control culture throughout the experiments. The tolans and BPEB have some structural similarities to retinoid ligands but they appear to be unable to bind and transactivate the

RAR receptors to induce differentiation. It is essential that the vehicle has no effect on cell behaviour. The eight-week old DMSO culture that had been trypsinised, then replated at a lower density and maintained for longer than before may have behaved differently to a culture grown undisturbed in DMSO enriched media for eight weeks. The putative neuronal colony observed under these conditions may be due to spontaneous differentiation independent of the presence of DMSO. This concentration of DMSO (0.1% v/v, 14 mM) has previously been demonstrated to be non-toxic and not induce differentiation in other EC cell lines.^{460,520} However, other studies suggest that DMSO can affect cell behaviour down to 0.1%.⁵¹⁹ Neuronal differentiation was reported in TERA2 huEC cells in response to a higher concentration, 1% DMSO.⁴⁶¹ The same concentration of DMSO should be used in all experiments to negate any effect on cell behaviour.

The three natural retinoids, ATRA, 9-*cis*-RA and 13-*cis*-RA, three synthetic retinoids prepared by the author (CEB16, CEB17 and CEB18) and the commercial retinoids Am580 and TTNPB all induced phenotypic changes consistent with differentiation. CEB16, CEB17 and CEB18 were less efficacious than the other compounds at this concentration (10 µM). These compounds are all known in the art: CEB18 has been used in a number of *in vitro* studies,^{238,496,499,521,522} and CEB17 binds to RAR-β and RAR-γ,⁴⁹⁵ but no biological data have been published for CEB16. CEB18 shows no activity towards the RXRs but does bind all three RAR isotypes weakly. It shows a preference for RAR-β and RAR-γ for which the binding affinities have been measured at 9.5 and 19 times weaker than ATRA respectively. CEB18 is consequently unable to transcriptionally activate RAR-α, but activates RAR-β and RAR-γ similarly to ATRA (Table 3-7).²³⁸ Whilst these data are specific to the cell type and assay used in this study, they are believed to be indicative of relative binding affinity. Conversely, CEB18 was found to be more than ten times as active as ATRA in the reversal of keratinisation in hamster tracheal organ culture, but up to 70% less active than ATRA in another assay.⁴⁹⁶

Table 3-7: Comparison of retinoid receptor transcriptional activation and competitive binding activity for ATRA and CEB18. Extracted from Gambone, 2002.²³⁸

Compound	Competitive binding activity, K_d (nM)			Receptor transcriptional activation, EC_{50} (nM)		
	RAR-α	RAR-β	RAR-γ	RAR-α	RAR-β	RAR-γ
ATRA	14	11	16	240	38	6
CEB18	600	103	301	>1000	103	301

Radial arrangements of columnar cells, described as rosette-like features, are observed in the differentiating cultures, as noted previously in this cell type and reported in human and mouse ES cells.^{505,523} These rosettes are described as the developmental signature of neural progenitors; they are nestin positive (see Section 3.6 and literature⁵⁰⁵) with broad differentiation potential towards CNS and PNS fates.^{523,524} Under appropriate conditions, rosettes derived from huES cells develop into neural tube-like structures,⁵⁰⁵ but in the absence of these signals, isolated neural rosette cells rapidly lose rosette organisation and progress to a more restricted stage of differentiation.⁵²³ Induction of

differentiation in TERA2.cl.SP12 huEC cells by natural and synthetic retinoids as described herein therefore appears to mimic a stage of the normal neuro-ectodermal development that takes place in the human embryo.⁵²⁵

Quantification of stem cell and neural antigens by flow cytometry

Differentiation was monitored and quantified by measuring the levels of characteristic cell surface markers: stem cell specific markers are down-regulated, whilst other markers of more differentiated phenotypes are induced. A number of markers are well established in studying EC cell differentiation.⁴²¹ Two stem cell markers, SSEA-3 and TRA-1-60, were chosen to assess loss of pluripotency,^{478,480} and two neural associated markers, VIN-IS-53 and A2B5,^{459,482} were measured to quantify differentiation towards neural lineages.

Flow cytometric analysis of these four extracellular antigens clearly distinguished between the compounds that were morphologically observed to induce differentiation and those that failed to affect these cells. The stem cell markers were rapidly down-regulated and neural associated antigens were concomitantly up-regulated. The small standard deviations in the data demonstrate the reproducible effects of the compounds on TERA2.cl.SP12 cells. Analysis of the kinetics of suppression/acquisition of immunophenotypic markers, in combination with the calculated statistically significant differences between conditions, can be used to quantify the relative potencies of the test compounds. From day 7 data, the order of loss of pluripotency markers and acquisition of neural antigens in response to retinoid treatment can be summarised as:

13-*cis*-RA>9-*cis*-RA=ATRA>CEB18>CEB17>>CEB16

This trend is in good agreement with the morphological data, although no tangible differences were observed between the phenotypes of the ATRA, 9-*cis*-RA and 13-*cis*-RA treated cultures. The enhanced differentiation-inducing ability of 9-*cis*-RA and 13-*cis*-RA compared to ATRA has also been noted in the neuroblastoma cell line SH-SY5Y.²⁸⁴ ATRA is only able to bind to the RARs, while 9-*cis*-RA is able to bind strongly to both the RARs and RXRs, and 13-*cis*-RA shows moderate affinity for both receptor families.¹⁶¹⁻¹⁶³ The differential effects of these isomers is believed to be due to additional or alternative mechanisms of action compared to ATRA. The RXR-binding affinity of 9-*cis*-RA may enable it to induce activity through additional pathways and 13-*cis*-RA is believed to act as a prodrug for ATRA.^{281,526}

Flow cytometry data of the DMSO cultures show some changes typical of cells undergoing slight differentiation, particularly after 28 days (Figure 3-11). This would seem to confirm the appearance of morphologically identifiable neurons in one mature culture. However, it is interesting to note that the data for LG100268 (dissolved in DMSO) is comparable to the control culture.



Proliferation

A tetrazolium colourimetric assay was employed to quantify the observed differences in proliferative capacity of TERA2.cl.SP12 cells undergoing retinoid induced differentiation. The MTS compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) is reduced by viable cells into a soluble coloured product which is measured by UV absorbance, and can thus be used to compare the effects of compounds on cell toxicity or proliferation.⁵²⁷⁻⁵³¹ Although it has been suggested that mitochondrial activity will be affected in cells undergoing differentiation,⁵¹⁹ others report that tetrazolium reduction capacity of a cell line are not altered by differentiation.⁵³² Preliminary studies determined that MTS absorbance results were in good correlation with total protein content of culture undergoing retinoid-induced differentiation (data not shown); furthermore, the MTS assay results were also more reproducible than protein measurement.

Dose response curves of cultures growing in 10 μ M retinoid were produced using the MTS assay data (Figure 3-14). The vehicle (DMSO) had no effect on the growth of TERA2.cl.SP12 huEC cells for the duration of the experiment and provides further confirmation of the morphological and flow cytometric data. This is important since DMSO can have anti-proliferative effects on cells, and may itself induce differentiation at higher concentrations.^{460,519,520}

At the first time point (3 days), treatment with CEB16, CEB17 and CEB18 was found to have slightly increased the number of viable cells in the culture whilst ATRA, 9-*cis*-RA and 13-*cis*-RA had all suppressed cell proliferation. After this initial period, when the CEB compounds appear to have promoted enhanced cell survival or mitosis, retinoid treatment resulted in reduced numbers of cells compared to the control. The CEB compounds and the natural retinoids formed two groups that were distinct in their effects on proliferation at all time points, though large standard deviations in the data (predominantly due to biological variation between batches) resulted in statistically insignificant differences.

In older and highly confluent cultures, particularly the controls, cells were easily dislodged during media changes and the PBS-wash prior to addition of MTS reagent. This increased the variation between samples at later time points. Further errors were introduced by the presence of cell debris in some samples, which affected the absorbance readings. Once this effect was noted, further samples were centrifuged briefly and the UV absorbance of the supernatant was measured. The solution of MTS reagent + culture media removed from cells after 4 hours' incubation was diluted 1:19 with dH₂O to ensure that the absorbance reading was within the linear range of the spectrophotometer. This dilution was performed in triplicate for all samples, at all time points for consistency.

Flow cytometric data showed that differentiation was induced more slowly in cultures treated with the synthetic CEB retinoids than in cultures treated with the natural retinoids. The maintained or even enhanced proliferation observed in the MTS assay appears to confirm that cells treated with the CEB compounds were slower to exit the cell cycle and hence remained proliferative for longer than the natural retinoids. Alternatively, the CEB compounds may be less toxic than the natural retinoids at this concentration, or may otherwise promote differentiation in TERA2.cl.SP12 cells. Natural and synthetic retinoids are known to exert their biological effects through a number of pathways and mechanisms. In addition to mediating differentiation, anti-proliferative and apoptosis-inducing effects are also being harnessed in retinoid therapy.¹²

This study would be complemented by an analysis of the expression of Ki-67 during differentiation of different retinoids. This antigen is expressed only in proliferating cells and is lost when cells exit the cell cycle (G0), *i.e.* when cells have reached an end stage in their development, for example commitment to development of differentiated phenotype. This data would provide an authentic indication of the induction potential of the different retinoids.

Immunocytochemical analysis of neural and non neural antigen expression

The expression of genes known to be involved in the formation of the vertebrate nervous system was examined during retinoid-induced differentiation of TERA2.cl.SP12 cells. Immunocytochemical localisation permits the positive identification of cells that were previously only putatively labelled from their characteristic morphology. A qualitative assessment of the intensity and maturity of the patterns of expression of each antibody provides another indication of the maturity of each of the cultures.

Cultures induced to differentiate by all active retinoids expressed neuron-specific genes in a developmentally controlled manner. Nestin was expressed at an early stage of differentiation but was down-regulated as the cultures matured. Young neurons expressed TUJ-1 and then NSE and MAP2 as they matured. NF68 and NF200 staining further established that the neurons in these retinoid-differentiated cultures were mature and similar in structure to naturally occurring neurons.^{427,533}

Non-neural antigens were also expressed, consistent with the heterogeneous pattern of differentiation typically observed in response to ATRA. CEB16, CEB17 and CEB18 induced the formation of a greater number of cytokeratin 8-positive epithelial colonies than was observed in the other cultures. These compounds may promote epithelial differentiation, be less effective at inducing neuronal differentiation or these colonies may develop as a consequence of the higher cell density. Low levels of fibronectin expression were detected in the undifferentiated culture, opposing previous reports that human EC cells in culture do not synthesize fibronectin until they differentiate.⁵³⁴ Western blot

analysis did not detect fibronectin in cells that had always been maintained at optimal culture densities. The immunocytochemical detection of fibronectin may therefore indicate that these EC cells were slightly sub-optimal after being seeded at 20 000 cells/cm³.

The strength of immunocytochemistry (ICC) as a technique for studying gene expression lies in its ability to detect *and* localise a specific antigen in a cell. Whilst other methods such as real-time PCR or flow cytometry are better suited to the accurate measurement of expression levels, for which ICC can only be semi-quantitative, the additional information about localisation within a cell can distinguish between different gene isoforms with potentially different functionality.⁵³⁵ For example a number of different C8-positive cell types were observed which will not be distinguishable by western blot analysis.

Western blot analysis

Western blot analysis supported the qualitative assessments of antigen expression determined by immunocytochemical staining and also examined expression of the retinoic acid receptors and OCT4. Although data are only presented for one sample of protein for each retinoid/time point, further batches of some samples were analysed during preliminary experiments and were found to have similar levels of expression. Protein samples were collected from cells grown under the same conditions as previous experiments and all cultures that were analysed developed in a manner that was phenotypically identical to other batches. It is therefore believed that the western blot data presented is representative of retinoid induced differentiation of TERA2.cl.SP12 cells.

Western blot analysis confirmed the absence of differentiation in the control and LG100268 cultures. Expression of the pluripotency marker OCT4 was maintained and antigens associated with differentiation were not significantly up-regulated in these cultures. Conversely, the retinoid treated cultures suppressed OCT4 and this was accompanied by increases in expression of neural and non-neural antigens. Protein expression levels of the differentiated phenotypes are consistent with immunocytochemical data, the previously observed order of differentiation-inducing efficacy and previously published data for this cell line.^{425,426,428}

The control cultures showed some changes in protein expression from the day 0 undifferentiated TERA2.cl.SP12 EC cells. Cells were seeded at a lower density for the retinoid experiments compared with routine propagation of the cell line, and were maintained beyond confluency (which was attained approximately 5 days into an experiment). The resulting cells are therefore sub-optimal and this is reflected in altered antigen expression by the cells.

Retinoic acid receptor expression

This study demonstrates for the first time that retinoid receptors are expressed in TERA2.cl.SP12 huEC cells and that treatment with retinoids can modulate their expression. The protein expression profiles for RAR- α and RAR- γ are not easily explained from these data and may reflect a complicated response to retinoid treatment. RAR- α was not detected in undifferentiated EC cells but was up-regulated in some of the retinoid cultures; however, protein levels are too low for a clear analysis of relative expression. RAR- γ is expressed at high levels in the control cultures and appears to be down-regulated in the retinoid cultures, but not in a manner consistent with the stage of differentiation. The levels of both RAR- α and RAR- γ appear to fluctuate between time points, which may suggest multiphasic expression of the receptors during differentiation, or indicate high levels of biological variation.

Changes in the expression of the retinoid receptors intimates at their involvement in the differentiation of these cells with the retinoids tested. There is extensive evidence to suggest that ATRA is able to directly modulate the expression of its own receptors.¹⁷⁸ All retinoids in the western blot study except CEB16 and LG100268 are known to bind to the RARs. Although it is feasible that the RARs may be regulated indirectly by other processes related to CEB16 differentiation, it seems highly probable that this compound is also a *bona fide* RAR agonist and is activating the classical retinoid pathway.

RAR- α , RAR- β and RAR- γ are differentially expressed in different cell types and differentially regulated during ATRA-induced differentiation, suggesting that they may regulate different sets of genes.⁵³⁶ Gene expression studies in a number of EC cell lines have revealed some differences in the regulation of *RAR* transcripts during ATRA-induced differentiation. Broadly speaking, ATRA treatment induces moderate up-regulation⁵³⁷⁻⁵³⁹ or no change^{31,540-542} in the level of *RAR- α* mRNA transcripts, rapid and dramatic increases in *RAR- β* mRNA^{31,198,538,540-542} and either suppression⁵³⁸ or elevation^{198,539,542-544} of *RAR- γ* mRNA. Underlying this generalisation is a more complex regulation of the *RAR* isoforms, which was not investigated in this study, but further adds to the multiplicity of the retinoid pathway. Specifically, *RAR- α_2* is induced by ATRA treatment whilst *RAR- α_1* expression is unaffected.⁵³⁷ *RAR- β_2* is the major isoform of this receptor to be activated and *RAR- β_1* and *RAR- β_3* are not modulated.⁵⁴⁵ Furthermore, induction of *RAR- β_2* has been reported to occur in a temporary⁵⁴⁶ or biphasic manner, suggesting that multiple factors and/or different mechanism are involved in controlling its expression.⁵³⁸ Finally, *RAR- γ_2* mRNA is strongly induced while *RAR- γ_1* can act as a transcriptional repressor and antagonise *RAR- β* expression.^{543,544,547,548} Maintained expression of *RAR- γ_2* may require continuous exposure to retinoid.⁵⁴⁸ These primary target genes for ATRA are unified by the presence of retinoic acid-responsive elements within their promoters which permit rapid and specific gene regulation.^{544,549-551}

The western blot data for retinoid differentiated TERA2.cl.SP12 cells appear to be in good agreement with the reported expression behaviour for RAR- α , which was moderately up-regulated. Similarly, because the RAR- γ antibody only recognises the RAR- γ_1 isoform, the results are as expected and agree with one report of suppression of RAR- γ . Almost all reports of ATRA regulation of the retinoic acid receptors analyse the regulation of mRNA transcripts while only a few measure protein expression, the real biological effector. While gene expression is correlated to protein expression, it is not directly related and cannot be used to predict protein levels.⁵⁵² Furthermore, western blot analysis of RAR expression has been reported to not always detect these proteins, even when mRNA regulation has been observed.⁵³⁹ The above data should therefore be interpreted carefully. It is unfortunate that RAR- β was not detected using this protocol as RAR- β_2 is of particular interest given its strong association with tumour suppression^{48,269} and its expression can be used as a cellular marker of retinoid activity.⁴⁸⁷

Synergism with a rexinoid

LG100268 (2-[1-(5,6,7,8-tetrahydro-3,5,5',8,8'-pentamethyl-2-naphthalenyl) cyclopropyl]-5-pyridinecarboxylic acid) is an RXR- β specific agonist with 3-4-fold higher affinity and 10-fold greater transactivation activity for the RXR receptors compared to 9-*cis*-RA.^{201,217} As observed in the TERA2.cl.SP12 cells, RAR ligand-mediated physiological responses are separable and distinct from RXR ligand activation functions.⁵⁵³ Retinoid agonists are able to induce co-activator recruitment to RXR but cannot dissociate co-repressors from the transcriptional complex, a phenomenon known as RXR subordination.^{199,200,202} Consequently, in common with other RXR agonists, LG100268 is unable to induce differentiation alone.¹⁶⁸ However, numerous reports have demonstrated that RXR ligands have the ability to synergistically increase the potency of RAR ligands.^{168,200,204,205,554-556} Two explanations for this observation have been proposed: RAR/RXR heterodimerisation is significantly improved when ligands for both receptors are available²⁵⁹ and secondly that when both RAR and RXR ligands are present, stability of the RAR- β promoter occupancy is increased.²⁰⁵

RXR-selective ligands have favourable therapeutic potential, being less toxic and more active in animal breast cancer prevention studies and less toxic than RAR ligands in clinical trials.^{300,557,558} Rat studies have demonstrated the ability of LG100268 to prevent and treat breast cancer, both as a single treatment agent and to synergistically enhance the efficacy of Arozoxifene[®] (Eli Lilly).⁵⁵⁹ Similar behaviour and effects have been reported for LGD1069, a pan-RXR agonist (Targretin[®], bexarotene, E).^{395,560,561}

A key feature of the RXR receptors is that they are able to heterodimerise and consequently activate a number of different signalling pathways, including the RAR receptors,¹⁸⁰ thyroid hormone receptors,^{159,187,188} vitamin D receptor¹⁸⁹ and peroxisome proliferator-activated receptor γ (PPAR- γ).¹⁹¹ It is proposed that the mechanism(s) of action in preventing and treating breast cancer may involve the

concerted activation of several signalling pathways, including enhanced RAR/RXR activation and activation of TGF- β signalling.⁵⁵⁹

Previous studies in EC,¹⁹⁹ cervical carcinoma cells⁵²¹ and neuroblastoma cells⁵⁵⁴ have demonstrated strong synergistic effects on cell differentiation and inhibition of proliferation respectively by combining an RXR selective ligand with an RAR selective ligand. The activity of LG100268 was therefore investigated in combination with the other retinoids in Table 3-1 (Section 3.8). These compounds were either known RAR agonists, or were believed to be RAR agonists based upon their differentiation-inducing ability. TERA2.cl.SP12 cells were cultured with either 5 μ M of the RAR agonist or LG100268 alone, or with a combination of 5 μ M RAR agonist and 5 μ M LG100268. The concentration of DMSO in the control culture and all retinoid treated cultures was maintained at 0.1% w/w, consistent with previous experiments.

The induction of cell differentiation using a concentration of 5 μ M was less efficient than 10 μ M retinoid, as shown by comparing the phenotypic observations and flow cytometric antigen analysis of this experiment with previous studies (Sections 3.3 and 3.4). Although synergistic modulation of gene expression has been observed in other studies without translation into promotion of differentiation,^{199,562} it was found that co-administration of LG100268 with RAR agonists synergistically enhanced differentiation in all cultures, as predicted. The effects were greatest in the cultures treated with CEB18 + LG100268 and less apparent in combinations of LG100268 with the natural retinoids. RXR synergism has previously been shown to have the greatest effect when compounds are used at sub-optimal concentrations.²⁰⁵ The data presented here suggest that 10 μ M of the natural retinoids and CEB16 is sufficient to induce optimal differentiation under these conditions, whereas CEB17 and CEB18 may demonstrate superior efficacy at a higher concentration. Preliminary experiments were performed in an attempt to define dose-response curves for these compounds but a satisfactory method could not be established within the available time. Developing an experiment to measure changes in a rapid-response antigen or gene (for example suppression of *OCT4*) would provide an improved indication of retinoid efficacy compared to long-term analysis of phenotypic changes and slowly acquired markers. This would enable retinoid compounds to be rapidly and efficiently screened over a range of concentrations.

SH-SY5Y studies

Studies using the SH-SY5Y human neuroblastoma cell line provide evidence of the ability of CEB16, CEB17 and CEB18 to activate differentiation in a second cell type of clinical relevance. Both experiments reveal the same order of activity, which is the complete opposite to that observed in TERA2.cl.SP12 cells. All compounds are also significantly less effective than ATRA at inducing neurite outgrowth and resisting the apoptosis inducing effects of fenretinide. These data confirms the retinoidal activity of the three synthetic compounds and highlights the different responses of different cells.

3.11 Conclusions

ATRA is used extensively by molecular biologists to investigate differentiation in a large array of cell models and this ability is well characterised in the TERA2.cl.SP12 EC cell line. 9-*cis*-RA and 13-*cis*-RA bind strongly to the RXR family of receptors in addition to the RAR binding activity of ATRA,¹⁶¹⁻¹⁶³ which enables these molecules to act *via* alternative additional molecular pathways. A number of RXR agonists have been identified as potent inducers of growth arrest and apoptosis in some cancer cells.⁴⁷ All three isomers are approved for the treatment of dermatological disorders and have also been investigated extensively in pre-clinical and clinical studies, primarily in chemotherapy and prevention, to utilise their differential pharmacological profiles (Section 1.5).²⁹⁷ All three of the “natural” retinoids effectively and reproducibly induced morphological differentiation in this investigation. Subtle differences in antigen expression suggest that the three compounds possess different efficacies that may be attributable to different mechanisms of action, mirroring the contrasting efficacy, toxicity and pharmacokinetics observed in clinical studies.⁴⁸⁷

Morphological features present in retinoid and control cultures were identified based on their characteristic morphology, for example neurons are distinctive in having a round dark soma with extending processes. Flow cytometric analysis of stem cell and neural cell type markers was used to analyse the differentiation biochemically, complementing and substantiating the observed morphological differentiation. Differentiation was further characterised by a proliferation study and the analysis of neural and non neural antigen expression by immunocytochemistry and western blot.

The three CEB synthetic retinoids are all known. Extensive data pertaining to CEB18 (TTNN) and its retinoidal activities have been published, whereas information on CEB16 and CEB17 is restricted to their structures and potential activities. (See references in Table 3-1.) It was therefore expected that these compounds should have differentiation inducing effects, so it was gratifying to confirm that this preparation of these compounds was effective in the cell line of interest. All three compounds induce neural differentiation, but with different potencies: CEB18>>CEB17>CEB16. In common with ATRA, 9-*cis*-RA and 13-*cis*-RA, epithelial colonies are also observed; large numbers are observed in CEB16 cultures. Cultures grown with CEB16-18 continue to be proliferative for longer and result in very confluent, multi-layered growths with high media demands. This might be due to a lower potency, relative to ATRA and its stereoisomers, lower toxicities or alternative mechanisms promoting mitosis.

Two commercially available synthetic retinoids, Am580 and TTNPB were also investigated that were both found to induce high levels of neural differentiation. To date, all compounds known to possess

RAR activity have successfully effected the morphological differentiation of TERA2.cl.SP12 cells. The nature of these synthetic retinoids will affect their potency: they are expected to show greater physical stability, so are likely to persist in cultures for longer. It is also known that TTNPB and Am580 are not metabolised by the CYP26 enzymes that are primarily responsible for the removal of ATRA from cells.^{273,563}

The structures of arotinoids such as CEB16, CEB17 and CEB18 were designed by cyclising elements of the labile polyene chain present in ATRA with the aim of improving retinoid receptor binding selectivity and also to improve compound stability.^{213,362} Specifically the trimethylcyclohexenyl group has been replaced by a tetrahydro-tetramethyl naphthalene moiety, and the carboxylic acid end of the polyene chain made rigid by substituting an acrylic acid group (CEB16 and CEB17) or a naphthalene carboxylic acid group (CEB18).

The effect of media-changing frequency appears to significantly affect differentiation, presumably due to a rapid loss of active retinoid in the media, and merits further investigation. These data, together with preliminary stability results,^{352,402} highlight the need to appreciate the instability of ATRA and its isomers, and the effects that this may have on cell behaviour. CEB16, CEB17 and CEB18 were stable during synthesis and the extensive manipulation involved in purification. This was confirmed in a unique stability study which also highlighted the moderate instability of the acrylic acid group present in CEB16 and CEB17 under UV light.⁴⁰² These synthetic retinoids are considerably more stable than their natural counterparts.

It is anticipated that CEB16, CEB17 and CEB18 will also be significantly more stable to store, handle and under culture conditions. The use of stable pharmacological agents will lead to more reproducible and reliable biological activity, which may facilitate superior cell culture and, ultimately, provide reliable entities suitable for therapeutic applications. Furthermore, stable synthetic retinoids offer easier handling and storage properties over ATRA, removing a variable from biological experiments. They are also likely to persist longer during cell culture, through a combination of increased stability and inferior cellular metabolism. Taken together, these factors will enhance and prolong the exposure of cell cultures to the retinoid, with reduced exposure to metabolites and other degradants that may have a heterogeneous affect on cell behaviour.

Stable synthetic retinoids offer further advantages to *in vivo* studies. The profile of metabolic products to be analysed during ADME studies would be simpler and biological samples would not require the laborious and scrupulous procedures required to limit isomerisation of ATRA and other labile retinoids after collection, during storage and sample preparation.

These improvements seem to offer a significant advance in cell culture techniques. However, ATRA has been extensively studied and its effects have been characterised in numerous cell lines, animal models and in clinical studies; substituting an alternative differentiation-inducing agent introduces an unknown – researchers have no standards to compare against. Careful validation of the effects of synthetic retinoids in this context is therefore required. An additional consideration in the adoption of stable retinoids is safety: these compounds possess similar (if not greater) toxicity than ATRA, yet their stability will prolong this hazard.

42

**CHAPTER 4: INVESTIGATING THE
MOLECULAR MECHANISM OF
SYNTHETIC AND NATURAL RETINOID
ACTION**

4.1 Introduction

Endogenous retinoids are small hydrophobic molecules that associate *in vivo* with a number of soluble proteins with high selectivity and affinity. Taken together these components comprise a pathway that transports retinoids to their nuclear receptors in order to effect gene transcription, whilst simultaneously regulating cellular exposure to these potentially toxic molecules. It is becoming clear that biologically active synthetic retinoids, of interest for potential therapeutic applications, do not necessarily invoke the same mechanism of activation. It is necessary to identify and understand the effect of retinoids on the body, and if they interact with the classical retinoid pathway, in order to responsibly harness the therapeutic promise that retinoids offer.

This chapter presents the results of a study undertaken to define the role of the molecular components of the classical retinoid signalling pathway that are involved in synthetic retinoid-induced differentiation of TERA2.cl.SP12 cells.

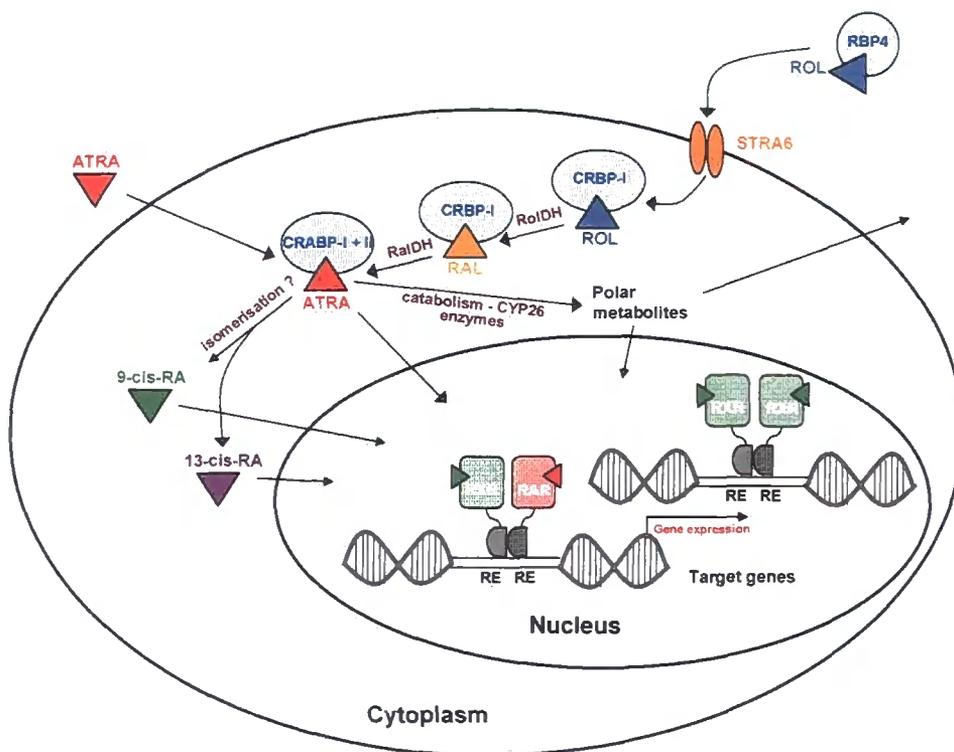


Figure 4-1: Schematic representation of the extra-visual retinoid signal transduction pathway. Abbreviations: ROL = retinol, RAL = retinal, RBP4 = serum retinol-binding protein, RolDH = retinol dehydrogenase, RalDH = retinaldehyde dehydrogenase, CRBP-I = cellular retinol-binding protein I, CRABP-I + II = cellular retinoic acid-binding proteins I and II, RE = DNA response element. Adapted from Klaassen and Braakhuis, 2002.¹⁰⁸ (Figure also presented as Figure 1-3 in Section 1.4, but included here to illustrate text.)

4.2 Retinoid-binding proteins: mediators of retinoid activity

Vitamin A is absorbed in the intestine, stored primarily in the liver and transported to target cells in blood, bound to serum retinol-binding protein (RBP4).⁴ Within the cell, retinol binds to cellular retinol-binding proteins (CRBPs) and undergoes a two-step oxidation process: first it is converted reversibly to retinaldehyde by retinol dehydrogenases and then irreversibly oxidised by retinaldehyde dehydrogenases to all-*trans*-retinoic acid (ATRA). The CRBPs possess a low affinity for ATRA, the major biologically active metabolite of vitamin A and it instead binds with cellular retinoic acid-binding proteins (CRABPs) which transport their ligands to two families of specific receptor within the nucleus, the retinoic acid receptors (RARs) and retinoic X receptors (RXRs). After binding with ligands, the RARs heterodimerise with the RXRs and together they bind to retinoic acid response elements (RAREs) in the promoter regions of primary target genes. This initiates a cascade of gene activation or repression that ultimately leads to a particular developmental or homeostatic event.⁶ The cellular retinoid-binding proteins, in combination with the cytochrome P450 ATRA hydroxylases, are also responsible for the control of ATRA catabolism, thus regulating cellular exposure to this essential vitamin. (Figure 4-1)

4.2.1 Cellular retinol-binding proteins

Retinol and ATRA circulate in the blood bound strongly to RBP4, whereas 9-*cis*-retinoic acid (9-*cis*-RA), 13-*cis*-retinoic acid (13-*cis*-RA) and synthetic retinoids are transported bound to serum albumin.⁹⁸ Retinol may then be transferred into target cells *via* STRA6 a specific glycoprotein cell membrane receptor,¹⁰⁵ while all other retinoids can presumably only enter cells by passive diffusion.¹⁰¹

Four human cellular retinol-binding proteins are currently known: CRBP-I¹⁰² and CRBP-II⁸⁰ have been thoroughly characterised and extensively investigated, while CRBP-III¹⁰⁹ (distinct from murine CRBP-III)⁵⁶⁴ and CRBP-IV¹¹⁰ have been identified as putative members of the CRBP sub-family. They are the cytoplasmic receptors of all-*trans*-retinol.^{80,110,565-567} The CRBPs are monomeric proteins of approximately 15.5 kDa, that possess a sequence homology of approximately 50% between any two CRBP.^{109,568} However each protein has significantly different affinities for all-*trans*-retinol: CRBP-I binds ~100 times more strongly than CRBP-II,⁵⁶⁹ ~600 times more strongly than CRBP-III,¹⁰⁹ and ~2000 more strongly than CRBP-IV.¹¹⁰ Considering that each protein also possesses distinct tissue expression patterns, these properties imply different roles for the different CRBP types. In humans, CRBP-I is found at low levels in a wide range of tissues with high levels of expression present in ovary, adrenal and pituitary glands, and testis.⁵⁶⁷ CRBP-II is localised to the enterocytes of the small intestine,⁸¹ where it absorbs retinol and facilitates its esterification by lecithin-retinol acyltransferase

(LRAT);⁷⁹ it is also able to bind retinal, which suggests that this protein may serve as a receptor for retinal formed during cleavage of β -carotene in the enterocyte. CRBP-III is expressed mainly in liver and kidney,¹⁰⁹ while CRBP-IV is expressed primarily in kidney, heart and transverse colon.¹¹⁰

The CRBPs possess both high specificity and binding affinity for retinol, their endogenous ligand, but discriminate against ATRA and *cis* forms of retinol.^{109,570} The CRBPs facilitate the enzymatic oxidation of retinol to either retinyl esters, for storage, or to retinal and subsequently ATRA. The CRBPs are involved in delivering retinol to microsomal NADP-dependent retinol dehydrogenase (RoldH), which it uses to form all-*trans*-retinal,^{111,112} and protect retinol from oxidation by other enzymes.²³⁵ Furthermore, the CRBPs facilitate the synthesis of ATRA by NAD-dependent cytosolic retinal dehydrogenases (RalDHs).¹¹⁴⁻¹¹⁶

4.2.2 Cellular retinoic acid-binding proteins

The cellular (or cytoplasmic) retinoic acid binding proteins, CRABP-I and CRABP-II are two homologous low molecular weight (~15 kDa) intracellular proteins that are found in all vertebrates and are highly conserved across species.^{118,119} As with the CRBPs, there is accumulating evidence to suggest that the two CRABP proteins perform different and potentially complimentary roles.

In the adult CRABP-I is almost ubiquitous, whereas CRABP-II is expressed only in skin, uterus, ovary and choroid plexus.¹¹⁹ During embryonic development both proteins are widely expressed although not in the same cells.¹¹⁹ The two proteins bind ATRA strongly, but with different affinities: CRABP-I binds almost twice as strongly as CRABP-II.¹²¹ The CRABPs bind only very weakly with 9-*cis*-RA and 13-*cis*-RA,^{163,571,572} presumably owing to their non-linear conformation,^{125,573} and the ATRA metabolites 4-oxo-ATRA and 4-hydroxy-ATRA also only bind weakly with the CRABPs.⁵⁷² They can also bind with a number of active synthetic retinoids with varying binding affinities, for example CD367 (compound **R** in Appendix 1) has a greater affinity than ATRA,⁵⁷¹ TTNPB (**D**) binds with similar affinity as ATRA and Am80 (**O**) has only a weak affinity.⁵⁷⁴ As with ATRA, CRABP-I and CRABP-II have different binding affinities for individual synthetic retinoids.⁵⁷⁴

Traditionally, these proteins were viewed simply as transport proteins, stabilising and protecting their lipophilic ligand from non-enzymatic oxidation and isomerisation.⁶ However, the CRABPs are also believed to play important roles in modulating the intracellular levels of ATRA in both the adult and developing embryo.^{118,119,575} Upon treatment with ATRA, CRABP-II, but not CRABP-I, translocates to the nucleus to deliver the ligand directly to the nuclear bound RARs.¹²⁰⁻¹²² This movement is facilitated by a conformational change upon retinoid binding, which permits the holo-form of CRABP-II to target the nucleus.¹²² CRABP-I is closely associated with regulating the metabolic fate of ATRA since differentiation rate, ATRA-degradation and rate of formation of polar metabolites have

all been shown to be proportional to the expression of CRABP-I.^{121,123,124,576,577} Furthermore, crystal structure studies have revealed that when ATRA is bound to the CRABPs the β -ionone ring of ATRA is accessible to solvent in a suitable orientation for presentation to metabolising enzymes.⁵⁷⁸

Elevated levels of CRABPs have been shown to be co-incident with the induced hypercatabolism observed during therapeutic application of retinoids, which leads to reduced drug sensitivity and resistance.^{274,325,326} However, it may be that CRABP regulation is a secondary factor in reducing plasma levels of ATRA during therapy, as indicated by a difference in the time-course of CRABP induction and reduction in ATRA levels, and that catabolic enzyme induction is more significant (see section 4.2.3).⁵⁷⁹

However, the biological functions of the CRABPs are not completely clear since they are not absolutely essential to the retinoid signalling pathway. The knockout of either one or both CRABP isoforms in mice resulted in essentially normal adult animals,^{580,581} yet mutant embryonal carcinoma (EC) cell lines that have little or no CRABP activity are differentiation-defective.^{582,583} Furthermore, despite low binding affinity to CRABP, synthetic retinoids Am80 (O), Am580 (P) and Ch55 (Q) regulate morphogenesis in the chick limb bud.⁵⁸⁴ Additionally, CRABP-I binding is also not required for retinoid reversal of keratinisation in hamster trachea,⁵⁸⁵ and HL-60 human promyelocytic leukaemia cells are induced to differentiate in response to ATRA but lack CRABP-I.⁵⁸⁶

4.2.3 CYP26: A family of retinoic acid specific P450 enzymes

In addition to the spatio-temporal regulation of all-*trans*-retinoic acid synthesis, co-ordinated by the CRBPs, CRABPs, RoLDHs and RaLDHs, the cellular concentration of ATRA is also controlled by metabolism and, it is proposed, isomerisation into its constitutional isomers 9-*cis*-RA and 13-*cis*-RA. ATRA is metabolised and subsequently deactivated by the specific and non-specific activity of cytochrome P450 (CYP) enzymes,¹²⁶ a diverse super-family of haemoproteins found in every class of organism that catabolise a wide range of both exogenous and endogenous compounds *via* mono-oxygenase enzymatic reactions.^{587,588}

The major CYP enzymes involved in the degradation of ATRA *in vivo* are the ATRA hydroxylases, CYP26,¹²⁷ currently divided into three isoforms in humans: CYP26A1,^{128,129} CYP26B1¹³⁰ and CYP26C1.¹³¹ A fourth member of the family, Cyp26D1, has also been identified in zebrafish.⁵⁸⁹ The CYP26 enzymes play essential roles in the regulation of ATRA exposure during embryogenesis and in adult tissues.¹³²⁻¹³⁷ The *CYP26* genes share extensive sequence similarity but differential spatial and temporal distribution suggesting individual roles in the catabolism of ATRA.^{128,131,590,591} All CYP26 enzymes catabolise ATRA in the adult and embryo to more polar metabolites.⁵⁹² CYP26A1 is also able to oxidise retinol to 4-oxo-retinol,¹⁴⁰ whilst CYP26C1 is more efficient at metabolising 9-*cis*-RA

than CYP26A1 or CYP26B1.¹³¹ Cyp26D1 can also metabolise 9-*cis*-RA and 13-*cis*-RA,⁵⁹¹ however a range of other cytochrome enzymes have been identified that are more effective at metabolising these isomers.¹⁴⁷ The metabolites of ATRA formed by CYP26A1 include 4-hydroxy-ATRA, 4-oxo-ATRA, 18-hydroxy-ATRA and 4-oxo-retinol.^{129,138-140} These intermediates can be further hydrolysed before they are converted to glucuronides and eliminated.^{6,141} The majority of these polar metabolites are inactive in the retinoid cycle, however exceptions include 4-oxo-retinol,¹⁴²⁻¹⁴⁴ 4-oxo-ATRA,^{28,142,593,594} 4-hydroxy-ATRA, 18-hydroxy-ATRA and retinoyl β -glucuronide.^{28,145,146} 9- and 13-*cis*-RA are similarly metabolised to their respective 4-hydroxy- and 4-oxo-metabolites (Figure 4-2).¹⁴⁷

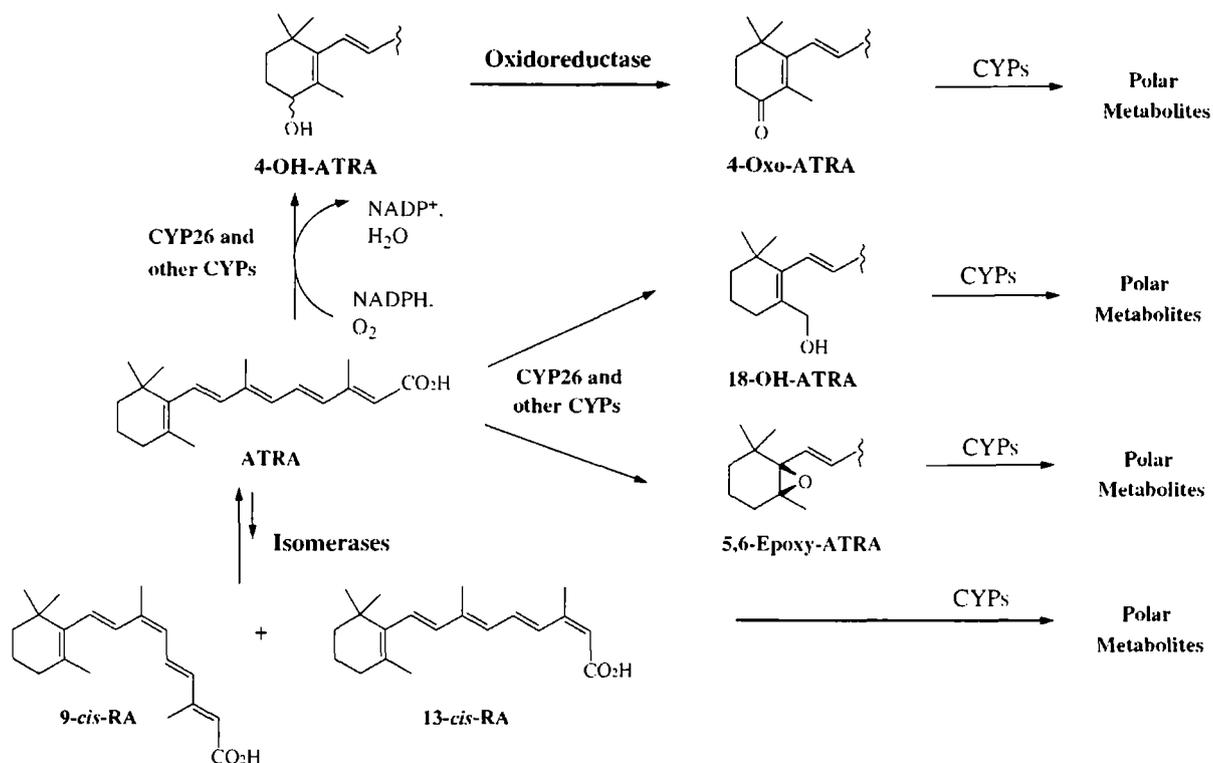


Figure 4-2: Metabolism of ATRA, 9-*cis*-RA and 13-*cis*-RA. Figure adapted from Bruon, 2007.¹⁴⁸ (Figure also presented as Figure 1-4 in Section 1.4, but included here to illustrate text.)

Expression of the CYP26s is induced by RAR/RXR heterodimer activation and can be blocked by RAR antagonists.^{327,595} Up-regulation of CYP26 mRNA is consequently observed in response to RAR agonists that are not metabolised by these enzymes, including Am580 (P) and CD367 (R), and results in a longer-lived up-regulation than is observed in response to ATRA.^{596,597}

Altered retinoid metabolism has been observed in a wide variety of human cancers, both in cell lines and in tumour tissue samples, which is thought to result in tumour cells that are ATRA-deficient.⁴⁸ The up-regulation of CYP26A1 accelerates the metabolism of ATRA in patients undergoing retinoid therapy and is likely to be a major factor in the development of reduced sensitivity or resistance to

retinoid therapy.^{325,327,333,598} Significant intra-patient variations in CYP26A1 expression are observed that correlate with efficacy of treatment. It is therefore proposed that pharmacokinetic monitoring during retinoid therapy may identify potentially receptive patients.³¹⁴ Employing a high-dose, intermittent regimen, rather than continuous low-dose treatment is one way to improve clinical outcomes.^{314,329}

A very promising alternative therapeutic area currently attracting a lot of research interest is the development of inhibitors of the CYP450 metabolism of endogenous and exogenous compounds.^{148,331} Retinoic acid metabolism blocking agents (RAMBAs) specifically block CYP26 activity, permitting an increase in endogenous retinoid levels for therapeutic benefit,³³² or alternatively act synergistically with ATRA or 13-*cis*-RA to inhibit the development of ATRA-resistance.³³³ This approach may yield effective agents for the treatment and/or prevention of cancers and dermatological diseases and will hopefully circumvent the extreme toxicities and resistance to traditional retinoid therapy.

Liarozole (Liazal[®] Johnson and Johnson Pharmaceutical Research and Development, S) is an imidazole derivate that has been clinically evaluated for dermatological disorders and prostate cancer, with further positive activity in cancer cell lines. Liarozole has been approved for the treatment of congenital ichthyosis; however it is a relatively weak and non-specific inhibitor of CYP26 and has consequently ceased anti-neoplastic clinical development.^{590,599,600} A large number of more specific azolyl RAMBAs have been developed, including Rambazole[®] (Talarozole, R115866, Barrier Therapeutics, T)⁶⁰¹ and R116010 (Barrier Therapeutics, U)^{602,603} which have been investigated for clinical dermatological effects⁶⁰⁴ and *in vitro* anti-cancer properties respectively.^{328,605} Other novel inhibitors have been developed as potential anti-cancer agents.^{331,606}

The atypical retinoid fenretinide is unusual in that CYP26A1 is involved in its metabolism,⁵⁹⁸ while other synthetic retinoids including CD367 (R), Tamibarotene (Am80, O), Am580 (P) and TTNPB (D) are not substrates for the CYP26 enzymes.^{273,563,596,597} Retinoids that are not recognised and hence not metabolised by this enzyme may therefore be more effective inhibitors of growth in susceptible cancer cells.⁵⁸⁷ This is one feature that enabled Tamibarotene to reintroduce complete remission in patients with acute promyelocytic leukaemia (APL) that had previously developed resistance to ATRA-treatment.^{275,330} Tamibarotene is also unable to bind to the CRABPs, yet is less toxic than ATRA partially because of the lack of binding affinity to RAR- γ .^{267,277} Poor metabolism is also a major factor in the remarkable toxicity of TTNPB, which also possesses reduced affinities for the CRABPs and prolonged nuclear receptor activation.⁵⁶³ Other enzymes may be involved in the metabolism of synthetic retinoids, for example Tamibarotene is metabolised by CYP3A4.²⁶⁷

4.2.4 Retinoic acid receptors

The retinoic acid receptors (RARs) play a pivotal role in the activation of retinoid-responsive genes. Regulation of receptor expression has consequently been extensively studied in an attempt to delineate the specific roles of the individual receptors and how they are modulated by retinoid treatment. For example, aberrant expression or regulation of the RARs is associated with various types of cancer,⁴⁶⁻⁴⁸ while expression of RAR- β_2 is of particular interest as it is strongly associated with tumour suppression.^{48,269,607} The general structure, features and functions of the two families of retinoid receptors, the RARs and the RXRs have been described previously (Section 1.5).

Crucially, the binding of ligand-bound RAR/RXR heterodimers to the response elements of a number of components of the retinoid pathway is responsible for inducing the transcription of these genes. This has been shown to be true for both ATRA and a number of synthetic retinoids.¹⁹⁸ Furthermore, *RAR- α* , *RAR- β* and *RAR- γ* are differentially expressed in different cell types and are differentially regulated during ATRA-induced differentiation, suggesting that they may regulate different sets of genes.^{536,608} For example, in some EC cell lines *RAR- α* is required for the induction of *RAR- β* , without which the cells fail to differentiate⁶⁰⁹ and the activation of the developmentally important homeobox *HOX* genes has been shown to be dependent on the induction of *RAR- α* ⁶¹⁰ and *RAR- β* .⁶¹¹ Specific receptors are also believed to act cooperatively in order to signal terminal growth suppression and maturation of NTERA2/D1 human EC (huEC) cells.⁶¹²

Novel synthetic retinoids have traditionally been assessed by comparing the affinity of the new compounds with that of ATRA for the retinoid receptors. It is also important to consider the ability of compounds to transactivate the receptors in functional assays, since many isotype-binding specific retinoids have subsequently been found to be less selective at activating the receptors.

4.3 HOX gene expression

The homeobox gene family are defined by the presence of an eponymous short DNA sequence which encodes a DNA-binding protein domain known as the homeodomain.⁶¹³⁻⁶¹⁵ The *Hox* gene family are the most studied and the most evolutionarily conserved group of the homeobox genes and the Hox proteins that they encode are putative master regulators of development, responsible for specifying the antero-posterior axis and controlling segmental patterning during embryogenesis.^{34,39-44}

The mammalian genome is currently known to contain thirty-nine *Hox* genes (designated *HOX* in humans⁶¹⁶) that are organised into four different clusters (*HOXA*, *HOXB*, *HOXC* and *HOXD*) found at four distinct chromosomal *loci* (7, 17, 12 and 2 respectively).⁶¹⁷⁻⁶²² Each gene is also assigned to one of thirteen paralogous groups based on sequence homology, analogous expression domains and occupation of similar positions along the chromosome (Figure 4-3).^{621,623}

Hox genes are activated and expressed in intricate spatial and temporal patterns during embryonic development.^{39,41,619,620} During initial stages, these expression events demonstrate spatial collinearity in that they form a sequential pattern of partially overlapping domains along the antero-posterior axis in coincidence with the 3' to 5' order of the genes in the *Hox* cluster. Additionally the genes are activated one after the other in the 3' to 5' order, which is termed temporal collinearity (Figure 4-3).^{42,624-626} The regulatory mechanism(s) responsible for this precise control of gene activation are poorly understood. It is believed that morphogenic signalling molecules including ATRA are responsible for the regulation of *Hox* gene expression, through direct and/or indirect control, which may involve graded sensitivities to ATRA concentrations.^{20,626-628} Furthermore, it is proposed that vertebrate *Hox* genes may synergistically co-operate in order to enhance or silence other *Hox* genes within the same complex.⁶²²

Aberration of the normal pattern of *Hox* gene expression results in homeotic transformations (duplication of head-tail axial structures) and malformations.^{39,623,629-633} Mutations in human *HOX* genes are observed to result in limb development defects; for example the duplication of a digit (polydactyly) occurs as a result of mutations in the *HOXD13* gene.⁶³⁴ *HOX* genes continue to be expressed in some normal adult tissues suggesting a role in maintenance,⁶²⁸ however the expression of *HOX* genes may be deregulated in cancers implicating the participation of *HOX* genes during carcinogenesis.⁴⁹⁻⁵⁴

Considerable experimental evidence strongly suggests that ATRA is an important modulator of *Hox* gene expression. Numerous *in vitro* studies have shown that homeobox genes are regulated during

ATRA-induced differentiation of cultured cells,^{540,635-641} *HOX* gene expression may be induced during retinoid therapy and thus contribute to tumour progression, potentially limiting its efficacy.⁶⁴² Both a deficiency and excess of ATRA are observed to disrupt normal vertebrate development in a similar manner to *Hox* gene mutations.^{21,32,643,644} Further evidence suggesting that ATRA has a normal role in embryogenesis comes from the restricted yet co-localised distribution of retinoic acid receptors,⁶⁴⁵⁻⁶⁴⁷ cytoplasmic retinoid binding proteins⁶⁴⁸ and areas of ATRA synthesis,^{26,649-653} which overlap with many of the sites in the embryo that are sensitive to ATRA.⁶³⁶ The gene transcription site of *Hoxa1* in F9 murine EC (muEC) cells contains an ATRA-inducible enhancer that contains an RARE.⁶⁵⁴ Similar response elements have also been found in human and murine *HOXB1/Hoxb1*⁶⁵⁵⁻⁶⁵⁹ and *HOXD4/Hoxd4* genes,^{611,660} suggesting that the expression of these genes may be directly responsive to ATRA-signalling via ligand-bound RAR-RXR heterodimers. This regulatory relationship is dynamic and has been shown to involve feedback regulation.^{610,661-664}

It is therefore suggested that endogenous retinoids may be vital for establishing the antero-posterior graded expression of *Hox* genes and thus influence antero-posterior identification along the body axis. The net effect of ATRA treatment can be said to recapitulate the differential *Hox* gene expression pattern seen in early embryogenesis and likely represents the posterior transformation signal in early central nervous system development.⁶²⁸

Of particular note for the study described in this chapter, ATRA has been demonstrated to induce *HOX* genes with gene-specific kinetics in the NTERA2/D1 human teratocarcinoma cell line.^{540,640,641,665} Detailed studies have established that within each cluster the gene located most 3' responds to low concentrations of ATRA, while increasingly higher concentrations are needed to activate successively more 5' positioned genes. At a given concentration of ATRA, the 3' genes are induced very rapidly, while transcripts from genes positioned successively more 5' respond after a few days of treatment.^{540,640,641}

In perfect accordance with collinearity, paralogue group 1 are the first members of their clusters to be activated and display the highest sensitivity to exogenous ATRA.^{623,666} *Hoxa1* is the first gene induced, and is required to initiate expression of the *Hoxb1* gene.⁶⁶⁷ Other *Hox* genes located in more 5' positions are not modulated directly by ATRA, it is hypothesised that they may be activated following expression of *Hoxa1* and *Hoxb1*, presumably through the protein products of these genes, leading to sequential activation of *Hox* genes.⁸ For example, activation of both *Hoxb2* and *Hoxb3* are regulated by *Hoxb1*,^{668,669} although knockout of the murine *Hoxa1* gene does not alter the ATRA-induced expression of other *Hoxa* cluster genes.⁶⁷⁰

In addition to their general patterning role during embryogenesis, specific functions for some of the *Hox* genes have been reported. For example, the *Hoxb* gene cluster is expressed in the developing

hindbrain and spinal cord, and may help to establish pattern and positional identity in neural development.^{8,671-673} *Hoxb1* is required for the specification of facial motor neurons and disrupting this gene results in facial paralysis in mice, a phenotype that resembles the human conditions of Bell's Palsy or Moebius Syndrome.⁶⁷⁴ *Hoxb1* also plays a critical role in the antero-posterior axis patterning of the gut.⁶⁷⁵

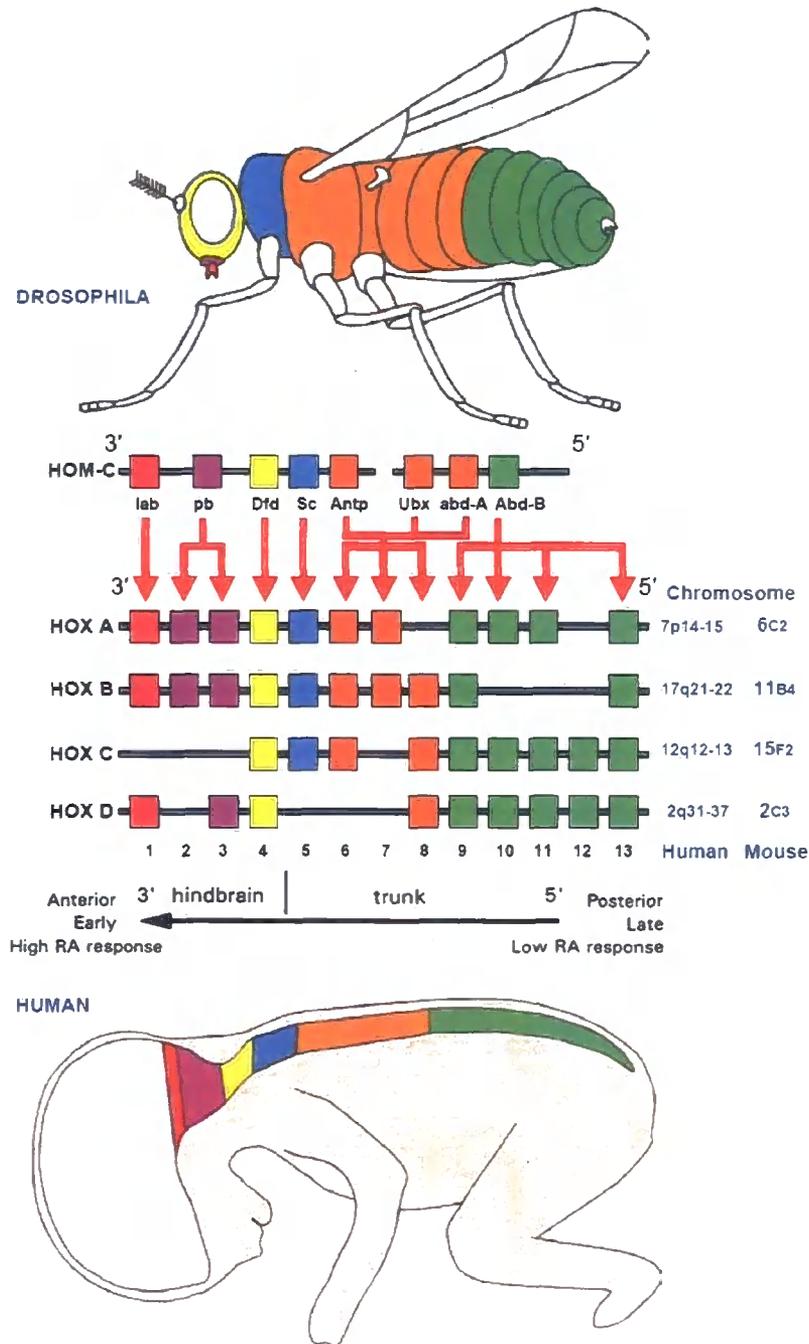


Figure 4-3: Conservation between the *Drosophila melanogaster* *Hom-C* and *Homo sapiens* *HOX* gene clusters. The four mammalian *Hox* gene clusters are conserved from the *Hom-C* complex in terms of sequence and co-linear expression. During embryonic development and ATRA-induced differentiation, the genes are expressed in a pattern that correlates with chromosomal positioning, namely the 3' genes are expressed both earlier and more anteriorly than the 5' genes. Figure prepared by combining and modifying figures from Lappin *et al.*, 2006 and Maconochie *et al.*, 1996.^{623,676}

4.4 POU5F1 (OCT4) transcription factor

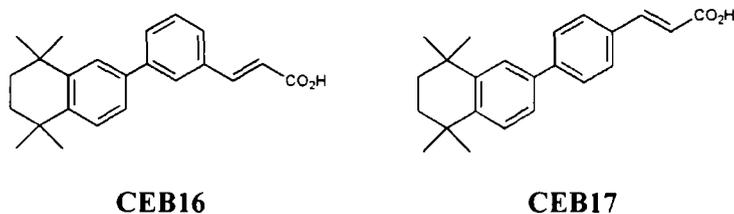
POU5F1 (*Pou5f1* in species other than *Homo sapiens*, previously known as OCT4 or OCT3) is known as the “guardian of pluripotent phenotype”.⁶⁷⁷ It is the earliest transcription factor, a DNA-binding protein required for the activation and regulation of expression of genes, known to be expressed during embryonic development. POU5F1 belongs to the sub-group of octamer-binding proteins which bind by the POU (Pit-Oct-Unc) domain to promote and enhance regions of various genes;⁶⁷⁸⁻⁶⁸² it is a key component in the molecular control of stem cell pluripotency and orchestrates the expression of genes associated with stem cell character, including *NANOG*^{683,684} and *SOX2*,⁶⁸⁵ and represses genes that promote developmental processes.⁶⁸⁶ Although the roles of these factors have not been fully elucidated, it is clear that their down-regulation correlates with the loss of pluripotency and self-renewal, and the initiation of differentiation.⁶⁸⁶⁻⁶⁸⁹

High expression of the *Pou5f1* gene is found in the blastocyst and epiblast of the developing mouse embryo but is subsequently restricted to primordial germ cells and silenced in all somatic cell lineages.^{677,681,690,691} During human development *POU5F1* mRNA is continuously expressed between the time of fertilization and 10+ cell stages.⁶⁹² Precise control of *Pou5f1* expression is essential for normal embryonic development.^{505,677,691,693} *Pou5f1* is also associated specifically with undifferentiated EC cells, embryonic germ cells, and embryonic stem (ES) cells^{680,681,693-695} and is rapidly down-regulated during differentiation of these cells, recapitulating *in vivo* observations.^{680,696-698} Furthermore, knockdown of POU5F1 leads to differentiation of EC and ES cells.⁶⁹⁹ *Pou5f1* has consequently become widely used as an important marker of undifferentiated pluripotent stem cells and has been identified as a potential marker in developmental toxicity testing.⁷⁰⁰

Two different isoforms have been identified: POU5F1_iA, or OCT3A/OCT4A and POU5F1_iB, or OCT3B/OCT4B.⁷⁰¹ POU5F1_iA is required for pluripotency, but the function of the POU5F1_iB isoform has yet to be identified.^{702,703} A number of pseudogenes are also known, these non-functional genes or gene fragments may be mistakenly identified as functional transcripts.⁷⁰⁴ *POU5F1* expression has also been demonstrated in terminally differentiated cells, suggesting that the presence of *POU5F1* is not sufficient to define a cell as pluripotent.^{535,701,705} However these data contradict previously published work and it has been proposed that the authors detected *POU5F1_iB*, not *POU5F1_iA*.^{706,707} The *POU5F1* gene is expressed in a number of adult stem cells, suggesting that these cells are potential pluripotent stem cells and could be the target cells for the initiation of the carcinogenic process.⁷⁰⁸ *POU5F1* is also expressed in human tumours, which may provide an opportunity for targeted cancer treatment.⁷⁰⁹

4.5 Experimental objectives

The aim of the study described in this chapter was to investigate the effects of ATRA and two synthetic retinoids, selected from those studied in the initial *in vitro* experiments (Chapter 3), on components of the retinoid pathway in TERA2.cl.SP12 human EC cells. The two cinnamic acids CEB16 and CEB17 were chosen to compare the effect of the position of the terminal carboxylic acid group on activity. In CEB17, the CO₂H group is in a *para* position to the tetrahydrotetramethyl naphthalene group, mimicking the configuration of all-*trans*-retinoic acid, whilst CEB16 is its *meta* isomer, bearing a similarity to 13-*cis*-retinoic acid. Both compounds were found to induce differentiation in TERA2.cl.SP12 cells, but at a slower rate than ATRA, and with moderately different proportions of terminal cell types.



TERA2.cl.SP12 cells were grown in the presence of either 10 μ M or 0.1 μ M of ATRA, CEB16 or CEB17 for up to 14 days. Cells were also grown with 0.1% v/v DMSO (14 mM) to control for the effects of the vehicle. The media was replaced with fresh retinoid- or DMSO-enriched media after 3 and 5 days' culture and then subsequently every day. Phenotypic changes in the cultures were observed and phase microscope images recorded after 7 and 14 days' culture. RNA was extracted from the cultures at ten time-points and the expression of eleven genes quantified by real-time reverse transcription-polymerase chain reaction. The expression of mRNA relative to a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) was profiled for *POU5F1*, *CRBP-I*, *CRABP-I*, *CRABP-II*, *CYP26A1*, *RAR- α* , *RAR- β* , *RAR- γ* , *HOXB1*, *HOXB2* and *HOXB3* genes. *POU5F1* was analysed in order to correlate the differentiation status of the cultures with the other genes of interest. The three genes located at the 3' end of the HOXB cluster were analysed to provide a further functional characterisation of the effects of synthetic retinoids.

Due to time limitations the data presented are from unique biological samples.

4.6 Results and discussion

4.6.1 Observation of changes to cell phenotypes

TERA2.cl.SP12 huEC cells were seeded at the standard density of 20 000 cells/cm³ and allowed to adhere for 24 hours before the normal media was replaced with media supplemented with either 0.1% v/v DMSO or freshly prepared solutions of 10 μM or 0.1 μM of ATRA, CEB16 or CEB17. The delayed addition of retinoid relative to the experiments reported in Chapter 3 was a practical necessity in such a large experiment. The cultures in this investigation were therefore slightly more confluent than in previous studies (compared to Section 3.3) and may consequently have developed at a slightly slower or different rate.

Phase images were recorded after 7 and 14 days' culture with retinoids to document the phenotypic changes that occurred during the experiment (Figure 4-4 and Figure 4-5). The DMSO cultures became significantly overgrown but did not develop the heterogeneity associated with cultures undergoing retinoid-induced differentiation nor develop any identifiably differentiated phenotypes. The cultures treated with 0.1 μM CEB16 developed identically to the control cultures: it would appear that the cells failed to differentiate. All other cultures underwent some phenotypic changes consistent with the induction of differentiation. As previously noted (Section 3.3), 10 μM ATRA induced differentiation in the TERA2.cl.SP12 cells more effectively than either 10 μM CEB16 or 10 μM CEB17. Treatment with 0.1 μM ATRA produced minor changes in the cells after 14 days' culture, including a reduction in cell number compared to the control and the development of several small epithelial colonies. The 0.1 μM CEB17 cultures became moderately heterogeneous by the end of the experiment and a number of epithelial colonies developed.

The effects of lower concentrations of ATRA and other retinoids on TERA2.cl.SP12 cells have not previously been reported. Concentrations of only 0.1 μM of ATRA and 0.1 μM CEB17 induced a degree of differentiation in the cells, although the changes were slower to appear than in the corresponding 10 μM cultures. Morphological observations suggest that 0.1 μM CEB16 was ineffective, which is consistent with this compounds previously observed low level of relative potency. No neuronal cells were observed in the 0.1 μM ATRA and CEB17 cultures at the 14 day time point but these cultures contained more epithelial colonies than their 10 μM counterparts. Different concentrations of retinoids may affect the differentiation propensity of these cells. Alternatively these phenotypes may have developed as a consequence of the increased cell density, which may also partly explain why the CEB compounds, in particular 10 μM CEB16, induce the formation of a greater number of epithelial colonies than the natural retinoids.

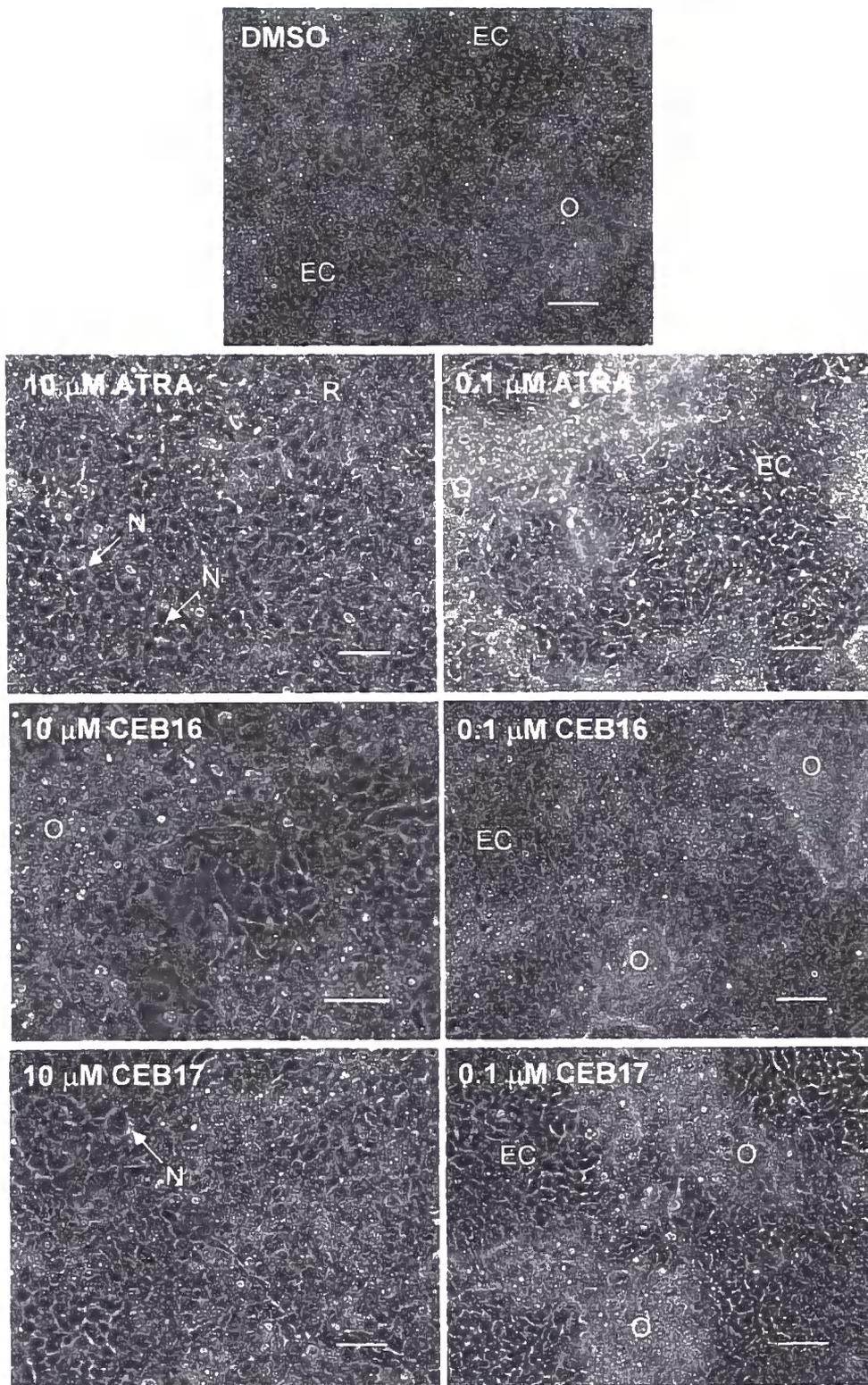


Figure 4-4: Phase images of 7 day old TERA2.cl.SP12 huEC cultures treated continuously with 0.1% v/v DMSO or either 10 μ M or 0.1 μ M of ATRA, CEB16 or CEB17. The DMSO control shows no phenotypic changes indicative of differentiation. All cells are EC-like (EC), possessing the typical high nuclear to cytosol ratio, and are starting to over-grow (O) as the culture has surpassed confluency. Cultures grown with 10 μ M retinoid are less dense than the control and consist of heterogeneous cell types. The 10 μ M ATRA culture is most developed; a few putative neurons (N) and a developing rosette (R) are visible. The 10 μ M CEB17 culture is slightly more developed than the 10 μ M CEB16 culture, consistent with previous efficacy observations. Of the lower concentration cultures, only the 0.1 μ M ATRA culture shows slight signs of heterogeneity, the other cultures resemble the DMSO culture and remain phenotypically undifferentiated. Scale bar represents 50 μ m.

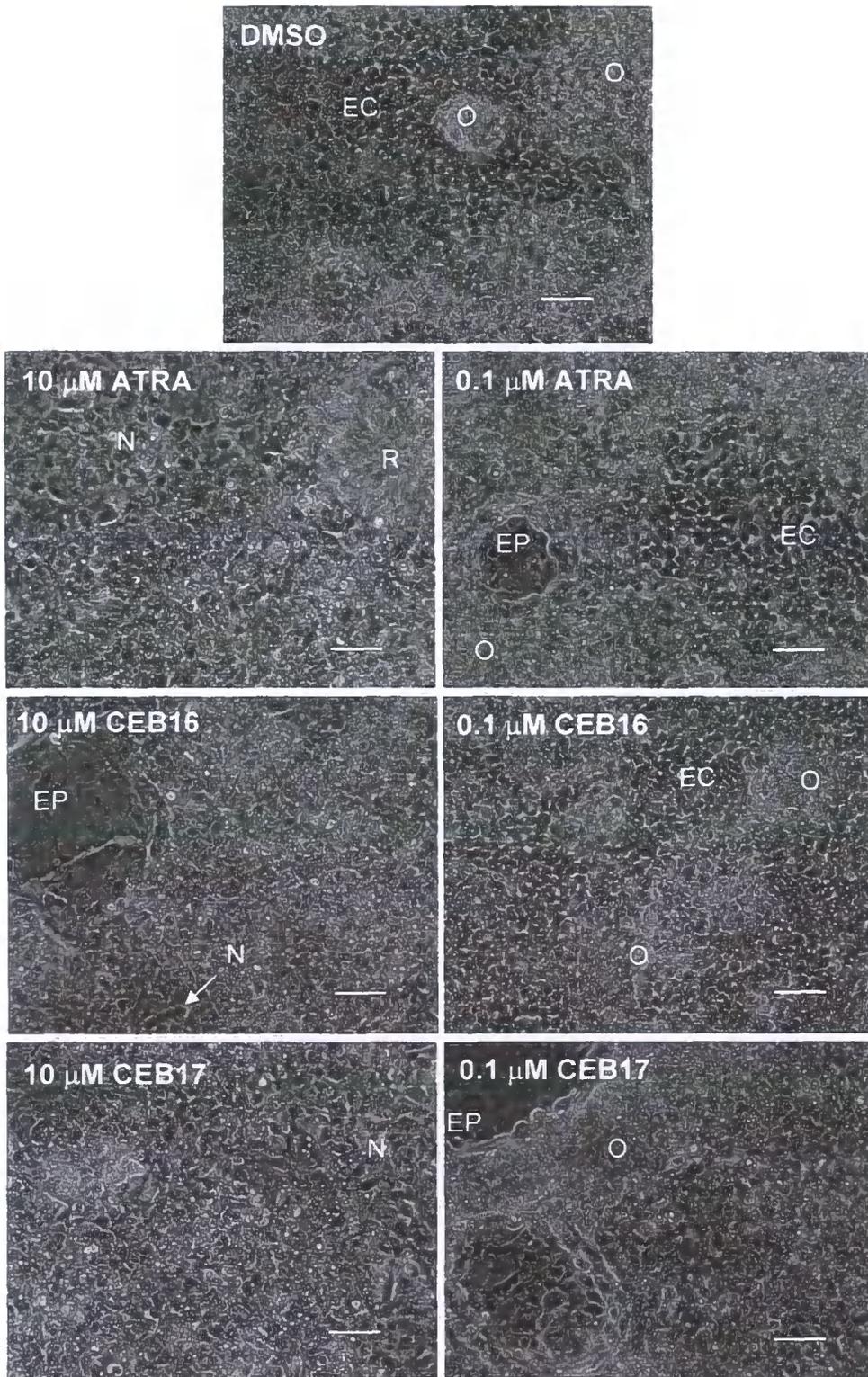


Figure 4-5: Phase images of 14 day old TERA2.cl.SP12 huEC cultures treated continuously with 0.1% v/v DMSO or either 10 μ M or 0.1 μ M of ATRA, CEB16 or CEB17. The control culture is very overgrown (O) but there are no differentiated phenotypes visible. The 10 μ M ATRA culture is the most differentiated: highly heterogeneous with a number of rosettes (R) and developing putative neuronal colonies (N). The cultures grown with 10 μ M of either synthetic retinoid are also undergoing morphological changes consistent with differentiation. Epithelial colonies (EP) and putative neurons are visible in the very dense cultures. The 0.1 μ M CEB16 culture is indistinguishable from the DMSO control culture. EC-like cells are visible amongst very overgrown areas. The 0.1 μ M ATRA and CEB17 cultures are heterogeneous and present a number of differentiated phenotypes but are significantly less developed than the 10 μ M cultures. Scale bar represents 50 μ m.

4.6.2 Induction of differentiation – POU5F1 expression

The transcription factor *POU5F1* is expressed in undifferentiated TERA2.cl.SP12 cells and down-regulated as the cells undergo differentiation, both in response to retinoid treatment and as a consequence of the sub-optimal culture conditions of highly confluent cultures (Figure 4-6). Expression of *POU5F1* mRNA in the control culture is suppressed after the time-point at 3.5 days' culture, gradually reaching basal levels between 7 and 14 days. These observations correspond with the culture reaching confluency (four days after the start of the experiment, five days after seeding) and beginning to overgrow. This process appears to have reduced the stem cell potential of the cells though the cells remain somewhat proliferative and no phenotypic features consistent with differentiation are observed. Changes in the expression of other genes investigated in this study are also observed in cells grown in control conditions, confirming that over 14 days these cells have genotypically changed from TERA2.cl.SP12 huEC cells. These observations must be considered when analysing the gene expression profiles of cells treated with retinoids; especially, the cultures grown in 0.1 μM CEB16 and 0.1 μM CEB17 that become significantly overgrown during the experiment.

The cultures treated with 10 μM of retinoid down-regulated *POU5F1* faster than the lower concentration of the same retinoid, consistent with phenotypic observations of differentiation. However, in contrast to phenotypic observations, the three retinoids modulated *POU5F1* at approximately similar rates, although ATRA was slightly more effective than CEB16 or CEB17. The 10 μM CEB16 and CEB17 cultures responded at approximately the same rate, but the CEB16 0.1 μM culture lost pluripotency at a similar rate to the control, whilst the CEB17 0.1 μM expression profile was very similar to that of 0.1 μM ATRA. All retinoid cultures, but not the control condition, showed an increase in *POU5F1* expression from the first time-point. This transient elevation has also been reported during the ATRA-induced differentiation of F9 muEC cells, but without explanation.⁷¹⁰

The 10 μM ATRA result is consistent with previously published data for TERA2.cl.SP12 cells⁴²⁵ and the 10 μM results for all retinoids correlate well with the levels of OCT4 protein detected by western blot (Section 3.7). The kinetics of *POU5F1* regulation are comparable to those observed in PCC7 muEC cell line,⁵³⁹ but slower than many other cultured cells. For example, the expression of *Pou5f1* mRNA in P19 muEC cells treated with 1 μM ATRA is significantly decreased within 24 hours and undetectable after 72 hours.^{697,711} NTERA2/D1 huEC cells also respond faster, with complete down-regulation, as detected by northern blot, within 72 hours.⁷¹² In F9 muEC cells protein levels are substantially suppressed after 48 hours' treatment.⁶⁷⁸ PCC7 cells have also been used to demonstrate the down-regulation of *Pou5f1* protein levels during synthetic retinoid induced differentiation.⁵³⁹

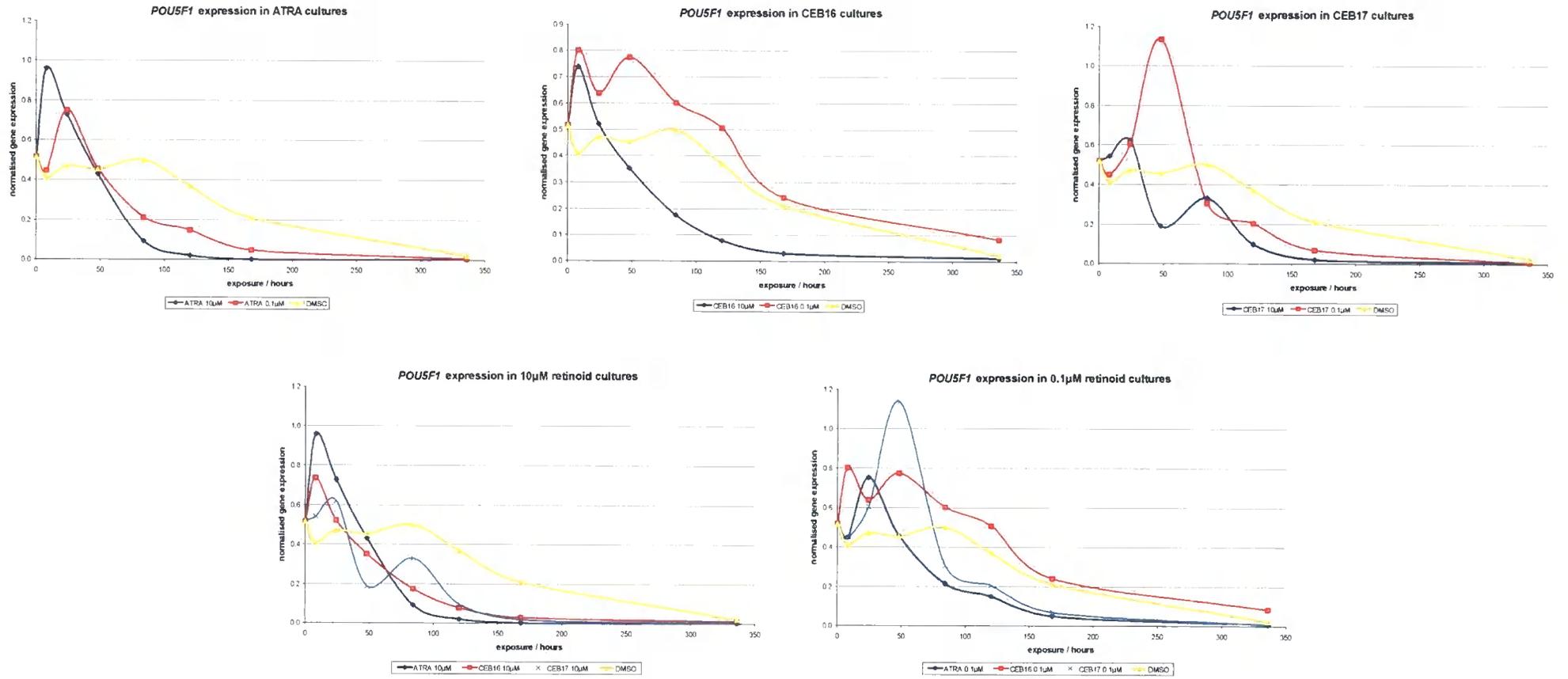


Figure 4-6: Concentration and time-dependent regulation of *POU5F1* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

It is suggested that some published studies may have erroneously identified the expression of *POU5F1* pseudogenes, rather than *POU5F1* itself, by relying solely on reverse transcription-PCR analysis.⁶⁹⁵ Whilst protein was not extracted from these cultures, other experiments conducted under identical conditions have demonstrated the presence of POU5F1 protein by positive immuno-labelling with POU5F1 specific antibodies (Section 3.7). It therefore seems reasonable to infer that the mRNA detected in this qRT PCR assay is translated into functional protein. It is hoped that analysis of more samples would smooth the *POU5F1* expression profiles whilst establishing the biological variation of this gene in TERA2.cl.SP12 cells.

4.6.3 Regulation of retinoid-binding protein expression

Expression of *CRBP-I*

Cellular retinol-binding protein (*CRBP-I*) transcripts are expressed at low levels in undifferentiated TERA2.cl.SP12 cells (Figure 4-7) and are only moderately up-regulated with retinoid treatment. This low level of expression was confirmed by the detection of only a faint band when a PCR product was analysed by electrophoresis (Figure 4-17). The fluctuations in the low levels of expression of this gene in the control culture during the first seven days highlights the limit of detection, such that from just one biological repeat the variations in expression observed for the majority of cultures can only be described as insignificant. Treatment with ATRA induced elevated levels of *CRBP-I* mRNA and this was the only retinoid for which a clear response was observed at 0.1 μM . This data is supported by the observation that CRBP-I protein is also up-regulated in these cells in response to ATRA treatment.⁷¹³

The kinetics of *CRBP-I* expression follow a complicated pattern, which may be partly explained as a response to the provision of fresh retinoid when the media was changed. Examining the 10 μM retinoid graph (Figure 4-7), an increase in gene expression is observed at the 3.5 day time-point in all retinoid conditions (after a media change at 72 hours) and a further increase is observed in the 10 μM CEB16 culture at the 7 day time-point (168 hours) after a media change at 120 hours. Depletion of retinoid in the culture media between media changes may have affected expression of this gene, which then responds in a magnified manner when new retinoid is supplied to the culture. Expression of *CRBP-I* in the 0.1 μM ATRA and CEB17 cultures appears to transiently increase at the initial time-points of the experiment before returning to basal levels after 5 days' culture. The ATRA 0.1 μM culture then appears to respond to the supply of fresh retinoid at 120 hours, before returning to low levels by the end of the experiment. A biphasic expression pattern has also been reported in ATRA-treated NTERA2/D1 huEC cells, without further explanation or correlation with culture conditions.⁵⁴²

Ostensibly the up-regulation of *CRBP-I* is intriguing: the protein is unable to bind to either ATRA or the synthetic retinoids and ATRA cannot be reduced to retinol *in vivo* so this modulation is unable to

have a direct effect. It is proposed that CRBP-I is a component of a feedback mechanism that is initiated by the presence of high cellular levels of retinoid; CRBP-I may be up-regulated to sequester excess retinol, the *in vivo* precursor of ATRA, and control cellular exposure to retinoids. The expression of *CRBP-I* is up-regulated by RAR activation of a functional RARE-related sequence within its promoter⁷¹⁴⁻⁷¹⁶ and cannot be blocked by cycloheximide, a protein synthesis inhibitor.⁷¹⁷ This is therefore a direct response to ATRA treatment.

ATRA-induced up-regulation of *CRBP-I* is also observed in F9 muEC cells,¹⁹⁸ P19 muEC cells,⁷¹⁷ NTERA2/D1 huEC cells⁵⁴² and rats,²²² however in all cases significant responses were observed within hours of treatment compared to the moderate changes that occur in TERA2.cl.SP12 over several days. Published data corroborate the modulation of *CRBP-I* in TERA2.cl.SP12 cells by synthetic retinoids. Treatment with 9-*cis*-RA (**B**) and synthetic retinoids Am80 (**P**) and Ch55 (**Q**) induces a strong up-regulation of *CRBP-I* in rats, comparable to the response observed with ATRA.²²² The apoptosis-inducing atypical retinoid fenretinide promotes a marked up-regulation in human ovarian tumour cells,⁵⁹⁸ which is of particular interest as dysfunctional regulation of CRBP-I may be observed in human cancers.^{55,56}

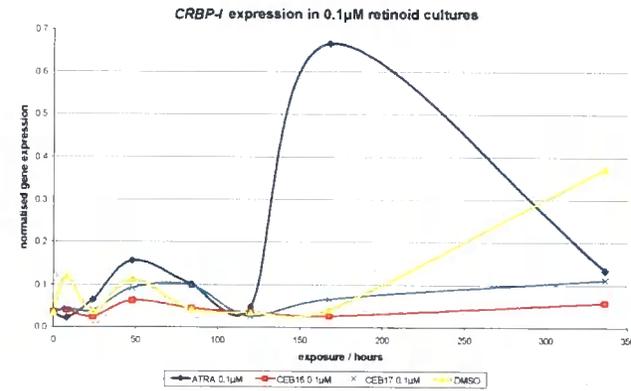
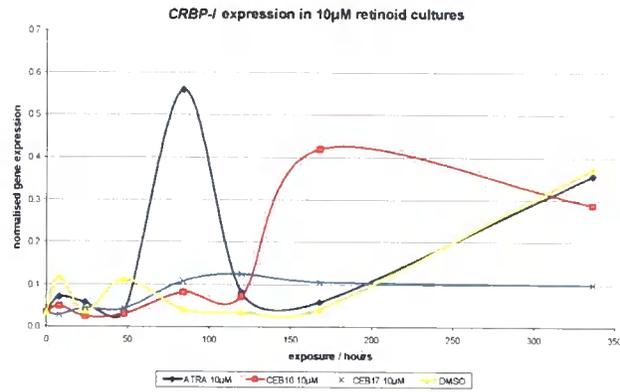
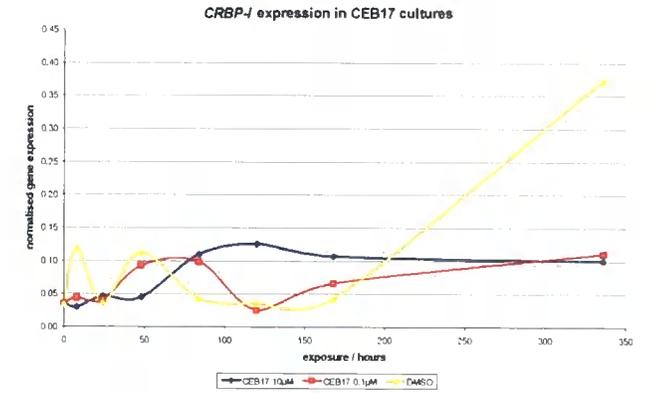
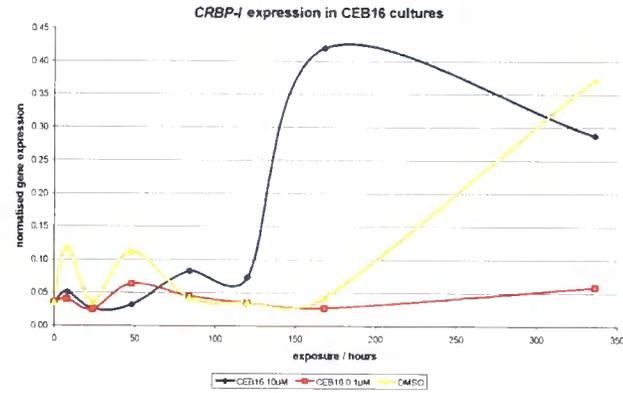
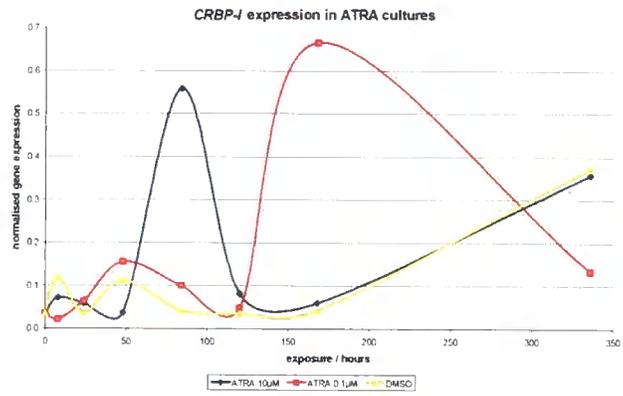


Figure 4-7: Concentration and time-dependent regulation of *CRBP-I* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

Expression of *CRABP-I*

Regulation of the intracellular ATRA carrier proteins, cellular retinoic acid-binding proteins I and II (CRABP-I and CRABP-II) was also investigated in response to ATRA, CEB16 and CEB17 (Figure 4-8 and Figure 4-9). *CRABP-I* and *CRABP-II* mRNA are both constitutively expressed in TERA2.cl.SP12 cells and are strongly up-regulated by all three of these retinoids, furthermore a moderate gradual increase is also observed in the DMSO control cultures over 14 days.

CRABP-I transcripts in the 10 μ M ATRA culture respond quickly reaching a maximum response after 96 hours before declining to basal levels; however the magnitude of this up-regulation is less than any of the other morphologically differentiating cultures (Section 4.6.1, *i.e.* excluding the CEB16 0.1 μ M culture). This unusual response is in good agreement with analysis of ATRA-treated AB1 muES cell line, in which *CRABP-I* mRNA and protein levels are induced at low concentrations of ATRA but diminished at higher concentrations.⁵⁷⁵

The expression pattern of *CRABP-I* in the cultures treated with 10 μ M CEB16, 10 μ M CEB17, 0.1 μ M CEB17 and 0.1 μ M ATRA are all very similar. Levels of mRNA initially increase very slowly for the first 48 hours and then, after a media change, rapidly accumulate high levels of transcripts to attain maximal expression at the seven day time point before levels decline to the same level as the control cultures at the end of the experiment. Except for the ATRA cultures, stronger responses are observed in the 10 μ M cultures compared to the 0.1 μ M cultures of the same retinoid.

The genes encoding CRABP-I are regulated by ATRA treatment in a number of different cell lines including P19 and F9 muEC cells and AB1 muES cells. *CRABP-I* is strongly induced in human keratinocytes, P19 and AB1 cells within 24 hours,^{71,717-719} whereas ATRA-treatment of F9 cells causes a 2-3 fold reduction in the expression of *CRABP-I* mRNA.^{720,721} A number of active synthetic retinoids including Am80 (O) and TTNPB (D) bind to CRABP-I;^{199,722} it is also known that CRABP-I binds retinoids with a higher affinity than CRABP-II.^{121,718} These data are believed to demonstrate that a high intracellular concentration of CRABP-I is able to modulate the effects of excess retinoid in the cell by sequestering both natural and synthetic retinoids to facilitate enhanced metabolism by CYP450 enzymes and simultaneously impede transport of retinoids into the nucleus by CRABP-II.^{123,124,576}

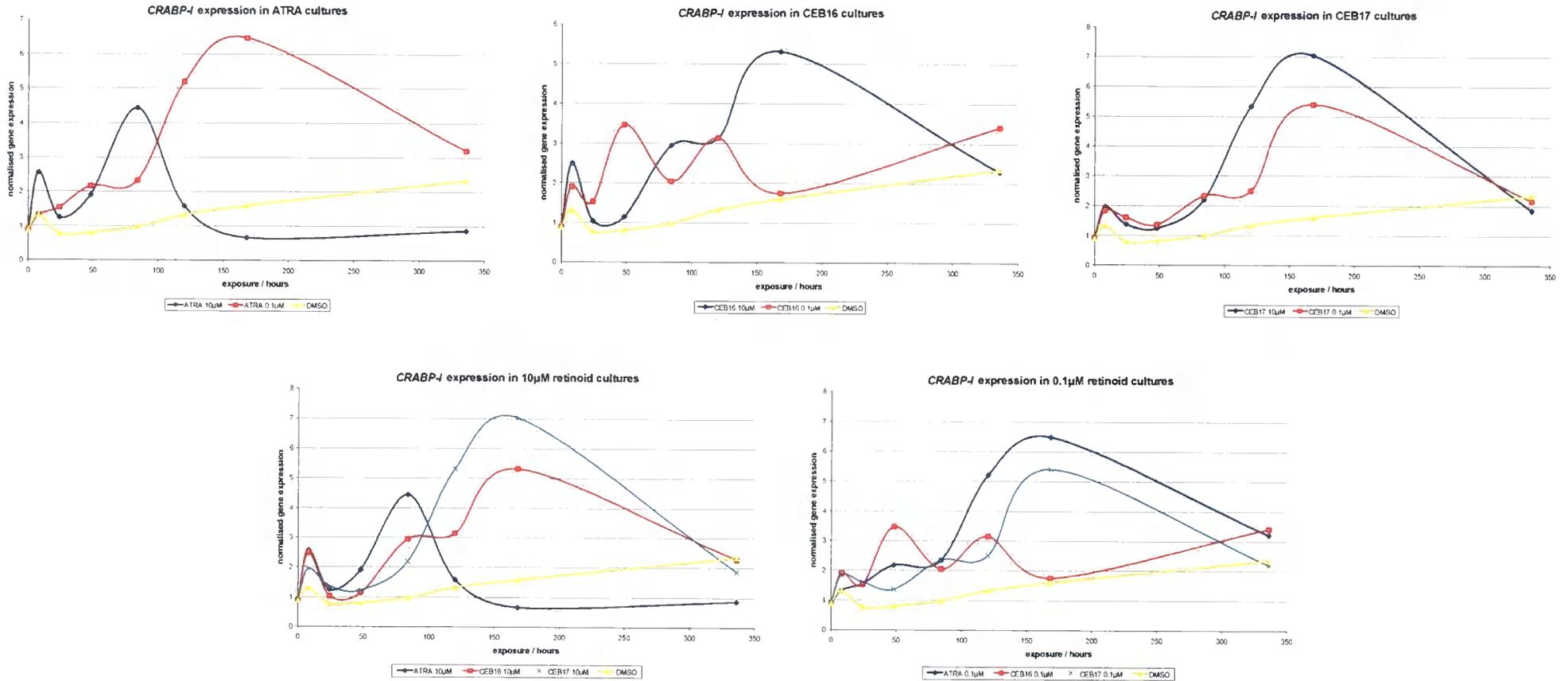


Figure 4-8: Concentration and time-dependent regulation of *CRABP-I* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

Expression of *CRABP-II*

CRABP-II, the gene encoding the cellular retinoic acid-binding protein involved in the transport of ATRA to the nuclear receptors, is expressed in undifferentiated TERA2.cl.SP12 cells at a very low level and is strongly up-regulated in all retinoid cultures except for those treated with 0.1 μ M CEB16 (Figure 4-9). The 10 μ M ATRA culture exhibits the fastest induction of gene expression; this expression is also biphasic in profile, unlike the synthetic retinoid 10 μ M cultures, with a second rapid accumulation of transcripts immediately after the first media change. This biphasic pattern has also been reported in human NTERA2/D1 EC cells.⁵⁴² Interestingly, the maximum response achieved in all three retinoid cultures is identical although only ATRA induces a sustained response; this may represent the maximum response of these cells. As with all the genes investigated in this study all fluorescence readings, except very low results which were effectively un-detectable, were within the standard curve generated during each assay. The maximum observed response is therefore not the upper limit of detection for this assay.

The 0.1 μ M retinoid cultures of ATRA and CEB17 also present biphasic temporal expression profiles. Although this cannot be directly associated with the media change, both of these compounds present a similar pattern suggesting that it is a genuine response in these cells. Furthermore, the levels of *CRABP-II* mRNA in the 0.1 μ M CEB16 culture show excellent agreement with the results of the DMSO culture lending a high degree of confidence to the data for this gene.

The gene encoding huCRABP-II is directly regulated by ATRA *via* an RARE that mediates the differential transcriptional transactivation by the RARs and RXRs.⁷²³ The rapid response of *CRABP-II* observed during these experiments is consistent with its identification as an early downstream target of ATRA during the commitment of NTERA2/D1 huEC cells to differentiation.⁷²⁴ *CRABP-II* transcripts are rapidly and strongly up-regulated by ATRA in F9, P19, neuroblastoma, NTERA2/D1, muES and human fibroblasts cells.^{487,542,546,575,719,725,726} A number active synthetic retinoids, including Am80 and TTNPB have been shown to bind to CRABP-II,⁵⁷⁴ and up-regulate *CRABP-II* mRNA,^{181,199,546,722} leading to the proposal that regulation of *CRABP-II* mRNA can be used as a retinoid bioassay.^{487,722} Affinity for the CRABPs may actually impede transport of ATRA to the nucleus compared with retinoids that cannot bind to these proteins, such that some low affinity synthetic retinoids may induce a faster genomic response.⁷²⁷ However, although active retinoids are able to induce a concentration dependent response to the levels of *CRABP-II*, the magnitude of response does not correlate with potency in some cell systems.^{722,728} Furthermore, whilst up-regulation of *CRABP-II* was observed in response to CD271 (adapalene, L) and LGD1069 (Targretin[®], E) this was not accompanied by induction of biological activity.^{562,729}

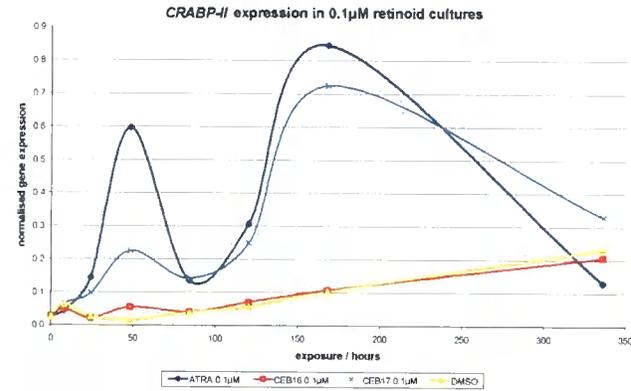
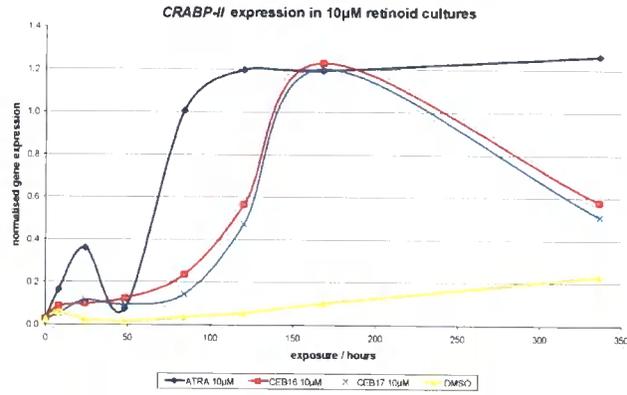
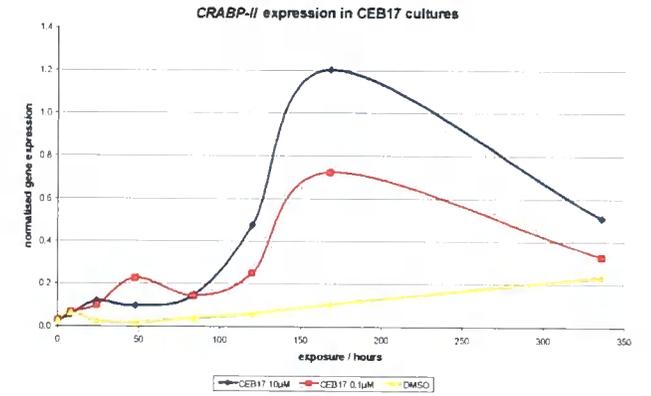
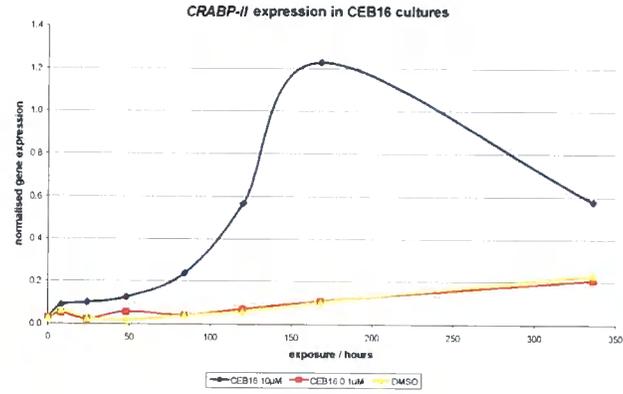
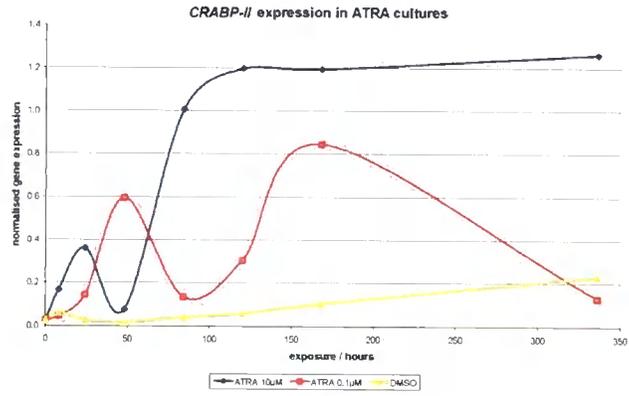


Figure 4-9: Concentration and time-dependent regulation of *CRABP-II* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

Expression of *CYP26A1*

CYP26A1 mRNA is also rapidly and strongly up-regulated in TERA2.cl.SP12 huEC cells after treatment with retinoids (Figure 4-10). Transcript levels in the DMSO culture remain unchanged throughout the 14 day experiment and demonstrate the consistency of the results of this gene assay, even though the data only represents one biological repeat. All three retinoids potentiate the expression of this ATRA 4-hydroxylase, a member of the family of CYP26 enzymes believed to be primarily responsible for the specific degradation of ATRA *in vivo*.

The fastest and strongest response is observed in the 10 μ M ATRA culture, which again presents a biphasic temporal expression profile consistent with hypothesis that a supply of fresh retinoid at 72 hours stimulates a further induction of gene expression. The results of the 10 μ M ATRA culture suggest that initial exposure to ATRA (during the first 48 hours) has primed the cells to respond to further stimulus with an augmented induction in a comparable manner to the development of clinical resistance in retinoid therapy. In addition to *in vivo* data,^{274,325,326} this hyper-catabolism has previously been reported in cultured cells^{730,731} and explains the decreased half-life of ATRA observed in pre-exposed cells.⁵⁷⁵ ATRA sensitivity is inversely correlated to the rate of ATRA metabolism. *CYP26A1* is part of a feedback loop involved in the auto-regulation of retinoid levels, such that when normal physiological levels are exceeded, induction of *CYP26A1* acts to normalise retinoid levels.⁷³¹

There are numerous reports of ATRA and a small number of reports of synthetic retinoid induced expression of *CYP26A1 in vitro*.^{327,328,587,598,732-734} The magnitude and speed of *CYP26A1* induction observed in the TERA2.cl.SP12 cultures is generally consistent with observed phenotypic differentiation. However ATRA induces a notably greater induction of this gene than the synthetic retinoids compared to any of the other genes investigated in this study. Given the lower sensitivity of *CYP26A1* to CEB16 and CEB17, this may indicate that *CYP26A1* is not an important metabolic enzyme for these compounds, particularly as it is known that other enzymes may be involved in the metabolism of other synthetic retinoids.^{267,735} Multi-phasic expression is observed in all the cultures suggesting that this gene is very sensitive to retinoid levels.

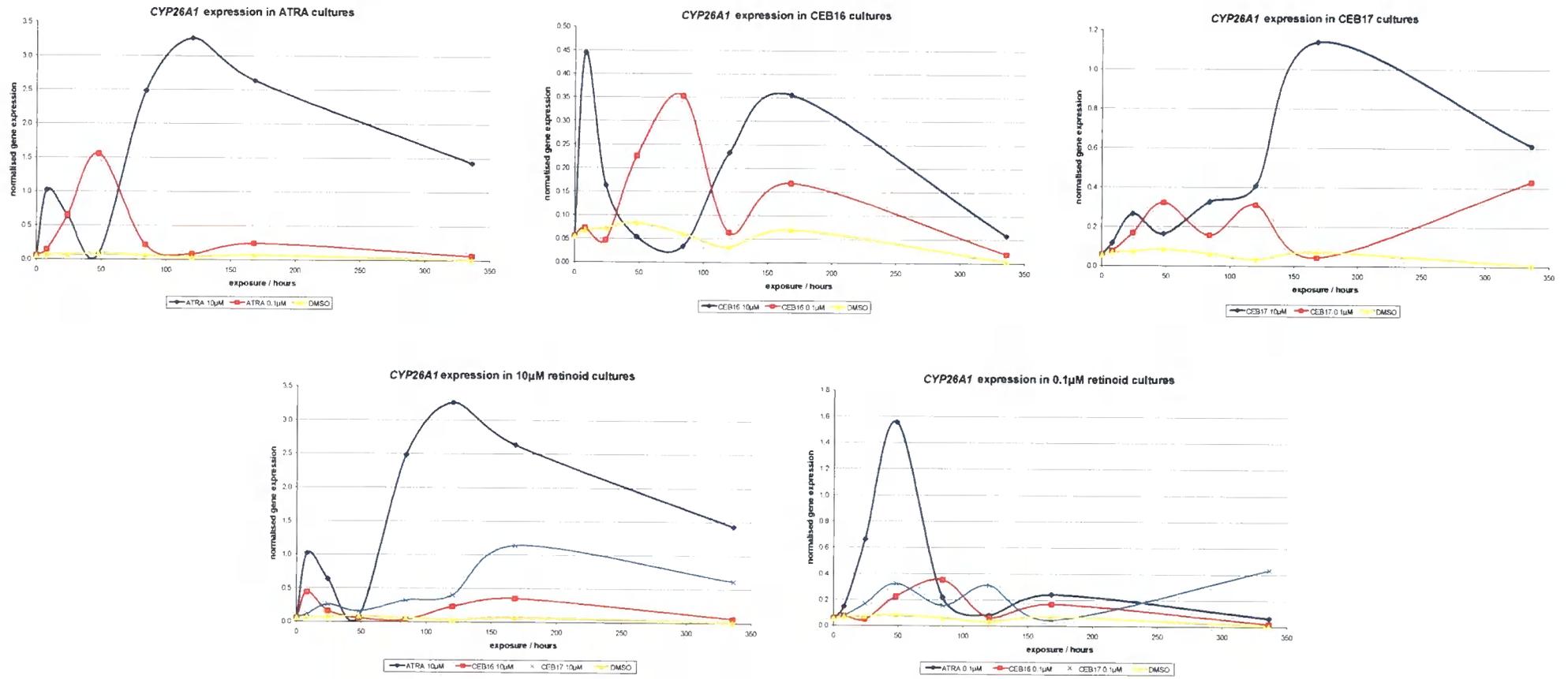


Figure 4-10: Concentration and time-dependent regulation of *CYP26A1* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

4.6.4 Analysis of retinoic acid receptor mRNA expression

The three retinoic acid receptors, RAR- α , RAR- β and RAR- γ , are the ligand-activated transcription factors responsible for mediating the biological response of natural and synthetic retinoids. Expression of these proteins is regulated in direct response to exogenous ATRA and changes in the expression of *RAR* transcripts can also be measured,¹⁷⁸ enabling the use of rt qPCR as a sensitive quantitative method to determine the ability of compounds to modulate the classical retinoid pathway.

The primers used in these assays amplify the DNA of all isoforms of each specific receptor. Low levels of each of the three *RAR* isotypes were detected in undifferentiated TERA2.cl.SP12 cells (Figure 4-11, Figure 4-12 and Figure 4-13). Culturing cells in media enriched with the same concentration of DMSO that was used as a vehicle in all retinoid cultures (0.1% v/v, 14 mM) had no effect on the expression of retinoic acid receptor transcripts, the 14 day DMSO time-point for *RAR- γ* appears to be anomalously high in comparison to all other cultures.

***RAR- α* expression**

RAR- α expression is strongly modulated by all three retinoids except in the generally unresponsive 0.1 μ M CEB16 culture for which the results show very close agreement with the control data. *RAR- α* transcripts respond rapidly to retinoid treatment, generally within 12 hours of initial exposure. This is particularly notable given the generally slow response of this cell line observed in other gene assays (Figure 4-11).

The temporal expression profiles of the 10 μ M ATRA culture and to a lesser extent the 0.1 μ M ATRA culture appear to respond to the supply of fresh retinoid on two occasions: after approximately 72 and 120 hours' culture. After this time the retinoid enriched media was replaced daily, providing a more consistent supply of retinoid. Further experiments are required to confirm this observation: analysis of additional biological samples and a specific investigation into the effect of the timing of media changes. Early transient accumulation of *RAR- α* transcripts is also observed in the cultures exposed to 10 μ M CEB16, 10 μ M CEB17 and 0.1 μ M CEB17. All cultures show a trend towards a slight increase in mRNA levels by the 14 day time-point, which may be a consequence of the extreme confluence of the cultures at this late stage of the experiment. The magnitude of response of *RAR- α* to 0.1 μ M retinoid follows the previously observed trend of differentiation-inducing efficacy, namely ATRA>CEB17>CEB16. Unexpectedly, the amount of gene up-regulation observed in the 10 μ M cultures is comparable for all three retinoids, although the elevated expression attained in the CEB16 and CEB17-treated cultures is short lived. This may indicate that *RAR- α* transcription is a general event that occurs in the early stages of differentiation of TERA2.cl.SP12 cells. Fluorescence levels of

RAR-α gene expression transcripts are the lowest of any of the genes examined in this study, however this is a function of primer efficiency and should not be confused with the level of gene regulation. Multiple SYBR green dye molecules may bind to single amplified products, though the amount is consistent for each gene. This has the major advantage of increasing sensitivity at low levels of gene expression, but the fluorescence signal is consequently dependent on the mass of the DNA product. The intensity of fluorescence is therefore only relative within single gene analysis and cannot be compared between genes.

Unlike *RAR-β* and *RAR-γ*, *RAR-α* is expressed ubiquitously,^{736,737} intimating a more general role in mediating the biological effects of ATRA. The expression of *RAR-α* mRNA correlates with the observed levels of *RAR-α* protein detected by western blot under identical experimental conditions (Section 3.7). *RAR-α* protein was undetected in undifferentiated TERA2.cl.SP12 cells and its expression was not induced in the control cultures, consistent with the lack of transcript regulation measured by rt qPCR. *RAR-α* protein levels increased slightly in all retinoid cultures over 28 days, consistent with the synthesis of mRNA, however the sustained expression of *RAR-α* in response to 10 μM ATRA did not have a measurable effect on protein levels.

Gene expression studies in other cell lines have previously been reported to induce moderate up-regulation⁵³⁷⁻⁵³⁹ or no change^{31,540-542} in the level of *RAR-α* transcripts during ATRA-induced differentiation, in good agreement with the data presented here. Furthermore, the different *RAR-α* isoforms are differentially regulated: *RAR-α₂* is induced by ATRA-treatment whilst *RAR-α₁* expression is unaffected.⁵³⁷ *RAR-α* modulation was observed in all cultures that also demonstrated *CRABP-II* activation consistent with the observation that this specific receptor is required to control *CRABP-II*.¹⁹⁸ Given that *RAR-α* is unresponsive to ATRA-treatment in a number of EC cell lines, it is un-surprising that *RAR-α* is also un-affected by synthetic retinoids in the same cell lines.^{244,738,739} Am580 (**P**) and fenretinide (**J**) have both been shown to up-regulated *RAR-α* transcripts in responsive murine and human cells respectively.^{246,539}

***RAR-β* expression**

Analysis of *RAR-β* expression following retinoid treatment is of particular interest given the rapid and dramatic increases in transcript levels that have been previously reported in response to ATRA^{31,157,198,487,538,540-542} and 9-*cis*-RA,²²² which have led to the proposal that *RAR-β* can be used as a biomarker for retinoid activity.^{487,546,740} Specifically, the expression of the *RAR-β₂* isoform, rather than *RAR-β₁* or *RAR-β₃* variants, is up-regulated,⁵⁴⁵ although up-regulation of *RAR-β₂* has also been shown to be redundant in F9 muEC cells.⁴⁶⁹ Alteration of *RAR* expression or function has been observed in a variety of cancers. Hypermethylation of the *RAR-β* gene, and specifically the *RAR-β₂* promoter, is

strongly associated with aberrant retinoid signalling and the development of numerous different tumour types including lung, squamous cell carcinoma, breast, stomach and prostate.^{48,741} Pharmacological reactivation of the RAR- β_2 promoter has been demonstrated *in vitro*, offering hope for novel chemotherapeutic strategies.⁴⁸

All three retinoids induced an up-regulation in *RAR- β* , though 0.1 μ M of CEB16 was insufficient to amplify transcription of this gene (Figure 4-12). Interestingly, gene expression in a number of the experimental conditions was very similar, both in terms of magnitude and profile, as observed in response to 10 and 0.1 μ M CEB17, 0.1 μ M ATRA and (to a lesser extent) 10 μ M CEB16. Levels of *RAR- β* mRNA were elevated almost immediately upon treatment (an increase is observed within 8 hours) and this moderate expression remained constant for the first five days' culture, whereupon transcription increased in an approximately exponentially manner to a very high level (to a maximal 200-fold increase upon control conditions), which was maintained for the remainder of the experiment. This response may be explained by a number of factors, which given the complex nature of the retinoid pathway may well operate in combination to achieve this fine level of control over gene expression.

The supply of fresh retinoid-enriched media at approximately 72 hours into the experiment did not have an immediately observable effect on the 3.5 day sample data, but may be responsible for initiating the large increase in expression visible in most of the cultures after 5 days' treatment. Further media changes after 120 hours and at 24-hour intervals thereafter could then be seen to permit the continued accumulation of *RAR- β* transcripts to a maximal level which is maintained by the continuous supply of retinoid. As was observed in the *CYP26A1* assay, TERA2.cl.SP12 cells may become sensitised to the presence of retinoid during the initial stages of the experiment and then respond more strongly upon further exposure to the retinoid. The cells may be said to be primed towards a magnified response and subsequent induction, which may itself cause cellular effects, or permit an opportunity for synergistic therapeutic activity.

Furthermore, the kinetics of the *RAR- β* response bears similarity to the *CRABP-I* and *CRABP-II* expression profiles and their maximal response. Given the co-dependence on these binding proteins with the retinoid receptors it is highly likely that regulation of all three factors are tightly related.

However, according to the above hypothesis, the data presented for the 10 μ M ATRA conditions are anomalous. As might have been predicted, the 10 μ M ATRA culture shows the fastest response of any of the treatment conditions in this assay and the multi-phasic expression appears to correlate very well with retinoid supply, yet the maximal response of *RAR- β* transcripts is considerably less than both concentrations of CEB17. It was expected that the retinoid that induced the greatest amount of neural

differentiation (*i.e.* 10 μ M ATRA) would also induce the highest levels of *RAR- β* transcription, given that several *RAR- β* isoforms are particularly abundant in the developing nervous system.⁷⁴² However the expression of *RAR- β* in this assay does not prescribe to this relationship. The observed expression of this receptor is likely to be regulated by feedback mechanisms of other components of the retinoid pathway and the obvious candidate for this behaviour in 10 μ M ATRA is CYP26A1. Indeed these two genes are observed to be co-ordinately controlled in P19 EC cells.⁷⁴³

With respect to synthetic retinoids, the retinoid-related molecules (also known as atypical retinoids) ST1926 (**K**) and CD437 (**I**) as well as 13-*cis*-RA (**C**), Ch55 (**Q**), Am80 (Tamibarotene, **O**), TTNPB (**D**), “acyclic retinoid” (**V**) and fenretinide (**J**) have all been demonstrated to induce a strong up-regulation in the levels of *RAR- β* expression.^{222,244,246,546,738,744} In good agreement with the data presented here, it has also been observed that synthetic retinoids may cause a slower accumulation of *RAR- β* , but ultimately significantly greater levels than the maximal mRNA levels induced by ATRA.⁵⁴⁶

***RAR- γ* expression**

Finally, the third retinoic acid receptor to be discovered, *RAR- γ* , is also modulated in TERA2.cl.SP12 cells by retinoid treatment (Figure 4-13). Aside from the 14 day time-point, which appears to be erroneously high, the expression of *RAR- γ* is un-affected by the control conditions. Both concentrations of all three retinoids regulated the expression of *RAR- γ* : the kinetics may be generalised to a rapid and intense response in the early stages of the experiment, followed by a rapid decline to a low level of stable transcript expression for the remainder of the study. A similar regulation has previously been observed in NTERA2/D1 huEC cells.⁵⁴²

Interestingly, the fastest and most intense response was measured in the CEB16 10 μ M culture; indeed expression in response to 10 μ M ATRA was also surpassed by 10 μ M CEB17 and 0.1 μ M ATRA. *In vivo*, *RAR- γ* is confined to lung and skin¹⁵⁷ and is believed to mediate the pharmacological activities of retinoids in the skin.⁷⁴⁵ It is therefore proposed that the expression of *RAR- γ* in response to 10 μ M CEB16 correlates with the observation that this compound induces a high proportion of epithelial cell types (Chapter 3). Furthermore, *RAR- γ* over-expression in NTERA2/D1 cells inhibits neuronal maturation, another characteristic of cultures grown in CEB16.⁶¹² Conversely, this theory would also explain the low proportion of epithelial differentiation observed in response to 10 μ M ATRA. Western blot analysis of *RAR- γ* protein (Section 3.7), where higher levels of protein were observed in response to treatment with the synthetic retinoids than either ATRA or its geometric isomers, corroborates the rt qPCR data. However, the high level of *RAR- γ* observed in the control cultures does not appear to require active transcription of *RAR- γ* .

Modulation of *RAR-γ* expression in TERA2.cl.SP12 cells was expected, given that terminal differentiation of NT2/D1 and F9 cells in response to ATRA has been reported to be triggered by an *RAR-γ* dependent pathway.^{212,661,746} In agreement with the results discussed for TERA2.cl.SP12 cells, ATRA treatment has been reported to either suppress⁵³⁸ or elevate^{198,539,542-544} *RAR-γ* mRNA. Specifically, beyond the scope of this experiment, *RAR-γ*₂ mRNA is strongly induced while *RAR-γ*₁ can act as a transcriptional repressor and antagonise *RAR-β* expression.^{543,544,547,548} Furthermore, induction has also been reported in response to a range of synthetic retinoids including ST1926 (K), CD437 (I), fenretinide (J) and Am580 (P).^{244,246,539,542} However, once again, transcript accumulation is reported in other cell lines at a much faster rate, for example *RAR-γ*₂ mRNA is strongly up-regulated in NTERA2/D1 cells within 3 hours of treatment.⁵⁴² These data do not support the suggestion that maintained expression of *RAR-γ*₂ may require continuous exposure to retinoid.⁵⁴⁸

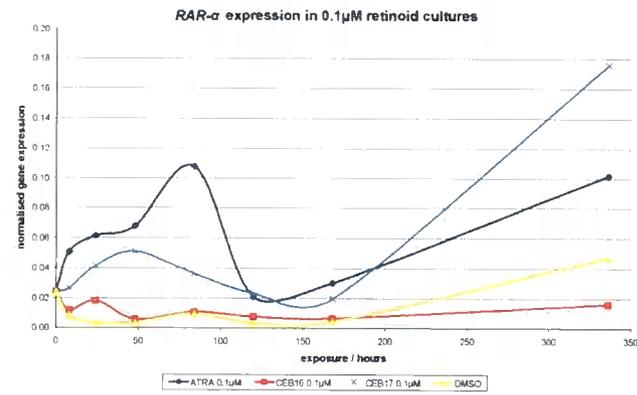
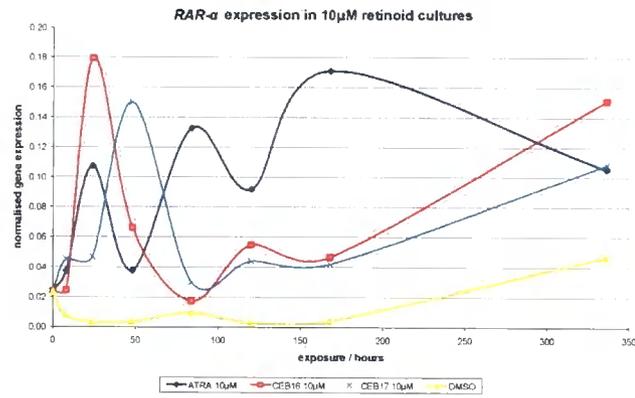
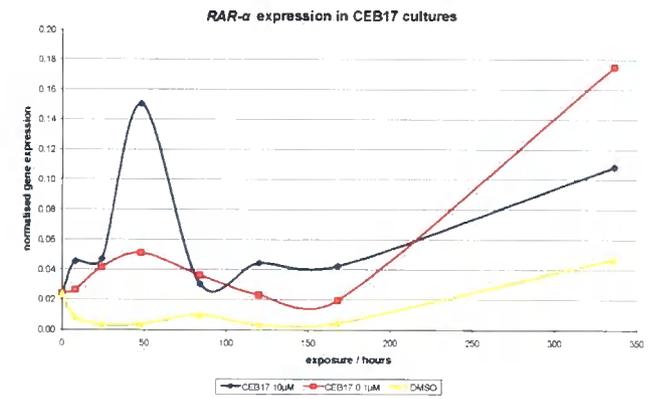
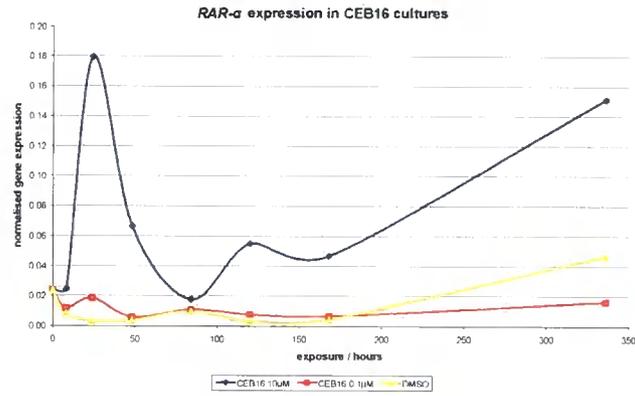
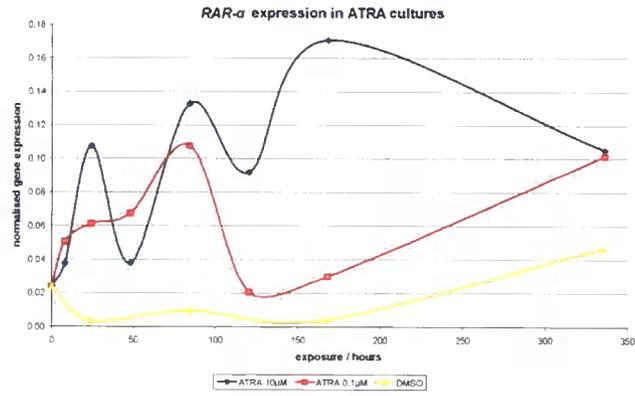


Figure 4-11: Concentration and time-dependent regulation of *RAR- α* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

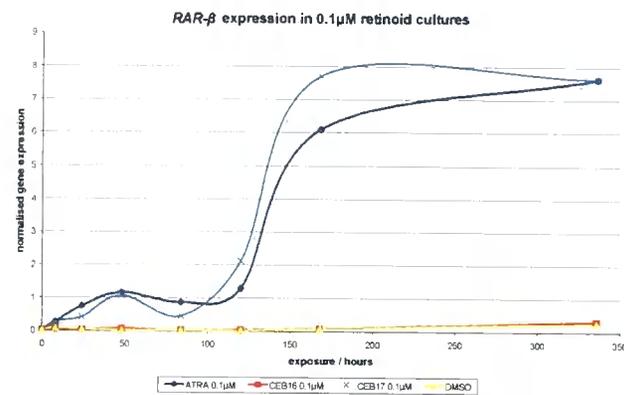
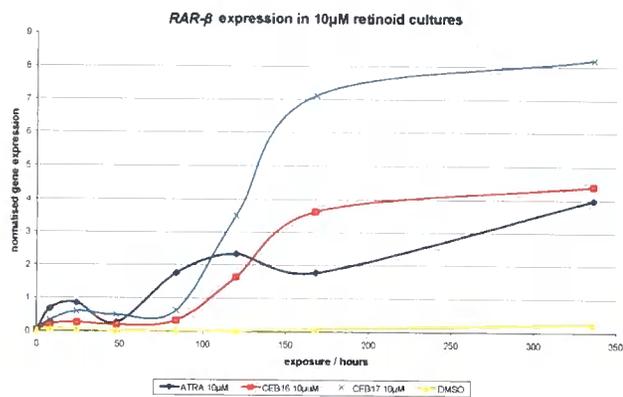
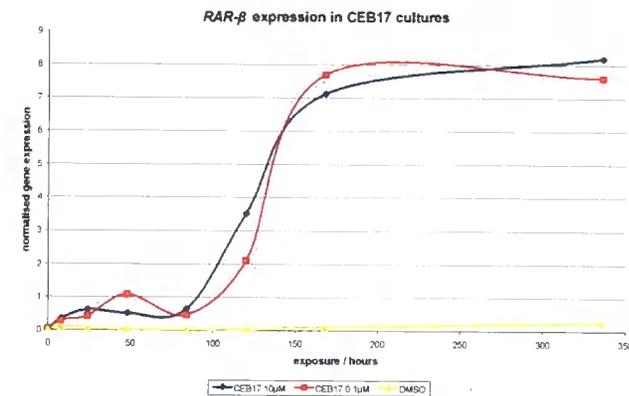
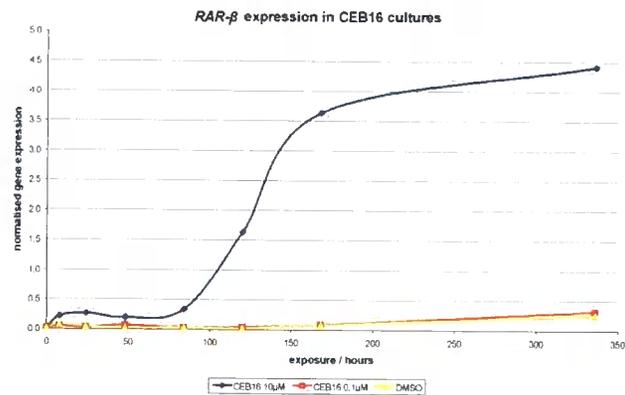
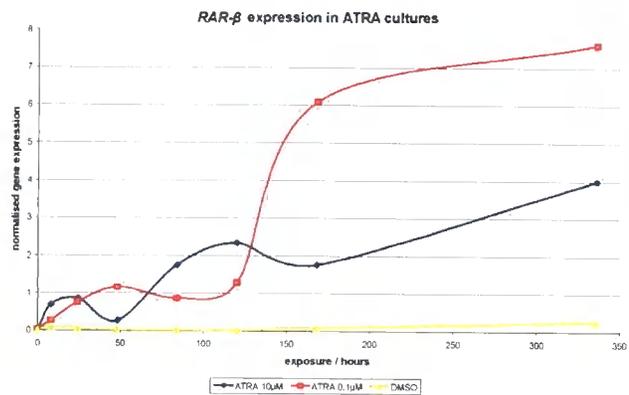


Figure 4-12: Concentration and time-dependent regulation of *RAR-β* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

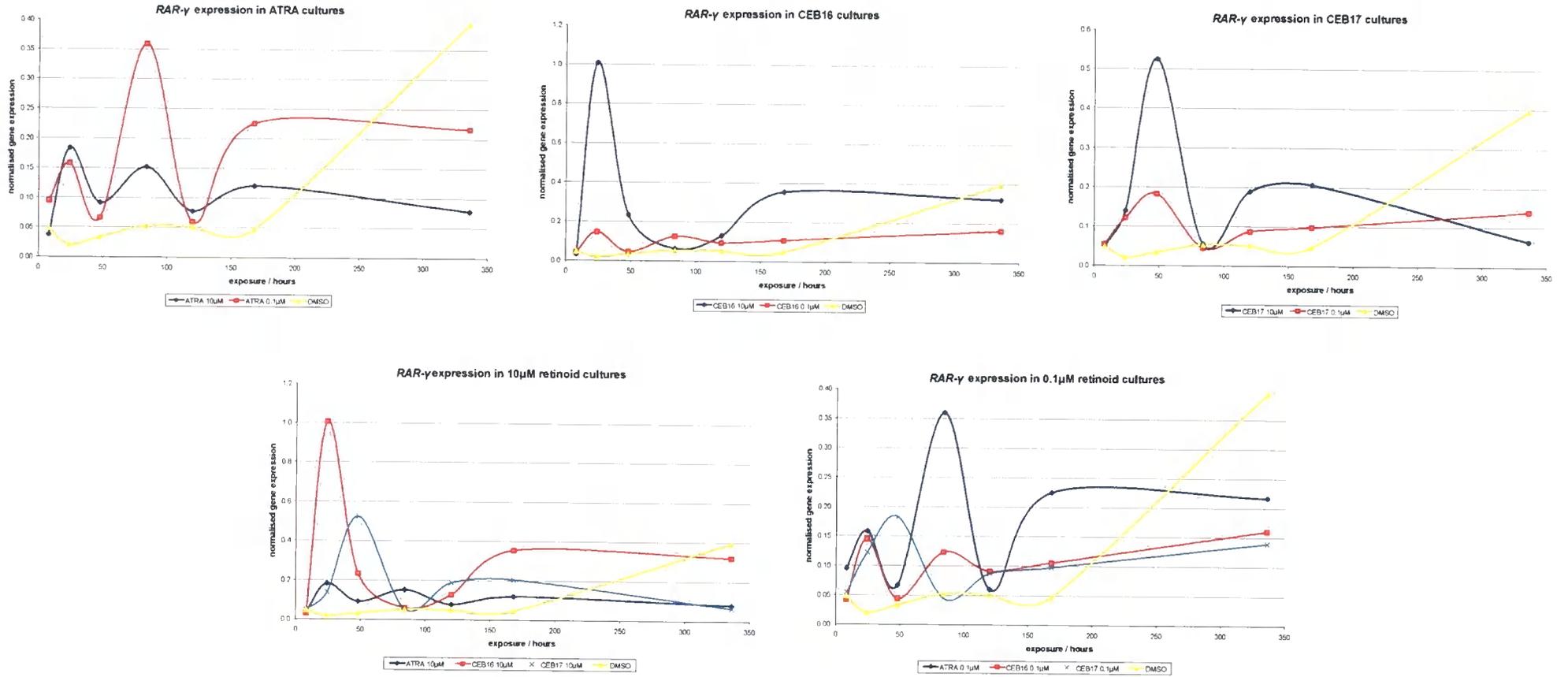


Figure 4-13: Concentration and time-dependent regulation of *RAR- γ* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

4.6.5 Analysis of HOXB expression

The mammalian HOX proteins are a large family of transcription factors that control cell identity, differentiation, and patterning in animal embryonic development. Given the important role that endogenous ATRA appears to play in controlling *HOX* expression, it is clearly important to understand if synthetic retinoid-induced differentiation affects *HOX* gene expression. Regulation of *HOX* genes also plays a role in modulating the expression of components of the retinoid pathway, as was recently demonstrated in muES cells: induction of *Hoxb1* resulted in a strong up-regulation of *CRABP-I* and *CRABP-II*.⁶⁶⁹

The three *HOXB* genes analysed in this study, *HOXB1*, *HOXB2* and *HOXB3* (previously known as *HOX2I*, *HOX2H* and *HOX2G* respectively, and corresponding to the murine genes previously known as *Hox2.9*, *Hox2.8* and *Hox2.7*) are barely detectable in undifferentiated TERA2.cl.SP12 cells. All three genes are strongly up-regulated in a co-linear and concentration dependent manner in response to ATRA and both synthetic retinoids, providing further evidence of the similarity of genotypic effects induced by CEB16 and CEB17 compared to the natural retinoid (Figure 4-14, Figure 4-15 and Figure 4-16). Once again, 0.1 μM CEB16 fails to modulate expression of these genes, mirroring the results of the DMSO treated culture.

Examining the *HOXB1* results in detail (Figure 4-14) reveals that transcript accumulation started to increase at approximately the same time in response to both ATRA and the synthetic retinoids and that expression levels appear to be un-related to the potency of the compounds. Gene expression appears to be a multi-phasic; this might be explained in the 10 μM cultures by a direct response to the provision of fresh retinoid, most notably in the 10 μM ATRA culture, consistent with its rapid degradation in culture. Accumulation of mRNA is slower in the 0.1 μM cultures, although interestingly treatment with 0.1 μM CEB17 results in sustained accumulation of *HOXB1* (seemingly in response to retinoid supply), producing the greatest expression of all cultures by the 14 day time-point. The least potent differentiating agent, CEB16, initially induces a slow response, but gradually accumulates mRNA throughout the study resulting in a higher level of gene expression than either of the other 10 μM conditions by 14 days.

The *HOXB2* and *HOXB3* expression profiles (Figure 4-15 and Figure 4-16 respectively) present simpler responses of TERA2.cl.SP12 cells to exogenous retinoid treatment. 10 μM ATRA induces the fastest and ultimately strongest expression of both genes; however both 10 μM CEB16 and 10 μM CEB17 modulate *HOXB2* accumulation similarly to 10 μM ATRA, whereas *HOXB3* expression follows the expected pattern of potency. At the lower concentration, 0.1 μM ATRA and 0.1 μM

CEB17 also induce very similar responses in both genes, which is of a lower intensity than the corresponding 10 μ M data. Induction of *HOXB1* and *HOXB2* appears to commence at approximately the same time in these cells, *i.e.* between 8 and 24 hours, at both concentrations and in response to all retinoids; however, the rate of transcript accumulation is fastest during these initial stages in *HOXB2*. Collection and analysis of more time points during this window would be expected to show an earlier induction of *HOXB1*. *HOXB3* is clearly regulated more slowly than either of the more 3' genes, in true co-linear fashion.

The co-linear induction of *HOXB* expression during ATRA-induced differentiation has previously been reported in a number of murine and human cell lines,^{540,636,747} and although this expression has not been reported as phasic, it has been established that a continual supply of retinoid is required for maintained expression.^{636,639} Gene regulation was faster in NTERA2/D1 cells compared to TERA2.cl.SP12 cells, for example an up-regulation of *HOXB1* was observed in NTERA2/D1 cells within just one hour in response to 10 μ M ATRA, although the expression of this gene in response to 0.1 μ M ATRA is more comparable.^{540,641} Human *HOXB* genes have previously been noted to require a longer induction period (up-regulation of *HOXB9* was observed after six days compared to just 24 hours for than the corresponding murine *Hoxb* gene) which have led to the suggestion that the induction may be directly linked to cellular differentiation rather than activation by ATRA.⁶³⁶

The gene transcription site of *Hoxa1* in F9 muEC cells contains an ATRA-inducible enhancer that contains an RARE.⁶⁵⁴ Similar response elements have also been found in human and murine *HOXB1/Hoxb1*⁶⁵⁵⁻⁶⁵⁹ and *HOXD4/Hoxd4* genes,^{611,660} suggesting that the expression of these genes may be directly responsive to ATRA-signalling *via* ligand-bound RAR-RXR heterodimers. Indeed, murine *Hox* expression has been demonstrated to probably be dependent upon the activation of RAR- α and RAR- β ,^{198,610,662,664} as part of a feedback circuit to ensure the correct expression of these powerful transcription factors.⁶⁶⁴

This study is believed to present the first investigation into the effects of synthetic retinoids upon human *HOX* gene expression. The synthetic retinoids ST1926 and CD437 have previously been shown to up-regulate *Hoxa1* in muF9 EC cells, but at a slower rate than ATRA.²⁴⁴ Interestingly, expression of the *HOXB* genes investigated in this study do not appear to be strictly related to the differentiation-potency of the retinoids.

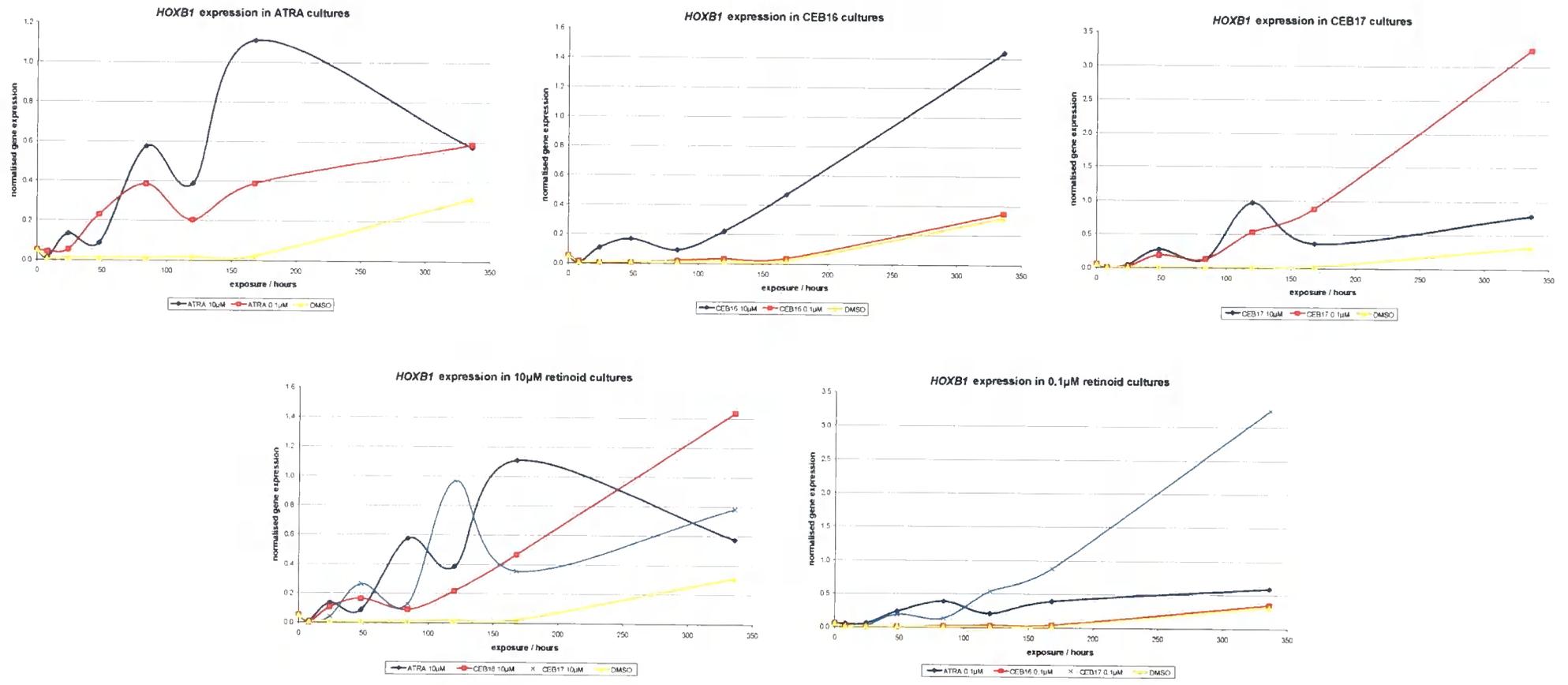


Figure 4-14: Concentration and time-dependent regulation of *HOXB1* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

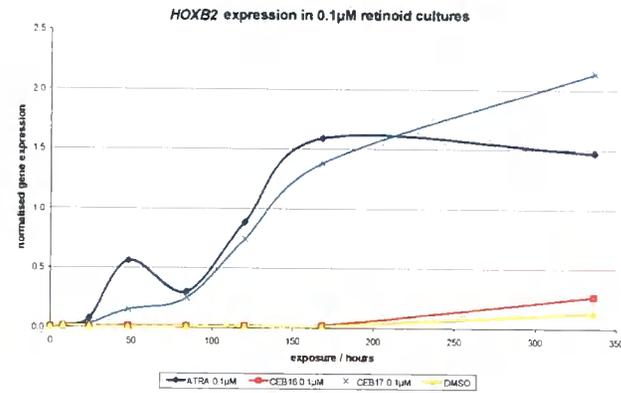
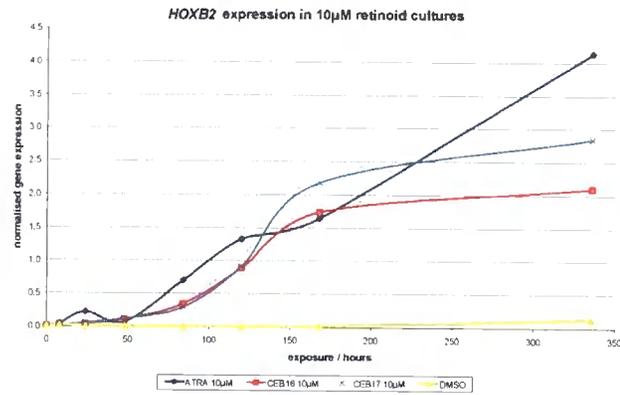
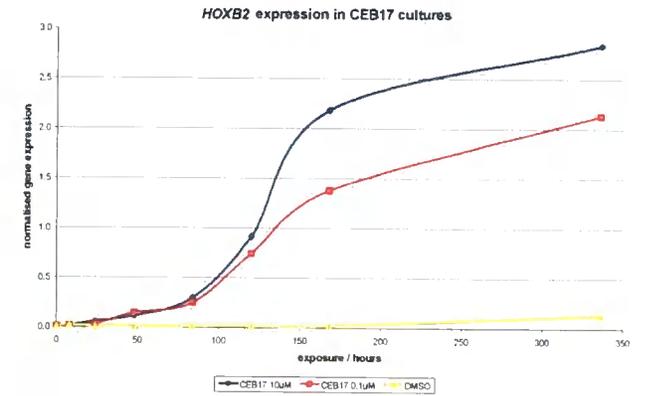
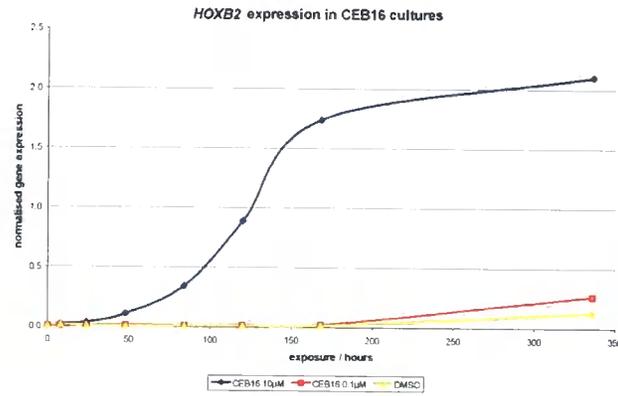
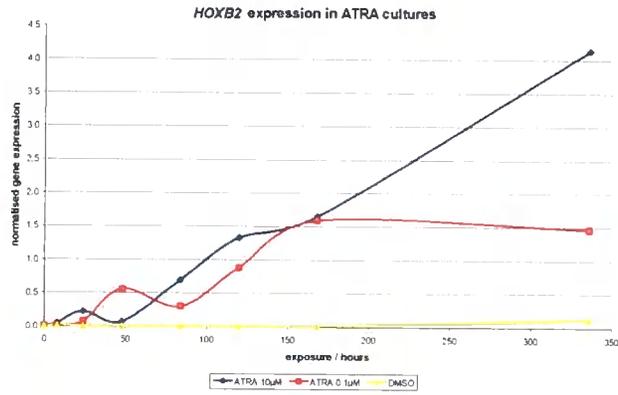


Figure 4-15: Concentration and time-dependent regulation of *HOXB2* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

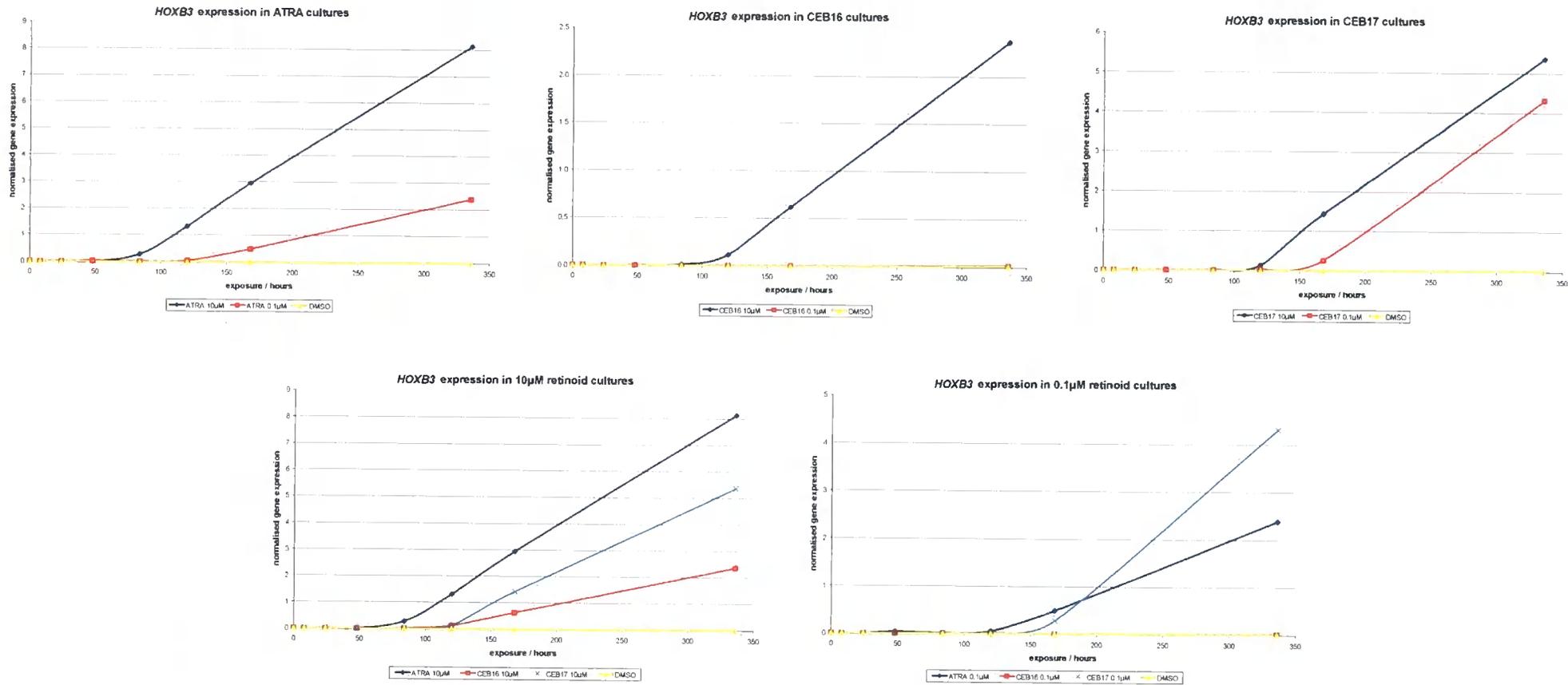


Figure 4-16: Concentration and time-dependent regulation of *HOXB3* expression in TERA2.cl.SP12 huEC cells by ATRA and two synthetic retinoids (n = 1).

4.6.6 Agarose gel electrophoresis of rt qPCR products

The major disadvantage of using SYBR green chemistry for real-time quantitative PCR analysis (rt qPCR) is that SYBR green binds non-specifically to double stranded DNA formed during the PCR reaction. However, if primers are well designed the AmpliTaq Gold[®] DNA polymerase in the mastermix will only amplify the target sequence, there will be no primer dimerisation and non-specific products will not be formed.

Initially, all primer sequences for the genes of interest were obtained from published sources (Table 6-4) and preliminary experiments were performed to test the performance of these primers. After each rt qPCR reaction, a final step in the analysis programme generated a dissociation curve. If only one peak is observed, and no peaks are visible in the no target control (NTC, sample DNA is replaced by H₂O to act as a blank), this demonstrates that a single product has been formed. Secondly, a PCR product from each gene was run out on a 1.5% w/v agarose gel and detected using ethidium bromide under UV light. Each PCR product produced a unique dissociation curve and was composed of only one band when run out on an agarose gel, which eluted at the correct molecular weight for the expected product (Figure 4-17).

The fidelity of transcription could be un-equivocally established by extracting the PCR products and sequencing. For three of the genes analysed in this chapter (*RAR-α*, *RAR-γ* and *HOXB3*) the initial choice of primer was either non-specific or failed to generate any product. Three pairs of additional primers for each problematic gene were sourced from either the literature or designed using Primer Express 3.0 (Applied Biosystems) software, tested as described above, and the best performing primers selected for use in rt qPCR analysis.

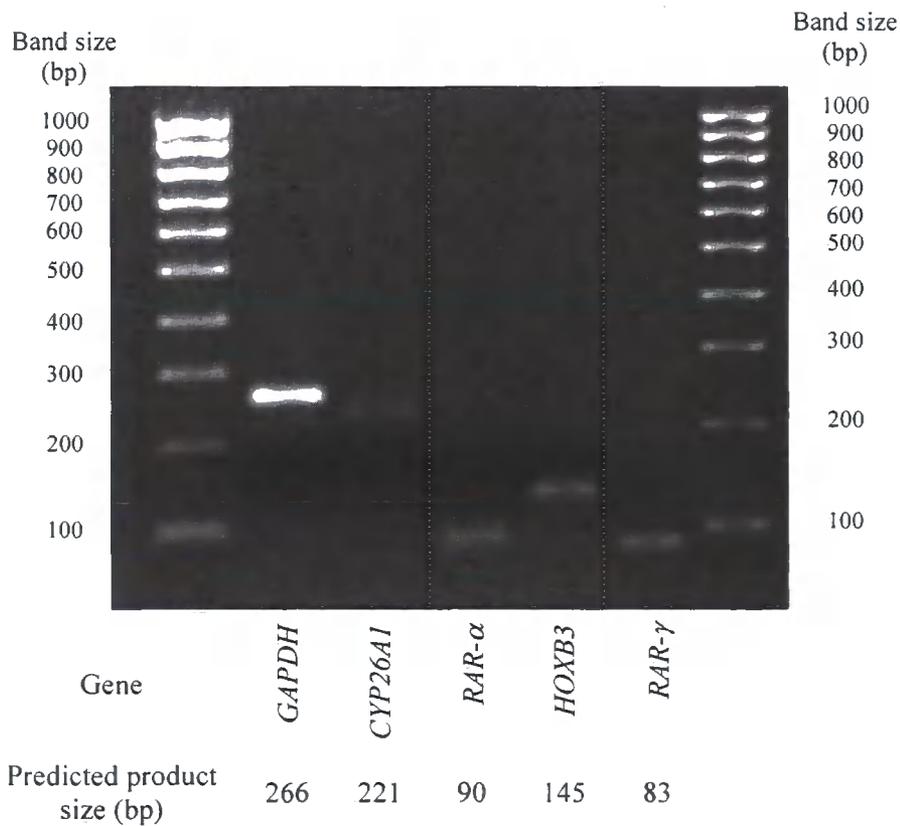
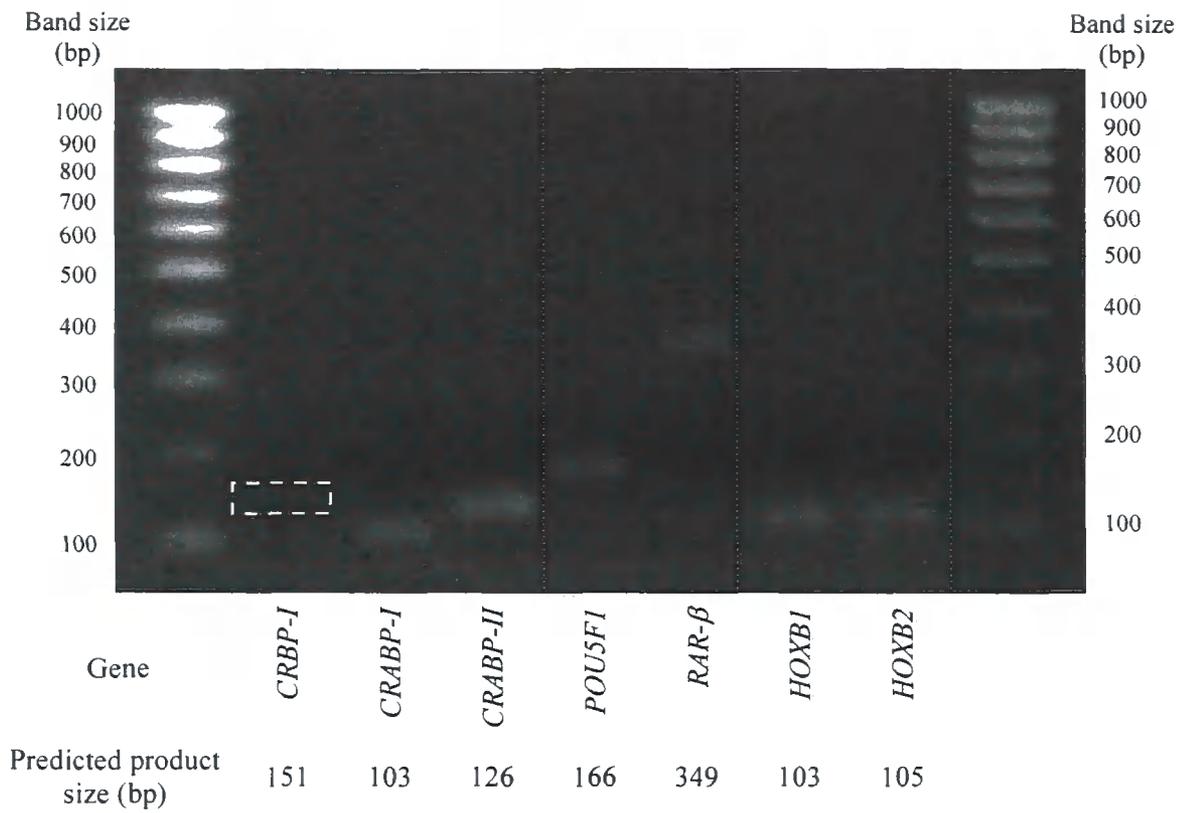


Figure 4-17: Images of PCR products eluted on 1.5% agarose gels, stained with ethidium bromide and visualised by UV. Each gene product produced only one band of the expected size confirming its identity.

4.7 Conclusions

The mechanistic pathway by which vitamin A exerts its activity has been studied extensively for more than fifty years and with renewed interest since the nuclear receptors for its major metabolite, all-*trans*-retinoic acid, were identified twenty years ago. It is of significant clinical interest to investigate whether synthetic retinoids also interact with the same retinoid proteins and receptors and to compare this behaviour to the activity of exogenous ATRA. The previous chapter demonstrated that some synthetic retinoids are able to induce differentiation in TERA2.cl.SP12 human EC cells in a phenotypically and seemingly genotypically analogous manner to ATRA. To date, limited data have been published on the effects of synthetic retinoids on the retinoid pathway during differentiation of human cells and no human studies have examined the activity of synthetic retinoids on the *HOX* genes. Delineating the biological behaviour of this powerful group of compounds to understand the fundamental mechanisms and signalling pathways that control and accompany the early stages of retinoid-induced differentiation should result in more effective therapies.

Studies of the ATRA-induced differentiation of cultured stem cells have permitted the detailed dissection of the differentiation pathway, such that more than 500 genes have been identified as regulatory targets of ATRA. Control may be exerted directly, driven by the binding of ligands to RAR/RXR heterodimers, or indirectly through intermediate factors or even more distant mechanisms.¹⁷⁸ The speed of gene response can vary from hours to weeks, and may be up-regulated, down-regulated or variable in their regulatory direction. However, few reported gene expression studies analyse the modulation of transcripts of the retinoid pathway in any detail or over an extended period; most arbitrarily select a small number of time-points to provide a simple snapshot. The data presented in this chapter suggest that gene modulation within the retinoid system is more complex than has previously been reported and highlights the limitations of other studies. This investigation was designed to measure these changes thoroughly over an extended period of differentiation and attempt to capture transient expression. It has also demonstrated the significant importance of timing of addition of inductive agent (*i.e.* retinoids), and an apparent unexpected consumption or degradation of “stable” synthetic compounds.

TERA2.cl.SP12 huEC cells were treated continuously with 0.1% v/v DMSO or either 10 μ M or 0.1 μ M of ATRA, CEB16 or CEB17 for a total of two weeks. After 8, 24 and 48 hours and 3.5, 5, 7, 10 and 14 days' culture mRNA was isolated from each of the different culture conditions and used to determine the expression of eleven transcripts involved in retinoid transport, activation and metabolism by quantitative real-time PCR (rt qPCR). A large number of time-points over an extended period of differentiation were analysed in order to try to capture the detailed fluctuations in mRNA expression

that previous studies have detected. The vehicle did not modulate the expression of any of the genes in this study, at the concentration used to deliver the retinoids, although it may interact with alternative pathways. These data confirms the results presented in Chapter 3 and further validates the use of this concentration of DMSO in TERA2.cl.SP12 cells.

As previously observed, ATRA, CEB16 and CEB17 induced phenotypic differentiation in this cell line (Section 4.6.1). At 10 μM the order of efficacy of morphological development towards mature neural cultures was ATRA \gg CEB17 $>$ CEB16. Evidence of differentiation was also observed in cultures grown with 0.1 μM ATRA and CEB17, but CEB16 was ineffective at this lower concentration. However, 0.1 μM CEB16 did modulate the expression of three of the genes investigated in this study: *CRABP-I*, *CYP26A1* and *RAR- γ* (Figure 4-8, Figure 4-10 and Figure 4-13). *CRABP-I* is involved in the transport of ATRA to the major specific metabolising enzyme *CYP26A1* so it is perhaps unsurprising that this pair of transcripts are modulated. Furthermore, activation of *RAR- γ* is consistent with the apparent propensity of CEB16 to induce epithelial differentiation.

All of the retinoic acid inducible genes examined in this study were found to be modulated in TERA2.cl.SP12 cells in response to both ATRA and two synthetic retinoids. It may therefore be inferred that these compounds are activating the same pathway as the natural metabolite in these cells and that this is responsible for at least some of the observed effects. The cellular behaviour of these synthetic retinoids may be largely analogous to the well studied actions of ATRA *in vitro* and *in vivo*. Gene up-regulation was typically greater in response to 10 μM compared to 0.1 μM of the corresponding retinoid, although the modulation does not always demonstrate a similar but concentration dependent profile. Furthermore, the order of phenotypic efficacy described above and in Chapter 3 is not observed as clearly in the gene expression assays. This final observation is consistent with the theory that geometrically constrained synthetic retinoids may function in a subtly differently manner compared to ATRA.

The induced retinoid-regulated genes show a range of different kinetic and temporal expression profiles. While the large fluctuations in gene expression were observed during the experiments do not represent the technical accuracy of the experiment (as exemplified by the consistent expression levels of the DMSO culture in many of the gene assays and a number of very similar results obtained with different retinoids, for example the 10 μM CEB16 and 10 μM CEB17 *CRABP-II* expression profiles, Figure 4-9), they may be less significant if further biological samples had been analysed.

Of the eleven genes analysed in this study, five have been established as direct targets of ATRA treatment: *CRABP-II*, *HOXB1*, *RAR- α* , *RAR- β* and *RAR- γ* .¹⁷⁸ Whereas the other genes are controlled indirectly. The quickest up-regulation of mRNA (within 8 hours of treatment) was observed in *RAR- β* ,

CRABP-I, *CRABP-II* and *CYP26A1*, genes which are involved in the modulation/attenuation of the retinoid signal.⁷²⁴

The large variations of individual gene expression during the course of these experiments, as a function of both retinoid concentration and retinoid structure, reveal an underlying complexity to the differentiation pathway(s). *In vivo*, it is well established that a fine spatial and temporal control of genes is required for cell growth, differentiation and development. Minor aberrations to the activity of the molecular signals and induction factors that underlie this control can cause lethal abnormalities. The developmental landscape is extremely complicated; whilst the scientific community understands only a small proportion of it, each new discovery provides a therapeutic potential.

The patterns of gene regulation observed in this study were compared with other published reports for cultured cells and *in vivo* data, where available. The multipotent murine embryonal carcinoma cell lines F9 and P19 have been used extensively as models of early embryonal differentiation.⁴²²⁻⁴²⁴ However, human EC cells appear distinct from murine EC cells in terms of genetic, phenotypic and biological markers,⁷²⁴ making human EC lines the best comparator for observations in TERA2.cl.SP12 cells. The TERA2.cl.SP12 and NTERA2/D1 (NT2) human embryonal carcinoma cell lines were both clonally derived from the human teratoma line TERA2 and have both retained the capacity for differentiation into diverse somatic lineages.^{425,471} However, differences in the method of isolation or simply as a consequence of each cell line originating from two different clones have led to previously observed differences between the cell lines.^{425,748} The data in this chapter further exemplifies the disparity between these cells.

A preliminary experiment determined that no significant changes in the expression of any of the genes occurred prior to 8 hours' retinoid exposure. The full gene analysis study presented in this chapter did not therefore examine any earlier time-points. Compared with the other cell lines discussed, which use similar retinoid concentrations and seeding densities, gene expression in TERA2.cl.SP12 cells respond relatively slowly to natural and synthetic retinoid treatment. Whilst it seems unlikely, it is possible that this discrepancy is somehow related to the protocol employed. In order to investigate this hypothesis it would be necessary to analyse other cell lines in the same manner and compare the results obtained with those in the published literature. Alternatively, these cells may be unusually inefficient at taking up retinoids from the media. This could be tested by comparing the intracellular concentrations of retinoids with those of other cell types treated similarly.

As with many previous studies, supra-pharmacological concentrations of the retinoids were applied to the cells, and may therefore not represent the *in vivo* behaviour of the retinoid molecular pathway under realistic therapeutic regimens. Furthermore, cultured cells are a simple but artificial model biological system, whereas animal models and the human body are likely to respond in a more

complicated and un-predictable manner. For example, *in vivo* the retinoids may differ in rate of cellular uptake and metabolic transformations. A large number of positive *in vitro* retinoid studies have promised effective treatments for a wide range of cancers; however, with the exception of acute promyelocytic leukaemia and neuroblastoma, clinical results have thus far been disappointing.³⁰¹

However, the up-regulation of mRNA observed in this study does not necessarily correlate directly with protein expression, the real effector in many biological processes, but does suggest that these genes are involved in the mediation of retinoid effects *in vitro*.⁵³⁹ Conversely, genes may be actively transcribed with measurable protein expression, but without accumulating high levels of RNA.^{539,636} The mRNA of different genes have widely ranging half-lives such that observed levels will vary significantly between genes and may not be detected if they are particularly transient.

The expression profiles of a number of the genes appear to respond to media changes, which are believed to demonstrate a direct response to the provision of fresh retinoid at these time-points. For example, expression of *HOXB1* in cells grown with 10 μ M ATRA declines just before the media is changed at approximately 72 and 120 hours and then increases rapidly immediately after this time (Figure 4-14). A more consistent level of expression thereafter is proposed to be a response to the subsequent supply of retinoids at daily intervals, providing a more consistent stimulus. Although further biological samples need to be analysed to confirm these observations, these results appear to represent a concentration dependent response to the amount of retinoid available to the cells. This relationship is rarely mirrored at both concentrations of the same retinoid as might be expected from this hypothesis, but the overall temporal expression profiles are more complicated and do not typically show a concentration dependent relationship. Similar responses are seen in some of the synthetic retinoid cultures (for example expression of *RAR- β* in response to 0.1 μ M CEB17, Figure 4-12), however these patterns are not clearly observed in response to all retinoids for a given gene analysis. A specific study to investigate the effect of the timing of media changes on gene expression is required to investigate this hypothesis.

All-*trans*-retinoic acid is notoriously unstable towards a range of physical, chemical and enzymatic triggers of oxidation, isomerisation, and metabolism. A small number of studies have examined the degradation of ATRA under cell culture conditions and although they highlight experiment-specific differences, such as cell type and presence of certain media supplements, they clearly demonstrate that the initial concentration of ATRA rapidly declines in culture.^{224,280,285,488} ATRA is predominantly isomerised into its 9- and 13-*cis* geometric isomers.⁴⁸⁸ In some instances these isomers induce phenotypically similar effects to ATRA (Chapter 3), but they are distinct in mechanism of action and in a clinical setting.^{281,491}

Stability studies need to be performed under typical TERA2.c1.SP12 culture conditions to determine the specific retinoid degradation kinetics of ATRA and the apparently more stable synthetic analogues under investigation. An almost complete loss of ATRA within 24 hours' culture has been reported,²⁸⁰ while two independent reports are in good agreement that after 24 hours 75% of the original concentration of ATRA remains, declining to 40-50% after 48 hours and 35-40% after 72 hours.^{285,488} Specific stability study results should be used to define a standard protocol on the frequency of media changes, to constrain retinoid concentrations within a defined range. Furthermore, given the dependence of effect on concentration, this parameter should be reported in all retinoid studies. Although a number of synthetic retinoids are more stable than ATRA outside the cell culture environment,^{352,402} their stability *in vitro* has yet to be tested.

The pleiotropic effects of retinoids may be explained by the existence of complex signal transduction pathways involving multiple forms of specific binding proteins, nuclear receptors and metabolic enzymes. These proteins have different expression patterns in the adult and during vertebrate embryogenesis suggesting that they have particular complementary functions that permit them to carefully control the concentration and effects of retinoids *in vivo*. Aberrations in the normal retinoid pathway have been identified in tumour cells, thus confirming the importance of timely regulated expression of components of the retinoid cycle and presenting the opportunity to use exogenous retinoids in clinical therapy. As further components of the retinoid cycle are identified and their physiological roles defined, it is anticipated that synthetic retinoids may be used to bypass non-essential parts of the system permitting the design of more efficacious and tolerable drugs.

**CHAPTER 5: FINAL CONCLUSIONS &
RECOMMENDATIONS FOR FUTURE
WORK**

5.1 Conclusions

Detailed conclusions have been presented at the end of Chapters 2, 3 and 4; the following is an overview of the key findings of this work.

Human EC stem cells have been used to screen and characterise the ability of a small library of synthetic retinoids to induce differentiation. Two isomeric compounds (CEB16 and CEB17) were further investigated to examine the capacity of synthetic retinoids to modulate the retinoid pathway. Three of these compounds, CEB16, CEB17 and CEB18 were prepared by the author using emerging C-H activation methodologies. Significant progress was also made towards the complete synthesis of two further series of compounds.

The retinoid and retinoid-like compounds shown in Table 3-1 and Table 3-2 were screened at a concentration of 10 μ M for the ability to induce differentiation in TERA2.cl.SP12 human EC cells. Phenotypic changes consistent with differentiation were observed in response to some of the compounds, in agreement with published data for a number of the compounds or as might be expected from comparison of the pharmacophore of previously un-tested compounds with that of *bona fide* retinoids. However prior to this study, the production of different proportions of cell types in response to alternative retinoid compounds had not been reported. Not only did the proportion of neuronal cell types observed vary, but CEB16, CEB17 and CEB18 induced a greater proportion of epithelial cell types to develop from TERA2.cl.SP12 cells than was observed with ATRA, 9-*cis*-RA, 13-*cis*-RA or two commercially available synthetic retinoids. The importance of subtle changes in ligand structure, in this case from *meta* to *para* substitution (CEB16 vs. CEB17), has subsequently been reported by our group for another isomeric pair of retinoids.³⁵² Flow cytometric analysis of stem cell and neural cell antigens confirmed the morphological observations and permitted the following order of differentiation efficacy to be established:



Differentiation was further characterised by immunocytochemical and western blot analysis of antigens associated with neural and non neural markers, proliferation studies and co-culture with a retinoid. Data are also presented that demonstrate, for the first time, the expression of retinoid receptors in TERA2.cl.SP12 huEC cells and that treatment with both natural and synthetic retinoids can modulate this expression. These data validate the use of this cell line for the study of retinoid effects. The synthetic retinoids are shown to induce the expression of RAR- α , RAR- β and RAR- γ and to have their efficacy potentiated by co-administration with an RXR ligand: two pieces of evidence that suggest these compounds are RAR ligands.

A further study was designed to investigate the effects of ATRA and two synthetic retinoids on components of the retinoid pathway. Real time PCR analysis was used to construct detailed temporal expression profiles of eleven genes involved in retinoid transport, metabolism and activation (*POU5F1*, *CRBP-I*, *CRABP-I*, *CRABP-II*, *CYP26A1*, *RAR- α* , *RAR- β* , *RAR- γ* , *HOXB1*, *HOXB2* and *HOXB3*). Although the data requires confirmation through analysis of further biological samples, CEB16 and CEB17 were clearly found to regulate the expression of a number of key components of the classical retinoid pathway, in a similar manner to ATRA. In many cases, gene expression patterns were complex, confirming the need for detailed expression analysis when comparing compounds of differing efficacy and emphasising the complicated nature of gene regulation in the retinoid pathway. Both CEB16 and CEB17 also induced the expression of three *HOX* genes. These results show that these synthetic retinoids are able to emulate some of the essential roles of ATRA in embryonic development.

Despite this similarity to ATRA and the observation of phenotypic changes consistent with neural and non-neural differentiation, it is unlikely that any of the other synthetic retinoids discussed in this thesis are able to offer the full differentiation potential and range of biological effects that are attributable to ATRA. However, this may actually be an advantage in the therapeutic application of retinoids: it is hoped that synthetic retinoids will induce specific biological effects and circumnavigate the toxicity associated with the pleiotropic nature of ATRA.

5.2 Further work

Complete and extend retinoid gene analysis

1. Analyse further independent biological samples for the expression of genes of the retinoid pathway to hopefully confirm the results presented in Chapter 4 and eliminate biological variation.
2. Investigate the effect of timing of media changes/supply of fresh retinoid on gene expression to validate the hypothesis that the temporal expression of a number of genes correlates with the strength of retinoid in the media.

Develop methods to measure changes in retinoid concentration *in vitro*

1. Develop suitable assay(s) to extract and then quantify natural and synthetic retinoid concentrations in culture media. A number of HPLC methods have been published which should provide a good starting point.^{487,749}
2. Investigate the stability of natural and synthetic retinoids under TERA2.cl.SP12 culture conditions and the effect that this has on cell behaviour. Are “stable” synthetic retinoids as un-labile *in vitro* as on the laboratory bench?
3. Attempt to correlate the expression of sensitive retinoic acid inducible genes (e.g. *CYP26A1* and *RAR-β*) with retinoid concentration in culture.
4. Define optimal media-changing protocol.

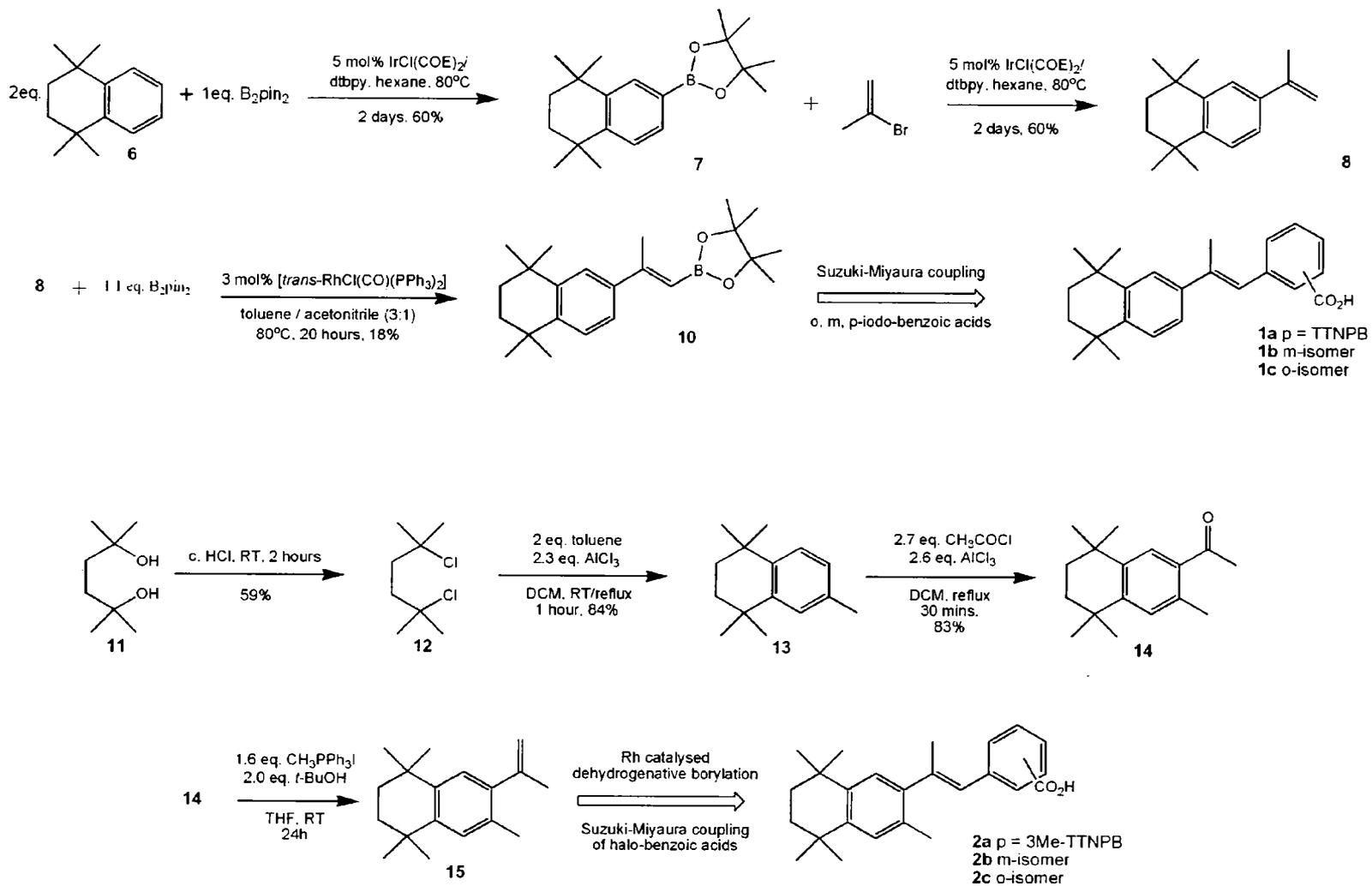
Develop retinoid screening assay

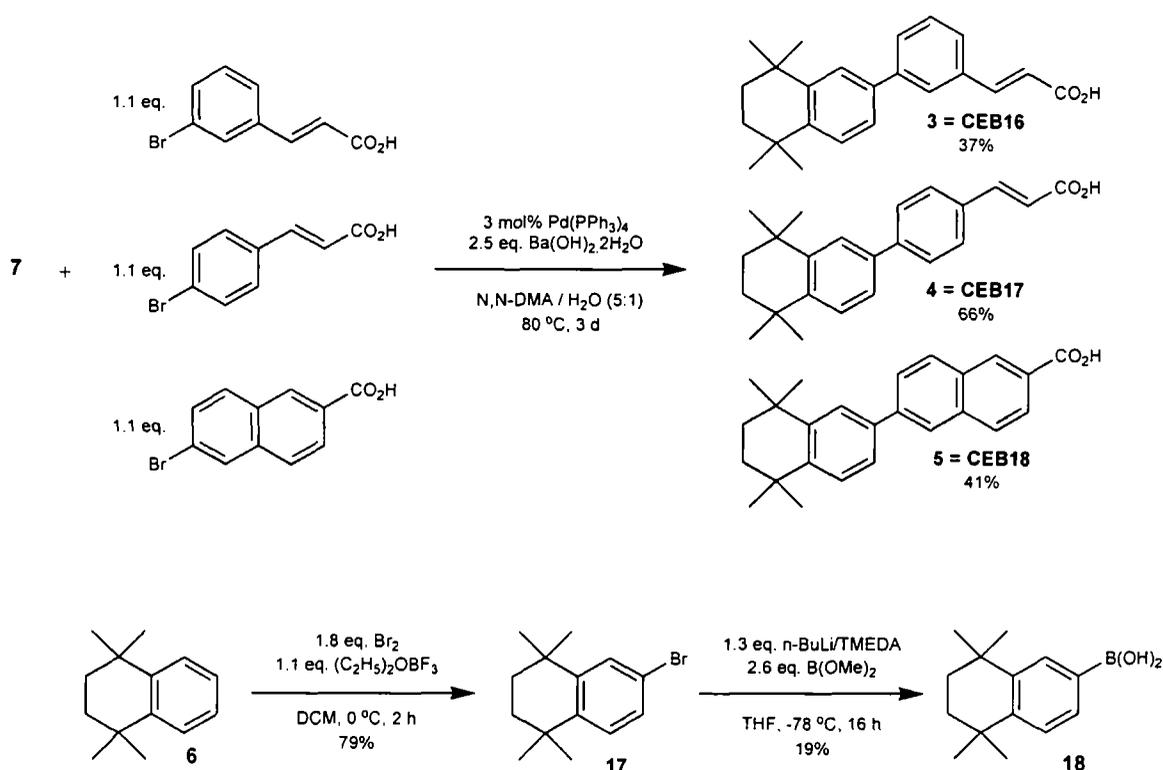
1. Develop a rapid and efficient method for screening synthetic retinoids in TERA2.cl.SP12 human EC cells.
2. Select appropriate marker(s): for example suppression of the sensitive stem cell marker SSEA-3 to investigate differentiation-inducing ability, or up-regulation of TUJ-1 to compare efficacy of neuronal differentiation induction. Construct dose response curves and calculate the EC₅₀ of novel and previously tested synthetic retinoids to provide a better analysis of retinoid efficacy in this system.
3. Use the determined optimal concentration(s) to differentiate TERA2.cl.SP12 stem cells.
4. Receptor binding assays will not capture non-retinoidal activation, so may be less appropriate than functional assays. However, this information would complement and extend the characterisation of retinoid ability and permit comparison with published affinity (K_d) data. Pharmacological antagonism of individual receptors could be alternatively or additionally used.

CHAPTER 6: EXPERIMENTAL SECTION

6.1 Chemistry

6.1.1 Synthetic scheme





6.1.2 General

All reactions were performed under dry nitrogen or argon atmospheres using either standard Schlenk techniques or in an Innovative Technology, Inc. System I glove box, except for compounds **12**, **13**, **14** and **17**. Once the reactions were complete, further procedures were carried out without any precautions against oxygen. Where appropriate, solvents were dried before use with appropriate drying agents. Reagents used in synthesis were purchased from commercial suppliers and used without further purification. B₂pin₂ was generously donated by Frontier Scientific Inc. TLC was performed with Merck Kieselgel 60 F-254 plates. Silica gel chromatography was conducted using 40 – 63 μM silica gel (Fluorochem). Evaporations were carried out at 20 mmHg using a Büchi rotary evaporator and water bath, followed by evaporation to dryness (< 2 mmHg).

NMR spectra were obtained using a Bruker 400 Ultrashield, Varian Mercury-200, Varian Unity-300 or Varian Inova-500 MHz Varian spectrometer. CDCl₃, C₆D₆ and d₆-DMSO solvents were used, the ¹H and ¹³C chemical shifts are recorded in ppm using partially deuterated solvent signals as the internal standards, and coupling constants are given in Hz.

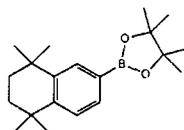
GC-MS analysis was performed on a Hewlett Packard 5890 Series II chromatograph equipped with a 5971A mass selective detector and a fused silica capillary column (10 m, 5% cross linked phenylmethylsilicone), using UHP helium as the carrier gas and under operating conditions similar to:

injector temperature 250 °C, detector temperature 300 °C, oven temperature ramped from 50 °C to 280 °C at a rate of 20 °C/min.

Mass spectra were obtained on a Micromass AutoSpec (EI) and Micromass-LCT (ES) spectrometers. The elemental analyses were performed using an Exeter Analytical Inc. CE-440 Elemental Analyser. FT-IR spectra were recorded as KBr discs using a Perkin-Elmer 1615 FT-IR spectrometer operating from a Grams Analyst1600. Melting point determinations were made using a Mel-Temp (Laboratory Devices) apparatus, using an external thermometer.

X-ray diffraction experiments were performed at 120 K on Bruker SMART 3-circle diffractometers with CCD area detectors, using graphite-monochromated Mo-K α radiation ($\lambda = 0.71073 \text{ \AA}$). The low temperature of the crystals was maintained by Oxford Cryosystems Cryostream open-flow nitrogen cryostats. All structures were solved by direct methods and refined by full matrix least squares against F^2 of all reflections using SHELXTL software. All non-H atoms were refined in anisotropic approximation; all H atoms were refined in isotropic approximation. Crystal data and experimental details are listed in Appendix 2.

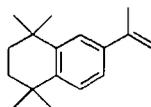
6-Pinacolboron-1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene (7).



A solution of 1,2,3,4-tetrahydro-1,1',4,4'-tetramethylnaphthalene (3.00 g, 16.0 mmol) in dry hexane (1ml) was added to B₂pin₂ (8.09 g, 32.0 mmol, 2.0 equiv.), IrCl(COE)₂ (1.42 g, 3.20 mmol, 20 mol%) and 4,4'-di-*tert*-butyl-2,2'-bipyridine (0.86 g, 3.20 mmol, 20 mol%). Dry hexane (24 ml) was added and the mixture shaken thoroughly, though the solids did not fully dissolve. The reaction was heated to 80 °C with stirring in a sealed tube. After 46 hours all starting material had been consumed (as determined by GC-MS). The solvent was removed *in vacuo* to give a dark-orange solid that was purified by silica gel chromatography (hexane:DCM, 1:1 as eluent) to afford 5.17 g (103%) of an off-white solid, which was shown by ¹H NMR to contain 10% of COE-Bpin by-product. Small-scale recrystallisation from hot hexane gave 7 as a white crystalline solid. Mp 116-118 °C; ¹H NMR (400 MHz, C₆D₆): δ 1.14 (s, 12H, pinacolato C(CH₃)₂), 1.18 and 1.22 (2s, 12H, C(CH₃)₂), 1.55 (s, 4H, CH₂CH₂), 7.28 (dd, 1H, $J = 1.3$ and 8.1 Hz, Ar-CH), 8.01 (dd, 1H, $J = 1.3$ and 8.1 Hz, Ar-CH), 8.23 (d, 1H, $J = 1.3$ Hz, Ar-CH); ¹H NMR (400 MHz, CDCl₃): δ 1.26 (s, 12H, pinacolato C(CH₃)₂), 1.30 (s, 6H, C(CH₃)₂), 1.31 (s, 6H, C(CH₃)₂), 1.66 (s, 4H, CH₂CH₂), 7.75 (d, 1H, $J = 1.2$ Hz, Ar-CH); ¹³C {¹H} NMR (101 MHz, C₆D₆): δ 25.0 (C(CH₃)₂), 31.8, 31.9, 34.3, 34.6, 35.6 (ring Cs of 1,1',4,4'-tetramethyl ring), 83.5 (C(CH₃)₂), 126.4, 132.7, 134.0, 144.2, 148.3 (C₆H₃-Bpin); ¹¹B {¹H} NMR (128 MHz,

C_6D_6) δ 31.19 (Bpin); IR (KBr disc): ν (cm^{-1}) *inter alia* 2966, 2926, 2855, 1609, 1364, 1145; GC-MS (EI) m/z : 314 $[M]^+$, 299 $[M-CH_3]^+$; Anal. calcd. (%) for $C_{20}H_{31}BO_2$: C, 76.44; H, 9.94; found: C, 76.64; H, 10.13.

Method 1: 6-Isopropenyl-1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene (8).



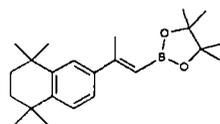
A solution of 2-bromopropene (260 μ l, 354 mg, 2.90 mmol), **7** (996 mg, 3.20 mmol, 1.1 equiv.), $Pd(dppf)Cl_2$ (121 mg, 0.16 mmol, 5.0 mol% w.r.t. **7**) and potassium phosphate dihydrate (2.01 g, 8.10 mmol, 2.5 equiv.) in DMF (15 ml) was heated to 80 °C in a sealed tube. After 17 hours the reaction mixture was cooled to room temperature and analysis by GC-MS showed total consumption of starting material. The reaction mixture was added to purified water (20 ml) in a separating funnel. Ether (20 ml) and brine (20 ml) were added, but the phase boundary could not be seen in the dark liquid. Further brine and ether were added, without improvement. The mixture was left to settle over weekend, and the layers separated. The organic layer was combined with ether washings (3 x 20 ml) of the aqueous phase, dried over Mg_2SO_4 and concentrated *in vacuo* to give a cloudy dark orange oil. To ensure all salt had been removed, the oil was dissolved in ether (10ml) and washed again with water (3 x 10 ml). Drying over Mg_2SO_4 and concentrating *in vacuo* gave a dark orange oil that was purified by silica gel chromatography (DCM:hexane, 1:9 as eluent) to give **8** as a yellow oil (approx. 0.35 g, 45% yield). 1H NMR (400 MHz, C_6D_6): δ 1.18 (s, 12H, $C(CH_3)_2$), 1.22 and 1.23 (2s, 4H, CH_2CH_2), 1.53 (s, 3H, $CH_2=CCH_3$), 4.98 (t, 1H, $J = 1.2$ Hz, $=CH_2$ cis to ring), 5.37 (t, 1H, $J = 0.8$ Hz, $=CH_2$ trans to ring), 7.09 (d, 1H, $J = 5.7$ Hz, Ar-CH), 7.19 (d, 1H, $J = 3.8$ Hz, Ar-CH), 7.46 (d, 1H, $J = 2.2$ Hz, Ar-CH); ^{13}C { 1H } NMR (101 MHz, C_6D_6): δ 22.0 ($CH_2=CCH_3$), 32.0, 32.0, 34.2 ($C(CH_3)_2$ and $C(CH_3)_2$), 35.5, 35.6 (CH_2CH_2), 111.6 ($CH_2=CCH_3$), 123.6, 123.9, 126.7, 128.5, 129.3, 139.1 ($C_6H_3-C(CH_3)=CH_2$), 144.2 (s, $CH_2=CCH_3$); GC-MS (EI) m/z : 228 $[M]^+$, 213 $[M-CH_3]^+$.

Method 2: 6-Isopropenyl-1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene (8).

A flask containing **7** (800 mg, 2.50 mmol), $Pd(PPh_3)_4$ (282 mg, 0.24 mmol, 10 mol% w.r.t. **7**) and $Ba(OH)_2 \cdot 8H_2O$ (2.01 g, 6.40 mmol, 2.6 equiv.) was evacuated / filled with nitrogen (x 3). A solution of 2-bromopropene (300 μ l, 410 mg, 3.40 mmol, 1.5 equiv.) in 25 ml of a 5:1 mixture of *N,N*-dimethylacetamide : purified water was freeze / thaw degassed (x 3) and transferred *via* syringe to the flask. The reaction mixture was heated at 80 °C for 65 hours, when analysis by GC-MS showed complete consumption of **7**. The reaction was quenched with dilute hydrochloric acid (2 ml). The resulting mixture was extracted with DCM (3 x 10 ml); the organic phase was washed with dilute

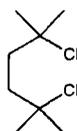
hydrochloric acid (3 x 10ml), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by silica gel chromatography (hexanes : DCM, 1:1 as eluent) gave **8** (0.62 g, 95% yield, >95% purity by GC-MS).

[2-(5,5',8,8'-Tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]pinacolboron (10).



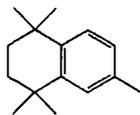
Dry toluene (3 ml) and dry acetonitrile (1ml) were mixed together and used to dissolve **8** (250 mg, 1.10 mmol), B₂pin₂ (278 mg, 1.10 mmol) and [*trans*-Rh(Cl)(CO)(PPh₃)₂] (23 mg, 33 μmol, 3 mol%). The reaction was heated to 80 °C with stirring, in a sealed tube, for 20 hours, when analysis by GC-MS showed complete consumption of the starting material. Water (5 ml) was added, and the aqueous layer washed with ethyl acetate (3 x 5 ml). The combined organic phases were dried over MgSO₄ and concentrated *in vacuo* to give a dark orange oil. The crude product was purified by silica gel chromatography (hexanes:DCM, 1:1 as eluent), solidified by washing with hot methanol and recrystallised from hot hexane to give **10** as an impure off white solid (91 mg, 77% purity by ¹H NMR = 18% yield). Mp 134-137 °C; ¹H NMR (400 MHz, CDCl₃): δ 1.25 and 1.26 (2s, 12H, C(CH₃)₂), 1.29 (s, 12H, pinacol C(CH₃)₂), 1.53 (s, 4H, CH₂CH₂), 2.37 (d, 3H, *J* = 0.8 Hz, CH=CCH₃), 5.71 (q, 1H, *J* = 0.8 Hz, CH=CCH₃), 7.23 (s, 1H, Ar-CH), 7.23 (s, 1H, Ar-CH), 7.45 (t, 1H, *J* = 1.3 Hz, Ar-CH); ¹¹B {¹H} NMR (128 MHz, CDCl₃) δ 30.42 (s, 1B, Bpin); GC-MS (EI) *m/z*: 354 [M]⁺, 339 [M-CH₃]⁺.

2,5-Dichloro-2,5-dimethylhexane (12).^{394,750}



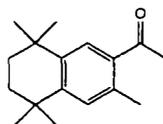
In a large conical flask 2,5-dimethyl-2,5-hexanediol (**11**) (33.5 g, 230 mol) was dissolved in c. HCl (450 ml) with stirring; a large quantity of white precipitate was formed immediately. The mixture was stirred for a further 2 hours and then poured carefully into ice-water (500 ml), filtered, and the white solid washed with more ice-water (2 x 250 ml). The aqueous filtrate was split into three and each portion was extracted by washing with ethyl acetate (2 x 75 ml). The white solid was dissolved in the combined organic washings, concentrated and dried *in vacuo* to give **12** as a white solid (24.8 g, 59%). Mp 63-65 °C (Lit. 63-65 °C³⁹⁴); ¹H NMR data were identical to those reported in the literature;³⁹⁵ ¹³C {¹H} NMR (101 MHz, CDCl₃): δ 32.5 (s, C(CH₃)₂), 41.2 (s, CH₂CH₂), 70.3 (C(CH₃)₂).

1,2,3,4-Tetrahydro-1,1',4,4',6-pentamethylnaphthalene (**13**).^{395,751-753}



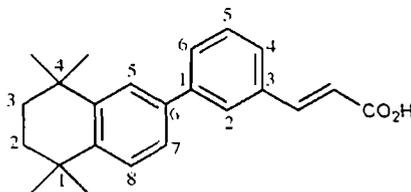
Compound **12** (20.1 g, 110 mol) was dissolved in HPLC grade toluene (20.3 g, 220 mol, 2.0 equiv.) and DCM (95 ml) and the apparatus flushed with nitrogen. The resultant pale yellow solution was stirred vigorously as AlCl_3 (202 mg, 1.50 mmol) was added in portions over 10 minutes to give a dark orange solution. The reaction was stirred at room temperature for 30 minutes, refluxed for 15 minutes, then allowed to cool to room temperature. Additional AlCl_3 was added (110 mg, 0.82 mmol) and the solution was stirred at room temperature for a further 30 minutes; the colour of the solution became dark orange-red and analysis by GC-MS showed that all the starting material had been consumed. Hydrochloric acid (20%, 20 ml) was added to the stirred solution, and the reaction mixture changed to a clear, pale yellow solution. The organics were extracted with hexane (3 x 100 ml), washed with purified water (100 ml) and brine (100 ml), then dried over MgSO_4 , filtered and concentrated *in vacuo*. The resultant pale yellow oil crystallised overnight when stored at $-20\text{ }^\circ\text{C}$ to give **13** as a pale yellow solid (23.4 g, 84% yield). Mp $27\text{--}28.5\text{ }^\circ\text{C}$ (Lit. $29\text{ }^\circ\text{C}$ ⁷⁵¹); all spectroscopic and analytical properties were identical to those reported in the literature.⁷⁵³

1-(5,6,7,8-Tetrahydro-3,5,5',8,8'-pentamethyl-2-naphthyl)ethan-1-one (**14**).



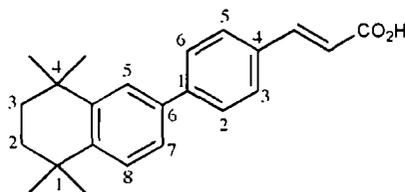
AlCl_3 (14.3 g, 110 mmol, 2.2 equiv.) was added slowly to a stirred DCM solution (50 ml) of **13** (10.0 g, 49.0 mmol) and acetyl chloride (10.0 g, 130 mmol, 2.7 equiv.). The reaction was refluxed for 15 minutes. Additional AlCl_3 (2.0 g, 15 mmol) was added to ensure the reaction was complete, and stirred for 15 minutes at room temperature. The reaction mixture was poured into vigorously stirred ice-water (200 ml), acidified with 20% hydrochloric acid (50 ml) and EtOAc added (100 ml). Stirring was continued until the organic layer was yellow (15 min). The organic layer was separated, and the aqueous layer extracted with ethyl acetate (2 x 100ml). The combined organic extracts were washed with water (100 ml) and brine (50 ml), then dried over MgSO_4 , filtered and concentrated *in vacuo* to give an off-white solid (11.5 g, 47.0 mmol). This was purified by Kugelrohr distillation to give **14** as white crystals (10.0 g, 83% yield). Mp $55\text{--}58\text{ }^\circ\text{C}$; ^1H NMR (400 MHz, CDCl_3): δ 1.26 and 1.28 (2s, 12H, $\text{C}(\text{CH}_3)_2$), 1.67 (s, 4H, CH_2CH_2), 2.47 (s, 3H, ArCH_3), 2.55 (s, 3H, $\text{O}=\text{CCH}_3$), 7.12 (s, 1H ArCH), 7.64 (s, 1H ArCH); ^{13}C { ^1H } NMR (101 MHz, CDCl_3): δ 21.4 (ArCH_3), 29.3 ($\text{O}=\text{CCH}_3$), 31.5, 31.8 ($\text{C}(\text{CH}_3)_2$), 33.9, 34.3 ($\text{C}(\text{CH}_3)_2$), 34.9, 34.9 (CH_2CH_2), 128.1, 130.1, 135.2, 135.3, 142.3, 149.0 (Ar-C_s), 201.3 (s, $\text{C}=\text{O}$); GC-MS (EI) m/z : 244 (46%, $[\text{M}]^+$), 229 (100%, $[\text{M}-\text{Me}]^+$); Anal. calcd. (%) for $\text{C}_{17}\text{H}_{24}\text{O}$: C, 83.55; H, 9.90; found: C, 83.29; H, 9.91.

3-[3-(5,6,7,8-Tetrahydro-5,5',8,8'-tetramethyl-2-naphthalenyl)phenyl] acrylic acid (3, CEB16).



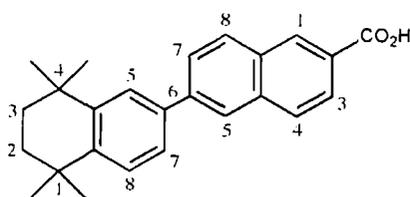
In a nitrogen atmosphere, **7** (534 mg, 1.70 mmol), 3-bromo cinnamic acid (406 mg, 1.80 mmol, 1.1 equiv.), Pd(PPh₃)₄ (57.8 mg, 50.0 μmol, 3.1 mol% w.r.t. **7**) and Ba(OH)₂·8H₂O (1.25 g, 4.00 mmol, 2.4 equiv.) were dissolved in a degassed mixture of *N,N*-dimethylacetamide / purified water (5:1, 17 ml). The solids did not dissolve completely. The reaction was heated at 80 °C in a sealed tube for 3 days when the reaction was quenched with dilute hydrochloric acid (2 ml) and extracted with DCM (3 x 20 ml). The organic phase was washed with brine (10 ml) and dilute hydrochloric acid (5 x 20 ml); the combined aqueous washings were back-extracted with DCM (2 x 20 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by silica gel chromatography (hexane : DCM, 1:1 as eluant), and recrystallisation first from EtOAc then acetone gave **3** as a white crystalline solid (280 mg, 37% yield). Mp 208-209 °C; IR (KBr disc) *inter alia*: 3436, 3029, 2955, 2858, 2682, 2595, 1679, 1626, 1434, 1271, 1217, 987, 873, 807, 701, 593, 538 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 1.31 and 1.34 (2s, 12H, C(CH₃)₂), 1.71 (s, 4H, CH₂CH₂), 6.50 (d, 1H, *J* = 15.9 Hz, CH=CH-CO₂H), 7.34 (dd, 1H, *J* = 2.0 and 8.1 Hz, 7-Ar-CH), 7.39 (d, 1H, *J* = 8.1 Hz, 8-Ar-CH), 7.45 (t, 1H, *J* = 7.7 Hz, 4/6-cinnamic-H), 7.49 (d, 1H, *J* = 1.8 Hz, 5-Ar-CH), 7.50 (dm overlapping 7.49, 1H, 5-cinnamic-H), 7.60 (dt, 1H, *J* = 1.5 and 7.6 Hz, 4/6-cinnamic-H), 7.71 (broad s, 1H, 2-cinnamic-H), 7.84 (d, 1H, *J* = 16.2 Hz, CH=CH-CO₂H); ¹H NMR (400 MHz, d₆-DMSO): δ 1.31 and 1.36 (2s, 12H, C(CH₃)₂), 1.71 (s, 4H, CH₂CH₂), 6.68 (d, 1H, *J* = 16.4 Hz, CH=CH-CO₂H), 7.44 (d, 1H, *J* = 8.3 Hz, 8-Ar-CH), 7.48 (dd overlapping 7.52, 1H, *J* = 1.6 and 8.6 Hz, 7-Ar-CH), 7.52 (t overlapping 7.48, 1H, *J* = 7.7 Hz, 5-Ar-CH), 7.67 (dm, 2H, *J* = 17.7 Hz, cinnamic-H), 7.73 (d, 2H, *J* = 16.2 Hz, cinnamic-H), 7.96 (broad s, 1H, CH=CH-CO₂H), 12.44 (s, 1H, CH=CH-CO₂H); ¹³C {¹H} NMR (101 MHz, CDCl₃): 31.9 and 31.9 (C(CH₃)₂), 34.2 and 34.4 (C(CH₃)₂), 35.1 and 35.2 (CH₂CH₂), 117.3 (CH=CH-CO₂H), 124.4, 125.3, 126.7, 127.2, 129.3, 129.5, 134.4, 137.5, 142.6, 144.7, 145.5 and 147.2 (Ar-C and cinnamic acid-C), 171.5 (CO₂H); ¹³C {¹H} NMR (101 MHz, d₆-DMSO): 32.0 and 32.1 (C(CH₃)₂), 34.2 and 34.5 (C(CH₃)₂), 35.0 and 35.2 (CH₂CH₂), 120.0 (CH=CH-CO₂H), 124.6, 125.2, 126.9, 127.2, 127.5, 128.8, 129.8, 135.3, 137.2, 141.6, 144.4 and 144.5 (Ar-C and cinnamic acid-C), 168.1 (CO₂H); MS (EI) *m/z*: 334 (21%, [M]⁺), 333 (100%, [M-H]⁺); HRMS (EI) calcd. for C₂₃H₂₆O₂ - H: 333.18600; found: 333.18582.

4-[4-(5,6,7,8-Tetrahydro-5,5',8,8'-tetramethyl-2-naphthalenyl)phenyl] acrylic acid (CEB17, 4).



In a nitrogen atmosphere, **7** (515 mg, 1.60 mmol), 4-bromocinnamic acid (396 mg, 1.70 mmol, 1.1 equiv.), Pd(PPh₃)₄ (58.7 mg, 51.0 μmol, 3.1 mol% w.r.t. **7**) and Ba(OH)₂·8H₂O (1.26 g, 4.00 mmol, 2.5 equiv.) were dissolved in a degassed mixture of *N,N*-dimethylacetamide / purified water (5:1, 17 ml). The solids did not dissolve completely. The reaction was heated at 80 °C in a sealed tube for 3 days when the reaction was quenched with dilute hydrochloric acid (2 ml) and extracted with EtOAc (50 ml). The organic phase was washed with brine (10 ml) and dilute hydrochloric acid (3 x 20 ml); the combined aqueous washings were back-extracted with EtOAc (2 x 50 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. The crude off-white product was purified by flash silica gel chromatography (THF as eluent), and recrystallisation first from hot THF then acetone at approximately -20 °C (freezer) to give **4** as a white crystalline solid (360 mg, 66% yield). Mp 276-278 °C; IR (KBr disc) *inter alia*: 2957, 2920, 1674, 1626, 1426, 1313, 1184, 1107, 821, 700, 500 cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 1.31 and 1.33 (2s, 12H, C(CH₃)₂), 1.71 (s, 4H, CH₂CH₂), 6.46 (d, 1H, *J* = 15.9 Hz, CH=CH-CO₂H), 7.37 (broad m, 2H, Ar-CH), 7.52 (broad m, 1H, Ar-CH), 7.60 (s, 4H, cinnamic acid Ar-CH), 7.79 (d, 1H, *J* = 15.9 Hz, CH=CH-CO₂H); ¹H NMR (400 MHz, d₆-DMSO): δ 1.31 and 1.35 (2s, 12H, C(CH₃)₂), 1.71 (s, 4H, CH₂CH₂), 6.60 (d, 1H, *J* = 16.0 Hz, CH=CH-CO₂H), 7.46 (s, 1H, 5-Ar-CH), 7.46 (m, 1H, 7-Ar-CH), 7.63 (bs, 1H, 8-Ar-CH), 7.65 (d, 1H, *J* = 15.7 Hz, CH=CH-CO₂H), 7.72 (d, 2H, *J* = 8.1 Hz, cinnamic-*H*), 7.80 (d, 2H, *J* = 7.6 Hz, cinnamic-*H*), 12.44 (s, 1H, CO₂H); ¹³C {¹H} NMR (101 MHz, d₆-DMSO): δ 32.4 and 32.5 (C(CH₃)₂), 34.7 and 35.0 (C(CH₃)₂), 35.4 and 35.6 (CH₂CH₂), 119.8, 124.9, 125.4, 127.8, 128.0, 129.7, 133.9, 137.4, 143.0, 144.4, 145.2 and 145.9 (Ar-C and cinnamic acid-C), 168.5 (CO₂H); MS (EI) *m/z*: 334 (23%, [M]⁺), 333 (100%, [M-H]⁺); HRMS (EI) calcd. for C₂₃H₂₆O₂ - H: 333.18600, found: 333.18586.

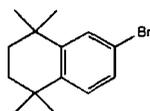
6-(1,1',4,4'-Tetramethyl-1,2,3,4-tetrahydro-6-naphthyl)-2-naphthalene carboxylic acid (CEB18, 5).⁴⁹⁶



In a nitrogen atmosphere, **7** (532 mg, 1.70 mmol), 6-bromo-2-naphthoic acid (439 mg, 1.70 mmol, 1.0 equiv.), Pd(PPh₃)₄ (58.9 mg, 51.0 μmol, 3.0 mol% w.r.t. **7**) and Ba(OH)₂·8H₂O (1.25 g, 4.00 mmol, 2.4 equiv.) were dissolved in a degassed mixture of *N,N*-dimethylacetamide / purified water (5:1, 17 ml). The solids did not dissolve completely. The reaction was heated at 80 °C in a sealed tube for 3

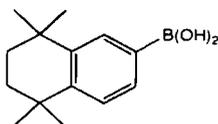
days when the reaction was quenched with dilute hydrochloric acid (15 ml) and extracted with EtOAc (3 x 50 ml). The organic phase was washed with water (3 x 20 ml); the combined aqueous washings were back-extracted with EtOAc (2 x 50 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was filtered through a silica gel pad (eluent = EtOAc). Re-crystallisation from acetone at -20 °C gave four crops of **5** as a white crystalline solid (250 mg, 41% yield) as colourless crystals. Mp 279-280 °C (Lit. 287.5-288.5 °C⁴⁹⁶); all spectroscopic and analytical properties were identical to those reported in the literature.⁴⁹⁶

6-Bromo-1,1',4,4'-tetramethyl-1,2,3,4-tetrahydronaphthalene (17).^{395,754,755}



A solution of **6** (25.0 g, 130 mmol) and Br₂ (38.1 g, 240 mol, 1.8 equiv.) in dry DCM (150 ml) under nitrogen was stirred in an ice-bath while boron trifluoride diethyl etherate (19.9 g, 140 mmol, 1.1 equiv.) was added drop-wise over 2 hours. Analysis by GC-MS showed no **6** remained. A 40% ethyl acetate / hexane mixture was added (350 ml), and the resultant solution washed sequentially with saturated aqueous sodium Na₂S₂O₃, NaHCO₃ and brine. The organic extracts were dried over MgSO₄, filtered and concentrated *in vacuo* to give a pale brown solid. The crude product was re-crystallised from methanol at approximately -20 °C (freezer) overnight to yield **17** as white needle-shaped crystals (27.9 g, 79%). Mp 47-48 °C (Lit. 35-36 °C⁷⁵⁴); all spectroscopic and analytical properties were identical to those reported in the literature;⁷⁵⁵ MS (EI) *m/z*: 268 and 266 (24% and 24%, [M]⁺), 253 and 251 (98% and 100%, [M-CH₃]⁺), 172 (49%, [M-MeBr]⁺).

5,6,7,8-Tetrahydro-5,5',8,8'-2-naphthaleneboronic acid (18).³⁵⁷



n-BuLi (15.5 ml of a 1.6 M solution in hexanes, 25.0 mmol, 1.3 equiv.) was added dropwise to a stirred solution of TMEDA (3.70 ml, 25.0 mmol) in dry THF (65 ml) at -78 °C under Ar. After 30 minutes a THF solution (35 ml) of **17** (5.00 g, 18.0 mmol) was added dropwise to the mixture and stirred for a further 75 minutes. The resultant yellow solution was treated with trimethylborate (5.30 ml, 47mmol, 2.6 equiv.) dropwise over 10 minutes. The solution was allowed to warm to RT and left to stir at RT for 15 hours under Ar. 3 M HCl was added slowly until the pH was 1. The organic and aqueous layers were separated and the aqueous layer washed with ethyl acetate/hexane (9:1, 3 x 75 ml). The combined organic fractions were dried over MgSO₄, filtered and concentrated *in vacuo* to give a dark orange oil. The crude product was purified in three stages by silica gel chromatography (ethyl acetate : hexane 1:4, then 1:1; 10% MeOH/90% CHCl₃ as eluent) to give **18** as a white

crystalline product (1.21 g, 19%). Mp 188-190 °C (Lit. 190-192 °C³⁵⁷); ¹H NMR data were identical to those reported in the literature;³⁵⁷ ¹³C {¹H} NMR (101 MHz, C₆D₆): δ 31.7 and 31.9 (C(CH₃)₂), 34.2, 34.7, 35.1 (ring Cs of 1,1',4,4'-tetramethyl ring), 126.2, 132.4, 134.2, 144.3, 149.8 (Ar-CH); ¹¹B {¹H} NMR (128 MHz, C₆D₆) δ 29.7 (s, B(OH)₂).

6.2 Biological testing

6.2.1 Cell culture techniques

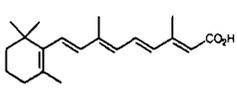
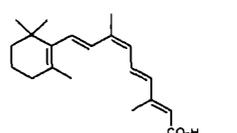
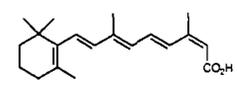
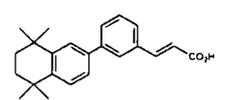
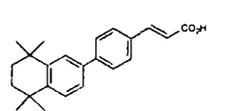
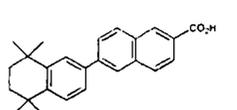
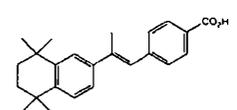
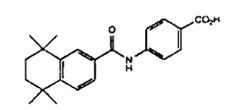
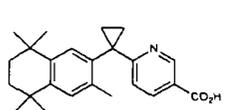
TERA2.cl.SP12 cell culture

TERA2 clone SP12 (TERA2.cl.SP12) human EC stem cells were maintained in Dulbecco's Modified Eagle's media (DMEM, Sigma or Gibco) supplemented with 10% heat deactivated foetal bovine serum (Gibco), 2 mmol L-glutamine (Cambrex), 1000 IU/ml penicillin and streptomycin (Gibco) (DMEM-FGA) at 37 °C in a humidified atmosphere of 5% CO₂ in air. TERA2.cl.SP12 cells were grown at high confluency as monolayers in tissue culture plastic flasks (Nunc), changing the medium every 2-3 days as necessary and passaging when fully confluent using acid-washed glass beads, typically in a 1:3 split. These conditions have previously been demonstrated to maintain the undifferentiated phenotype of this cell line.⁴²⁵

In preparation for differentiation induction, confluent cultures of TERA2.cl.SP12 cells were gently disassociated by incubating with 2 mg/L trypsin-EDTA (Cambrex) for up to 2 minutes to produce a suspension of single cells. The number of cells harvested was counted using a haemocytometer and then seeded at a density of 20 000 cells/cm² into an appropriate vessel for the experiment with DMEM-FGA containing 10⁻⁵ M retinoid or an equal volume of vehicle (0.1% v/v DMSO). Media was refreshed every 2-3 days with retinoid or DMSO-enriched media and the cultures maintained for up to 4 weeks.

Differentiated cells were harvested by disassociating with 2 mg/L trypsin-EDTA, however it was noted that while some cell types were loosely attached to the tissue culture plastic, some cells (particularly epithelial cells) adhered strongly. To capture the whole population in the best condition it was found that an initial wash with PBS prior to trypsinisation aided disassociation; if necessary the cells should be harvested in two stages. Cultures should be treated with trypsin-EDTA for up to 2 minutes, the trypsin neutralised and all suspended cells removed from the flask. The flask should be washed with PBS (and added to harvested cells), and the culture trypsinised again.

Table 6-1: Retinoid and retinoid-like compounds.

Compound		Synonym(s)
all-trans-retinoic acid		ATRA tretinoin
9-cis-retinoic acid		9-cis-RA alitretinoin
13-cis-retinoic acid		13-cis-RA isotretinoin
CEB16		CD2908
CEB17		—
CEB18		TTNN AGN 191650
TTNPB		TTNPB Ro 13-7410
Am580		CD366
LG100268		—

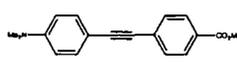
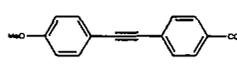
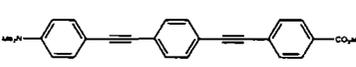
Compound		Synonym(s)
NMe₂ tolan		—
OMe tolan		—
BPEB		—

Table 6-1 contains lists of the retinoids and other compounds tested during in the project. All-*trans*-retinoic acid (ATRA), 9-*cis*-retinoic acid (9-*cis*-RA), 13-*cis*-retinoic acid (13-*cis*-RA), (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5',8,8'-tetramethyl-2-naphthalenyl) propen-1-yl] benzoic acid (TTNPB) and 4-

(5,6,7,8-tetrahydro-5,5',8,8'-tetramethyl-2-naphthalenecarboxamido)-benzoic acid (Am580) were purchased from Sigma-Aldrich. Compounds CEB16, CEB17 and CEB18 were prepared by the author, Jonathan Barnard prepared additional samples of CEB16 and CEB17. A sample of LG100268 (6-[1-(3,5,5',8,'8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-cyclopropyl]) was kindly provided by Dr. Chris Redfern. Compounds identified as OMe tolan, NMe₂ tolan and BPEB were prepared by Dr. Tolu Fasina. Samples were dissolved in sterile dimethylsulfoxide (DMSO, Sigma) to a concentration of 10 mM, protected from light and stored in aliquots at -18 °C. The stock solutions were diluted in DMEM-FGA to give a final concentration of approximately 10 µM of retinoid or control substance, and filter sterilised through a 0.22 µm membrane filter (Millex-LG, Millipore).

The effects induced in the TERA2.cl.SP12 cells were studied by:

1. Morphological observation over 28 days, recorded by phase microscopy;
2. Changes in expression of established immunophenotypic antigens by indirect flow cytometry after 7, 14 and 28 days' culture;
3. Immunocytochemical (ICC) localisation of neural and non-neural antibodies after 14 and 28 days' culture on glass coverslips;
4. Investigating the effects on cell proliferation, measured by tetrazolium dye reduction assays for up to 14 days' culture;
5. Western blots were used to profile the protein expression of samples harvested after 7, 14 and 28 days' culture;
6. Temporal variations of selected components of the retinoid pathway was measured using real-time PCR analysis over 14 days' culture.

SH-SY5Y culture

Experiments using SH-SY5Y neuroblastoma cells were performed by Dr. Chris Redfern's research group (Northern Institute for Cancer Research, Medical School, Newcastle University). The SH-SY5Y cells were kindly supplied by Professor Robert Ross (Fordham University, NY, USA). SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma or Gibco) supplemented with heat-deactivated foetal bovine serum (10% v/v), L-glutamine (2 mM) and antibiotics (penicillin and streptomycin, 1000 IU/ml) at 37 °C in a humidified atmosphere (5% CO₂ in air).

6.2.2 Morphological changes

Cell cultures were monitored carefully for the duration of each experiment, using a Nikon Eclipse TS100 phase-contrast microscope. Typical observations for a culture, in isolation and in relation to other cultures, included:

1. Colour of phenol red indicator in medium;
2. Using the naked eye: opacity of the culture, areas of the flask where cells are clumping, absent, or adopting other notable features;
3. Amount of cell debris;
4. The level of culture confluence;
5. Individual cell and colony morphologies.

Representative phase photographs were captured during this time using Nikon Coolpix 5000 and Nikon DMX1200 digital cameras.

6.2.3 Immunofluorescent flow cytometry

Cell surface antigen expression was determined on TERA2.cl.SP12 stem cells and their retinoid-induced derivatives by indirect immunofluorescence using an EPICS XL cytometer (Coulter). Primary monoclonal antibodies specific to the antigens associated with pluripotent stem cells (globoseries glycolipid SSEA-3 and keratan sulfate-related TRA-1-60) and neural cell types (VIN-IS-53 and A2B5) were used to investigate the differentiation status of EC cells and their derivatives grown in media enriched with test retinoids. Undifferentiated TERA2.cl.SP12 cells were analysed before treatment and after 7, 14 and 28 days' treatment with retinoids.

Cell cultures were disassociated with 2 mg/L trypsin-EDTA trypsin EDTA to create a single cell suspension and the number of cells counted using a haemocytometer. All subsequent procedures were performed on ice to preserve cell viability. An appropriate number of cells were washed twice in chilled wash buffer (WB) consisting of PBS supplemented with 0.1% w/w bovine serum albumin (BSA, Sigma) and 200 000 cells for each antibody were transferred into one well of a 96-well round-bottomed plate (Nunc). The plate was centrifuged and supernatant removed.

Cell pellets were re-suspended in 50 μ l of one of the following primary antibodies diluted in WB: P3X (IgG, a generous gift of Prof. P. Andrews, University of Sheffield, 1:10), SSEA-3 (IgM, Developmental Studies Hybridoma Bank, 1:5), TRA-1-60 (IgM, a generous gift of Prof. P. Andrews, University of Sheffield, 1:6), VIN-IS-53 (IgG, Developmental Studies Hybridoma Bank, 1:5), A2B5

(IgM, R & D Systems, 1:100) and incubated for between 20 and 40 minutes. Excess antibody was removed with three washes of WB. The cells were re-suspended in 50 μ l of the appropriate FITC-conjugated secondary antibody diluted in WB: α -mouse IgG (Cappel, 1:100) or α -mouse IgM (Cappel, 1:100) and incubated shielded from light for a further 20 to 40 minutes. The cells were washed with WB three times, then re-suspended in 800 μ l WB and transferred to cytometer tubes for analysis. 10 μ l propidium iodide solution (1.0 mg/ml, Sigma) was added to each sample to label dead cells.

Single, live cells were examined by indirect immunofluorescence using a Coulter XL flow cytometer fitted with a 15 mW Ar ion air-cooled laser with a 488 nm excitation wavelength and a 10 mW HeCd air-cooled laser exciting at 325 nm. Dead cells and cell debris were excluded from the analysis by gating with forward scatter and side scatter.

Data analysis

Data were analysed using SPSS version 12.01 for Windows (SPSS Inc.). Data were normally distributed (one sample Kolmogorov-Smirnov, $P > 0.05$), but since the variance increased with the mean, and the SSEA-3 and A2B5 data sets were skewed, the % positive cells data was arcsin transformed for statistical analysis. Univariate analysis of variance (ANOVA) was used to test for significant differences between compounds, followed by Bonferroni correction post-hoc analysis. Significance levels were set at: * P -value < 0.05 , ** P -value < 0.01 and *** P -value < 0.001 .

6.2.4 Tetrazolium proliferation assay

TERA2.cl.SP12 cells were seeded at a density of 20 000 cells/cm² in 12-well plates (Nunc) in retinoid-enriched media, media containing the vehicle (0.1% v/v DMSO) or fresh proliferative media. Media was changed every two days, or more frequently as required, with fresh media containing the appropriate retinoid or control reagent. After 0, 3, 7, 10 and 14 days' exposure to the test conditions the cell cultures were subjected to a tetrazolium colourimetric assay to measure the number of proliferative cells.

Media was gently aspirated from the wells and replaced with fresh DMEM-FGA (1500 μ l) and 300 μ l MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, Promega).[§] An additional empty well was also prepared in this manner to act as a UV blank. The plates were incubated for four hours and the resulting solutions were mixed gently.

[§] Optimum volumes were determined in a preliminary experiment. These volumes provide an excess of MTS reagent at the greatest cell density expected in this experiment (data not shown).

50 μ l of each solution was diluted in 950 μ l dH₂O** and the UV absorbance of these solutions measured at 490 nm. The dilution and UV measurement step was performed in triplicate and the result averaged to control for pipetting accuracies.

Data analysis

Data were analysed using SPSS version 12.01 for Windows (SPSS Inc.). Data were normally distributed (one sample Kolmogorov-Smirnov, $P > 0.05$), but since the variance increased with the mean the % UV absorbencies were arcsin transformed for statistical analysis. Univariate analysis of variance (ANOVA) was used to test for significant differences between compounds, followed by Bonferroni correction post-hoc analysis. Significance levels were set at: * P -value < 0.05 , ** P -value < 0.01 and *** P -value < 0.001 .

6.2.5 Immunocytofluorescence

Cells were grown on poly-D-lysine coated coverslips in 12-well plates (Nunc) or 8-well chamber slides (BD Falcon) using culture techniques as previously reported. After 14 and 28 days' culture, the cells were fixed with either 4% $\%$, paraformaldehyde in PBS (20 minutes, RT) or 5% $\%$, glacial acetic acid in methanol (for MAP-2 staining: 20 minutes, -20 °C). Cells were blocked for 30 minutes in wash diluent (WD: 5% normal goat serum (Invitrogen) and 0.2% Triton X-100 (Fisher Scientific) in PBS) to limit background staining, then incubated for 60 minutes with one or two of the primary antibodies shown in Table 6-2 diluted in WD. Cells were then washed three times in WD and subsequently conjugated with the appropriate secondary antibodies (α -mouse IgG-FITC conjugated, α -rabbit IgG-Cy3 conjugated, α -mouse IgM-Texas Red conjugated, or α -rabbit IgG-FITC conjugated, all diluted 1:600 in WD) for 1 hour at room temperature. Unbound secondary antibodies were removed by three PBS washes. Control slides were also prepared for each retinoid: the slides were prepared as above, except WD alone was used in place of the primary antibodies.

Stained coverslips were inverted and mounted on microscope slides in Vectashield Mounting Medium (Vector Laboratories) containing 1% of Hoechst 33342 (10 ng/ml aqueous solution, Molecular Probes) to retard photobleaching and the coverslips sealed using nail varnish. Stained coverslips were examined using a fluorescence microscope (Axiovert 135). Phase contrast and corresponding fluorescence images were collected using a Nikon Coolpix 5000 digital camera.

** To give absorbencies within the linear range of the spectrophotometer.

Table 6-2: Antibodies used in immunocytochemical analysis.

Antibody anti-	Raised in	Dilution	Supplier
β -III-tubulin (clone TUJ-1)	rabbit	1:100	Covance
nestin	mouse	1:200	Chemicon
neurofilament-68 (NF68)	mouse	1:200	Sigma
cytokeratin 8 (C8)	mouse	1:300	Sigma
fibronectin	rabbit	1:500	Sigma
Glial fibrillary associated protein (GFAP)	mouse	1:500	Sigma
Neuron specific enolase (NSE)	mouse	1:200	Chemicon
A2B5	mouse	1:200	R & D Systems
Microtubule associated protein-2 (MAP-2)	mouse	1:200	Sigma
Growth associated protein-43 (GAP-43)	mouse	1:400	Sigma

6.2.6 Western blots

Protein samples were prepared from TERA2.cl.SP12 EC cells and their retinoid-induced derivatives by lysing harvested cells for 30 minutes on ice, in a lysis buffer prepared from six parts Igepal lysis buffer (1% Igepal CA-630 (Sigma), 5% 1 M Tris (pH 8.0), 5% 3 M NaCl, 1% 1 M MgCl₂, 89.6% dH₂O) and one part protease inhibitors (Complete Mini protease inhibitor cocktail tablets, Roche Diagnostics). The resulting protein solutions were centrifuged at 14 500 rpm for 5 minutes to remove cytoskeletal debris. Protein concentrations of the supernatants were quantified using the Bradford method,⁷⁵⁶ then snap-frozen in liquid N₂, and stored at -80 °C until required.

Sodium dodecyl sulfate (SDS)-polyacrylamide gels were prepared according to the method of Laemmli.⁷⁵⁷ Using the Bio-Rad minigel system (Bio-Rad Laboratories), separating gel (10% or 5% polyacrylamide) with a loading gel (4% polyacrylamide) was poured to a thickness of 0.75 mm. Samples were prepared as follows: 12 µg of each protein sample was diluted in dH₂O to 12 µl and denatured in 3 µl of reducing sample loading buffer (22% 1 M Tris-HCl (pH 6.8), 44% glycerol, 63 mM sodium dodecyl sulfate, 11% 0.5 M EDTA, 8% β-mercaptoethanol, 5% bromophenol blue, 5% phenol red and 5% xylene) at 95 °C for 3 minutes and vortex mixed.

15 µl of each sample and 3 µl of ECL Plex Fluorescent Rainbow Marker (GE Healthcare) were loaded onto the gels and electrophoresed at 70 V until the dye bands were compressed at the gel junction (approx. 12 minutes), then at 200 V until the coloured bands at the SDS front dropped off the bottom of the gel (approx. 45 minutes). Resolved gels were immediately transferred onto Hybond-P (PVDF) membranes (GE Healthcare) using the Bio-Rad mini-gel transfer apparatus at 100 V, at 5 °C for 1 hour. Confirmation of protein transfer was achieved by staining blots with 0.2% w/v Ponceau S in 5% acetic acid; however, Ponceau staining resulted in elevated background fluorescence when using the Typhoon scanner so this step was generally omitted.

Membranes were blocked in 5% BSA in PBS, with rocking, for one hour at RT and subsequently incubated with the primary mouse and goat antibodies listed in Table 3-6 diluted in 0.1% Tween 20 in PBS (PBS-T) for 1 hr at RT or overnight at 5 °C. Blots were rinsed in PBS-T twice and washed for 2 x 5 minutes in PBS-T rocking at RT. The membranes were then incubated for 1 hour at RT, protected from light and with rocking, with ECL Plex CyDye α-mouse and/or α-goat conjugated secondary antibodies as appropriate (1:2500, GE Healthcare) diluted in PBS-T. Unbound secondary antibodies were washed off by rinsing the membranes three times in PBS-T, followed by 4 x 5 minutes in PBS-T with shaking at RT and finally rinsed three times with PBS. Blots were dried and viewed using a Typhoon 9400 Variable Mode Imager (Amersham Biosciences).

Table 6-3: Antibodies used in western blot analysis.

Gel	Antibody anti-	Predicted MW (kDa)	Raised in	Dilution	Supplier & product code
1	β -actin	42	mouse	1:10 000	Sigma A5441
	TUJ-1	50	rabbit	1:1000	Covance PRB-435P
	NF68	68	mouse	1:1000	Sigma N5139
2	NSE	45	mouse	1:1000	Sigma MAB324
	RAR- β	51	rabbit	1:500	Santa Cruz sc-552
	NF160	160	mouse	1:1000	Sigma N2787
3	C8	52.5	mouse	1:500	Sigma C5301
	OCT 4	43	rabbit	1:500	Abcam ab19857
	RAR- γ	60	rabbit	1:500	Santa Cruz sc-550
	RAR- α	50 / 55	rabbit	500	Santa Cruz sc-551
4	NF200	200	mouse	1:400	Abcam ab7795
	fibronectin	220-240	rabbit	1:500	Sigma F3648
5	nestin	220-240	mouse	1:1000	Chemicon MAB5326
	MAP-2	2 x 280 & 70	mouse	1:1000	Sigma M9942

6.2.7 Real-time PCR

RNA extraction and reverse transcription (RT)

Cultures were washed gently with PBS prior to extracting total RNA with TRI reagent[®] (Sigma) according to the manufacturer's suggested protocol for monolayer cell extraction for rt qPCR. The RNA pellet was dissolved in DEPC-treated water (Sigma) and stored at -80 °C. Purified RNA was quantified and assessed for purity by UV spectrophotometry using a Nanodrop[®] ND-1000 (Thermo Fisher Scientific).

Single stranded cDNA was synthesised from 4 µg of total cellular RNA by reverse transcription in a 20 µl reaction using oligo-d(T)₁₆-primers and MultiScribe™ reverse transcriptase (TaqMan[®] Reverse Transcription Reagents, Applied Biosystems) following the manufacturer's instructions. The RT reaction was performed in an Eppendorf Mastercycler gradient programmed with the parameters: incubation at 25 °C for 10 minutes, reverse transcription at 48 °C for 30 minutes followed by RT inactivation at 95 °C for 5 minutes. cDNA was stored at -80 °C until use.

Absolute quantitative real-time PCR (rt qPCR)

Quantitative PCR was used to measure the relative changes in RNA gene expression after treatment of TERA2.cl.SP12 with retinoids. Real-time PCR (rt qPCR) was performed using Applied Biosystems 7500 Fast Real-Time PCR Systems instrument, with Sequence Detection Software Version 1.4 (Applied Biosystems) and SYBR[®] GREEN chemistry.

PCR reactions were performed in a volume of 10 µl containing 1 µl cDNA, forward and reverse oligonucleotide primers (150 nM of each), SYBR[®] GREEN PCR master mix (x2, Applied Biosystems, 5 µl) and dH₂O. After incubating the plate at 50 °C for 2 minutes, rt qPCR amplification was performed in a two-step procedure: denaturation at 95 °C for 10 minutes and between 40-60 cycles (as required for full amplification of the gene of interest, Table 6-4) of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 1 minute. Acquisition of the fluorescent signal from the samples was carried out at the end of annealing step. Finally, a disassociation programme was run: the plate was heated to 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds.

Primer sequences (Table 6-4) were obtained from published references, some of which were sourced from the Real Time PCR Primer and Probe Database (RTPrimerDB),^{758,759} or designed using Primer Express 3.0 (Applied Biosystems). All oligonucleotides were custom synthesised by VH Bio. Primer sequences were confirmed to be specific to only the sequence(s) of interest using the Basic Alignment

Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov/blast/blast.cgi>)⁷⁶⁰ and Netprimer (<http://www.premierbiosoft.com/netprimer/index.html>) was used to assess the quality of primer design and likelihood of primer dimerisation.

The purity and identity of the PCR products was confirmed by establishing the presence of a single peak in the dissociation curve, and subjecting a sample to electrophoresis on a 1.5% w/v agarose gel stained with ethidium bromide and visualised by UV, to confirm a single product of the expected size.

Table 6-4: rt qPCR primer sequences

Gene	Primer Sequences (5' to 3') F, R	Product length (bp) and position(s)	Number of cycles	Sequence Ref.	Standard
CRBP-I	TAGAGATGAGAGTGGAAAGGTGTGGT GGGGTGGCTGGACATTTTTG	151 (628-478)	45	761	5d 10 µM ATRA
CRABP-I	AGGTCGGAGAAGGCTTTGAG AGAAGAGTTTGCCTGCAGTG	103 (376-274)	40	56	5d 10 µM ATRA
CRABP-II	TGCAGGGTCTTGCTTTCTTT GGGCTAGGACTGCTGACTTG	126 (698-823)	40	762	5d 10 µM ATRA
CYP26A1 (variants 1 & 2)	GGAGGACACGAAACCAC TCAGAGCAACCCGAAACC	221 (1143-923, 1070-1290)	60	763	5d 10 µM ATRA
RAR-α (variants 1 & 2)	GGGACAAGAAGTGCATCATCAA CCTTGGACATGCCCACTTC	90 (832-921, 977-1066)	50	self- designed	14d 10 µM ATRA
RAR-β (variants 1 & 2)	ATTCCAGTGCTGACCATCGAGTCC CCTGTTTCTGTGCATCCATTTCC	349 (722-1070, 1079-1427)	60	764	14d 10 µM ATRA
RAR-γ (variants 1 & 2)	TGTGCGAAATGACCGGAAC CTAACTGAGGGCTCAGCTCATAGC	83 (906-988, 805-887)	50	216	d0
POU5F1 (OCT 3/4) (variants 1 & 2)	GAGAACCGAGTGAGAGGCAACC CATAGTCGCTGCTTGATCGCTTG	166 (768-933, 528-693)	45	765	d0
HOXB1	CTCCTCTCCGAGGACAAGGAA CTGTCTTGGGTGGGTTTCTCTTAA	103 (481-583)	50	51	5d 10 µM ATRA
HOXB2	TCCTTGGCCGTCTACTGGAA AGTGGATTAACGCTAATTCAGTAATACC	105 (1228-1332)	50	51	5d 10 µM ATRA
HOXB3	CCTGGCCTGAGAGGTTGCT TCCCGGGCGTGGAATT	145 (2304-2448)	50	51	5d 10 µM ATRA
GAPDH	ATGGGGAAGGTGAAGGTCGGAG TCGCCCCACTTGATTTGGAGG	266 (103-368)	40	RTPrimer DB ID: 2072	d0

Data processing

Data were analysed using Sequence Detection Software Version 1.4 (Applied Biosystems). Standard curves were constructed for each PCR analysis, consisting of six serial dilutions of cDNA analysed in triplicate. The cDNA sample selected as the standard for each gene is shown in Table 6-4. An example calibration curve is shown in Appendix 3. The average correlation coefficients (R^2) was 0.99 (range = 0.999-0.958). Greater than 99% of samples with detectable levels of gene expression were within the standard range (>90% of all samples).

To account for variations in RT efficiency, individual gene expression values were normalised using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. This gene has been previously demonstrated to be stably expressed in this cell line under similar experimental conditions.⁴⁷³

Bibliography

- 1 M. B. Sporn, N. M. Dunlop, D. L. Newton and J. M. Smith, *Fed. Proc.*, 1976, **35**, 1332-1338.
- 2 IUPAC-IUB Joint Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 1982, **129**, 1-5.
- 3 M. B. Sporn and A. B. Roberts, *Ciba Foundation Symposia*, 1985, **113**, 1-5.
- 4 R. Blomhoff and H. K. Blomhoff, *J. Neurobiol.*, 2006, **66**, 606-630.
- 5 C. Liebecq, *J. Mol. Biol.*, 1998, **275**, 527-537.
- 6 J. L. Napoli, *Clin. Immunol. Immunopathol.*, 1996, **80**, S52-S62.
- 7 J. L. Napoli, in *Chemistry and Biology of Synthetic Retinoids*, ed. M. I. Dawson and W. H. Okamura, CRC Press, Boca Raton, Fla, 1990, 229-249.
- 8 S. A. Ross, P. J. McCaffery, U. C. Drager and L. M. De Luca, *Physiol. Rev.*, 2000, **80**, 1021-1054.
- 9 G. Wald, *Nature*, 1968, **219**, 800-807.
- 10 M. Clagett-Dame and H. F. DeLuca, *Annu. Rev. Nutr.*, 2002, **22**, 347-381.
- 11 R. D. Semba, *J. Nutr.*, 1999, **129**, 783-791.
- 12 A. L. Fields, D. R. Soprano and K. J. Soprano, *J. Cell. Biochem.*, 2007, **102**, 886-898.
- 13 G. H. Travis, M. Golczak, A. R. Moise and K. Palczewski, *Annu. Rev. Pharmacol. Toxicol.*, 2007, **47**, 469-512.
- 14 M. Golczak, A. Maeda, G. Bereta, T. Maeda, P. D. Kiser, S. Hunzelmann, J. von Lintig, W. S. Blaner and K. Palczewski, *J. Biol. Chem.*, 2008, **283**, 9543-9554.
- 15 H. Meinhardt, *Dev. Dyn.*, 2006, **235**, 2907-2919.
- 16 A. Lumsden and R. Krumlauf, *Science*, 1996, **274**, 1109-1115.
- 17 C. Tickle, *Semin. Cell Dev. Biol.*, 1999, **10**, 345-351.
- 18 E. M. De Robertis and H. Kuroda, *Annu. Rev. Cell Dev. Biol.*, 2004, **20**, 285-308.
- 19 A. E. Schier and W. S. Talbot, *Annu. Rev. Genet.*, 2005, **39**, 561-613.
- 20 F. Mavilio, *Eur. J. Biochem.*, 1993, **212**, 273-288.
- 21 A. L. Means and L. J. Gudas, *Annu. Rev. Biochem.*, 1995, **64**, 201-233.
- 22 E. Marti and P. Bovolenta, *Trends Neurosci.*, 2002, **25**, 89-96.
- 23 L. Wilson and M. Maden, *Dev. Biol.*, 2005, **282**, 1-13.
- 24 M. Maden, *J. Neurobiol.*, 2006, **66**, 726-738.
- 25 E. Dessaud, L. L. Yang, K. Hill, B. Cox, F. Ulloa, A. Ribeiro, A. Mynett, B. G. Novitsch and J. Briscoe, *Nature*, 2007, **450**, 717-720.
- 26 K. Niederreither, V. Subbarayan, P. Dollé and P. Chambon, *Nature Genet.*, 1999, **21**, 444-448.
- 27 P. McCaffery and U. C. Drager, *Cytokine Growth Factor Rev.*, 2000, **11**, 233-249.
- 28 S. Reijntjes, A. Blentic, E. Gale and M. Maden, *Dev. Biol.*, 2005, **285**, 224-237.
- 29 M. Uehara, K. Yashiro, S. Mamiya, J. Nishino, P. Chambon, P. Dollé and Y. Sakai, *Dev. Biol.*, 2007, **302**, 399-411.
- 30 R. J. White, Q. Nie, A. D. Lander and T. F. Schilling, *PLoS Biol.*, 2007, **5**, 2522-2533.
- 31 H. de The, A. Marchio, P. Tiollais and A. Dejean, *EMBO J.*, 1989, **8**, 429-433.
- 32 C. Thaller and G. Eichele, *Nature*, 1987, **327**, 625-628.
- 33 Y. Kawakami, A. Raya, R. M. Raya, C. Rodriguez-Esteban and J. C. I. Belmonte, *Nature*, 2005, **435**, 165-171.
- 34 R. Keynes and R. Krumlauf, *Annu. Rev. Neurosci.*, 1994, **17**, 109-132.
- 35 M. Maden, *Proc. Nutr. Soc.*, 2000, **59**, 65-73.
- 36 M. Maden, *Nat. Rev. Neurosci.*, 2002, **3**, 843-853.
- 37 J. C. Glover, J. S. Renaud and F. M. Rijli, *J. Neurobiol.*, 2006, **66**, 705-725.
- 38 M. Maden, *Nat. Rev. Neurosci.*, 2007, **8**, 755-765.
- 39 E. B. Lewis, *Nature*, 1978, **276**, 565-570.
- 40 A. Giampaolo, D. Acampora, V. Zappavigna, M. Pannese, M. Desposito, A. Care, A. Faiella, A. Stornaiuolo, G. Russo, A. Simeone, E. Boncinelli and C. Peschle, *Differentiation*, 1989, **40**, 191-197.
- 41 W. McGinnis and R. Krumlauf, *Cell*, 1992, **68**, 283-302.
- 42 R. Krumlauf, *Cell*, 1994, **78**, 191-201.
- 43 G. Gellon and W. McGinnis, *BioEssays*, 1998, **20**, 116-125.
- 44 D. Lemons and W. McGinnis, *Science*, 2006, **313**, 1918-1922.
- 45 L. Colucci-D'Amato, V. Bonavita and U. di Porzio, *Neurol. Sci.*, 2006, **27**, 266-270.
- 46 S. Y. Sun and R. Lotan, *Crit. Rev. Oncol./Hematol.*, 2002, **41**, 41-55.
- 47 L. Altucci, M. D. Leibowitz, K. M. Ogilvie, A. R. de Lera and H. Gronemeyer, *Nat. Rev. Drug Discovery*, 2007, **6**, 793-810.
- 48 N. P. Mongan and L. J. Gudas, *Differentiation*, 2007, **75**, 853-870.
- 49 D. Flagiello, A. Gibaud, B. Dutrillaux, M. F. Poupon and B. Malfoy, *FEBS Lett.*, 1997, **415**, 263-267.

- 50 C. Abate-Shen, *Nat. Rev. Cancer*, 2002, **2**, 777-785.
- 51 Y. Takahashi, J. Hamada, K. Murakawa, M. Takada, M. Tada, I. Nogami, N. Hayashi, S. Nakamori, M. Monden, M. Miyamoto, H. Katoh and T. Moriuchi, *Exp. Cell Res.*, 2004, **293**, 144-153.
- 52 K. N. Chen, Z. D. Gu, Y. Ke, A. Y. Li, X. T. Shi and G. W. Xu, *Clin. Cancer Res.*, 2005, **11**, 1044-1049.
- 53 S. Samuel and H. Naora, *Eur. J. Cancer*, 2005, **41**, 2428-2437.
- 54 O. Takahashi, J. Hamada, M. Abe, S. Hata, T. Asano, Y. Takahashi, M. Tada, M. Miyamoto, S. Kondo and T. Moriuchi, *Oncol. Rep.*, 2007, **17**, 753-760.
- 55 Y. K. Jing, J. Zhang, I. J. Bleiweiss, S. Waxman, A. Zelent and R. MiraYlopez, *FASEB J.*, 1996, **10**, 1064-1070.
- 56 J. Kwong, K. W. Lo, L. S. N. Chow, K. F. To, K. W. Choy, F. L. Chan, S. C. Mok and D. P. Huang, *Neoplasia*, 2005, **7**, 67-74.
- 57 C. Funk, *J. State Med.*, 1912, **20**, 341-368.
- 58 J. E. Dowling and G. Wald, *Proc. Natl. Acad. Sci. U. S. A.*, 1958, **44**, 648-661.
- 59 O. Wallach, *Justus Liebigs Ann. Chem.*, 1887, **239**, 1-54.
- 60 O. Isler, *Pure Appl. Chem.*, 1979, **51**, 447-462.
- 61 J. F. Arens and D. A. Van Dorp, *Nature*, 1946, **157**, 190-191.
- 62 O. Isler, W. Huber, A. Ronco and M. Kofler, *Helv. Chim. Acta*, 1947, **30**, 1911-1927.
- 63 J. G. Wilson, C. B. Roth and J. Warkany, *Am. J. Anat.*, 1953, **92**, 189-217.
- 64 J. N. Thompson, J. M. C. Howell, G. A. J. Pitt and C. I. McLaughlin, *Br. J. Nutr.*, 1969, **23**, 471-490.
- 65 P. J. McCaffery, J. Adams, M. Maden and E. Rosa-Molinar, *Eur. J. Neurosci.*, 2003, **18**, 457-472.
- 66 E. J. Lammer, D. T. Chen, R. M. Hoar, N. D. Agnish, P. J. Benke, J. T. Braun, C. J. Curry, P. M. Fernhoff, A. W. Grix, I. T. Lott, J. M. Richard and S. C. Sun, *N. Engl. J. Med.*, 1985, **313**, 837-841.
- 67 D. R. Soprano and K. J. Soprano, *Annu. Rev. Nutr.*, 1995, **15**, 111-132.
- 68 H. M. Dolk, H. Nau, H. Hummler and S. M. Barlow, *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 1999, **83**, 31-36.
- 69 Expert Group on Vitamins and Minerals, Safe Upper Levels for Vitamins and Minerals, 2003.
- 70 X. D. Wang, *Nutr. Rev.*, 1999, **57**, 51-59.
- 71 J. Bi, X. L. Hu, F. C. Zhou and L. N. Wei, *Dev. Growth Differ.*, 2001, **43**, 553-561.
- 72 A. P. Brogan, T. J. Dickerson, G. E. Boldt and K. D. Janda, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 10433-10438.
- 73 World Health Organisation, Global prevalence of Vitamin A Deficiency. Micronutrient Deficiency Information System Working Paper No. 2, 1995.
- 74 M. Mark, N. B. Ghyselinck and P. Chambon, *Annu. Rev. Pharmacol. Toxicol.*, 2006, **46**, 451-480.
- 75 A. B. Goodman, *J. Cell. Physiol.*, 2006, **209**, 598-603.
- 76 S. B. Wolbach, *J. Bone Joint Surg. Am.*, 1947, **29**, 171-192.
- 77 Food Standards Agency, <http://www.food.gov.uk/>.
- 78 E. H. Harrison, *Annu. Rev. Nutr.*, 2005, **25**, 87-103.
- 79 F. M. Herr and D. E. Ong, *Biochemistry*, 1992, **31**, 6748-6755.
- 80 D. E. Ong, *J. Biol. Chem.*, 1984, **259**, 1476-1482.
- 81 J. A. Crow and D. E. Ong, *Proc. Natl. Acad. Sci. U. S. A.*, 1985, **82**, 4707-4711.
- 82 E. Li and A. W. Norris, *Annu. Rev. Nutr.*, 1996, **16**, 205-234.
- 83 N. Noy, *Biochem. J.*, 2000, **348**, 481-495.
- 84 W. S. Blaner, J. C. Obunike, S. B. Kurlandsky, M. Alhaideri, R. Piantedosi, R. J. Deckelbaum and I. J. Goldberg, *J. Biol. Chem.*, 1994, **269**, 16559-16565.
- 85 R. Blomhoff, M. H. Green, J. B. Green, T. Berg and K. R. Norum, *Physiol. Rev.*, 1991, **71**, 951-990.
- 86 D. S. Goodman, H. S. Huang and Shirator, T., *J. Lipid Res.*, 1965, **6**, 390-396.
- 87 R. Blomhoff, M. H. Green, T. Berg and K. R. Norum, *Science*, 1990, **250**, 399-404.
- 88 B. A. Underwood, J. D. Loerch and K. C. Lewis, *J. Nutr.*, 1979, **109**, 796-806.
- 89 N. B. Ghyselinck, C. Bavik, V. Sapin, M. Mark, D. Bonnier, C. Hindelang, A. Dierich, C. B. Nilsson, H. Hakansson, P. Sauvart, V. Azais-Braesco, M. Frasson, S. Picaud and P. Chambon, *EMBO J.*, 1999, **18**, 4903-4914.
- 90 E. H. Harrison and M. Z. Gad, *J. Biol. Chem.*, 1989, **264**, 17142-17147.
- 91 D. E. Ong, *Arch. Dermatol.*, 1987, **123**, 1693-1695.
- 92 R. Blomhoff, M. Rasmussen, A. Nilsson, K. R. Norum, T. Berg, W. S. Blaner, M. Kato, J. R. Mertz, D. S. Goodman, U. Eriksson and P. A. Peterson, *J. Biol. Chem.*, 1985, **260**, 3560-3565.
- 93 H. Senoo, *Med. Electron. Microsc.*, 2004, **37**, 3-15.
- 94 M. Kanai, A. Raz and D. S. Goodman, *J. Clin. Invest.*, 1968, **47**, 2025-2044.
- 95 Y. Fierce, M. D. Vieira, R. Piantedosi, A. Wyss, W. S. Blaner and J. Paik, *Arch. Biochem. Biophys.*, 2008, **472**, 126-138.
- 96 J. O. Alvsaker, F. B. Haugli and S. G. Laland, *Biochem. J.*, 1967, **102**, 362-366.
- 97 G. Zanotti and R. Berni, *Vitam. Horm.*, 2004, **69**, 271-295.
- 98 G. Siegenthaler and J. H. Saurat, *Biochem. Biophys. Res. Commun.*, 1987, **143**, 418-423.

- 99 M. T. P. Ho, J. B. Massey, H. J. Pownall, R. E. Anderson and J. G. Hollyfield, *J. Biol. Chem.*, 1989, **264**, 928-935.
- 100 N. Noy and Z. J. Xu, *Biochemistry*, 1990, **29**, 3878-3883.
- 101 A. R. Moise, N. Noy, K. Palczewski and W. S. Blaner, *Biochemistry*, 2007, **46**, 4449-4458.
- 102 D. E. Ong and F. Chytil, *Nature*, 1975, **255**, 74-75.
- 103 C. C. Chen and J. Heller, *J. Biol. Chem.*, 1977, **252**, 5216-5221.
- 104 M. Sundaram, A. Sivaprasadarao, M. M. DeSousa and J. B. C. Findlay, *J. Biol. Chem.*, 1998, **273**, 3336-3342.
- 105 R. Kawaguchi, J. M. Yu, J. Honda, J. Hu, J. Whitelegge, P. Ping, P. Wiita, D. Bok and H. Sun, *Science*, 2007, **315**, 820-825.
- 106 F. Pasutto, H. Sticht, G. Hammersen, G. Gillessen-Kaesbach, D. R. FitzPatrick, G. Nurnberg, F. Brasch, H. Schirmer-Zimmermann, J. L. Tolmie, D. Chitayat, G. Houge, L. Fernandez-Martinez, S. Keating, G. Mortier, R. C. M. Hennekam, A. von der Wense, A. Slavotinek, P. Meinecke, P. Bitoun, C. Becker, P. Nurnberg, A. Reis and A. Rauch, *Am. J. Hum. Genet.*, 2007, **80**, 550-560.
- 107 J. W. Crabb, S. Goldflam, S. E. Harris and J. C. Saari, *J. Biol. Chem.*, 1988, **263**, 18688-18692.
- 108 I. Klaassen and B. J. M. Braakhuis, *Oral Oncol.*, 2002, **38**, 532-542.
- 109 C. Folli, V. Calderone, S. Ottonello, A. Bolchi, G. Zanotti, M. Stoppini and R. Berni, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 3710-3715.
- 110 C. Folli, V. Calderone, I. Ramazzina, G. Zanotti and R. Berni, *J. Biol. Chem.*, 2002, **277**, 41970-41977.
- 111 K. C. Posch, M. Boerman, R. D. Burns and J. L. Napoli, *Biochemistry*, 1991, **30**, 6224-6230.
- 112 M. Boerman and J. L. Napoli, *J. Biol. Chem.*, 1996, **271**, 5610-5616.
- 113 M. Lidén and U. Eriksson, *J. Biol. Chem.*, 2006, **281**, 13001-13004.
- 114 K. C. Posch, R. D. Burns and J. L. Napoli, *J. Biol. Chem.*, 1992, **267**, 19676-19682.
- 115 P. A. Dockham, M. O. Lee and N. E. Sladek, *Biochem. Pharmacol.*, 1992, **43**, 2453-2469.
- 116 L. C. Hsu, W. C. Chang, L. Hiraoka and C. L. Hsieh, *Genomics*, 1994, **24**, 333-341.
- 117 P. N. Macdonald and D. E. Ong, *J. Biol. Chem.*, 1987, **262**, 10550-10556.
- 118 J. L. Napoli, in *Vitamin A in Health and Disease*, ed. R. Blomhoff, Marcel Dekker, New York, 1994.
- 119 D. E. Ong, M. E. Newcomer and F. Chytil, in *The Retinoids: Biology, Chemistry and Medicine*, ed. M. B. Sporn, A. B. Roberts and D. S. Goodman, Raven Press, Ltd., New York, 2nd edn., 1994, 283-318.
- 120 M. P. Gaub, Y. Lutz, N. B. Ghyselinck, I. Scheuer, V. Pfister, P. Chambon and C. Rochette-Egly, *J. Histochem. Cytochem.*, 1998, **46**, 1103-1111.
- 121 D. Dong, S. E. Ruuska, D. J. Levinthal and N. Noy, *J. Biol. Chem.*, 1999, **274**, 23695-23698.
- 122 R. J. Sessler and N. Noy, *Mol. Cell*, 2005, **18**, 343-353.
- 123 J. F. Boylan and L. J. Gudas, *J. Cell Biol.*, 1991, **112**, 965-979.
- 124 J. F. Boylan and L. J. Gudas, *J. Biol. Chem.*, 1992, **267**, 21486-21491.
- 125 P. D. Fiorella and J. L. Napoli, *J. Biol. Chem.*, 1994, **269**, 10538-10544.
- 126 J. Marill, T. Cresteil, M. Lanotte and G. G. Chabot, *Mol. Pharmacol.*, 2000, **58**, 1341-1348.
- 127 P. M. Petkovich, *J. Amer. Acad. Dermatol.*, 2001, **45**, S136-S142.
- 128 J. A. White, B. Beckett-Jones, Y. D. Guo, F. J. Dilworth, J. Bonasoro, G. Jones and M. Petkovich, *J. Biol. Chem.*, 1997, **272**, 18538-18541.
- 129 W. J. Ray, G. Bain, M. Yao and D. I. Gottlieb, *J. Biol. Chem.*, 1997, **272**, 18702-18708.
- 130 J. A. White, H. Ramshaw, M. Taimi, W. Stangle, A. Q. Zhang, S. Everingham, S. Creighton, S. P. Tam, G. Jones and M. Petkovich, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 6403-6408.
- 131 M. Taimi, C. Helvig, J. Wisniewski, H. Ramshaw, J. White, M. Amad, B. Korczak and M. Petkovich, *J. Biol. Chem.*, 2004, **279**, 77-85.
- 132 G. MacLean, S. Abu-Abed, P. Dollé, A. Tahayato, P. Chambon and M. Petkovich, *Mech. Dev.*, 2001, **107**, 195-201.
- 133 S. Abu-Abed, P. Dollé, D. Metzger, B. Beckett, P. Chambon and M. Petkovich, *Genes Dev.*, 2001, **15**, 226-240.
- 134 Y. Sakai, C. Meno, H. Fujii, J. Nishino, H. Shiratori, Y. Saijoh, J. Rossant and H. Hamada, *Genes Dev.*, 2001, **15**, 213-225.
- 135 K. Yashiro, X. L. Zhao, M. Uehara, K. Yamashita, M. Nishijima, J. Nishino, Y. Saijoh, Y. Sakai and H. Hamada, *Dev. Cell*, 2004, **6**, 411-422.
- 136 S. Reijntjes, A. Rodaway and M. Maden, *Int. J. Dev. Biol.*, 2007, **51**, 351-360.
- 137 S. Langton and L. J. Gudas, *Dev. Biol.*, 2008, **315**, 331-354.
- 138 J. A. White, Y. D. Guo, K. Baetz, B. Beckett-Jones, J. Bonasoro, K. E. Hsu, F. J. Dilworth, G. Jones and M. Petkovich, *J. Biol. Chem.*, 1996, **271**, 29922-29927.
- 139 H. Fujii, T. Sato, S. Kaneko, O. Gotoh, Y. Fujii-Kuriyama, K. Osawa, S. Kato and H. Hamada, *EMBO J.*, 1997, **16**, 4163-4173.
- 140 M. A. Lane, A. C. Chen, S. D. Roman, F. Derguini and L. J. Gudas, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 13524-13529.
- 141 J. V. Chithalen, L. Luu, M. Petkovich and G. Jones, *J. Lipid Res.*, 2002, **43**, 1133-1142.

- 142 W. W. M. Pijnappel, H. F. J. Hendriks, G. E. Folkers, C. E. Vandenbrink, E. J. Dekker, C. Edelenbosch, P. T. Vandersaag and A. J. Durston, *Nature*, 1993, **366**, 340-344.
- 143 C. C. Achkar, F. Derguini, B. Blumberg, A. Langston, A. A. Levin, J. Speck, R. M. Evans, J. Bolado, K. Nakanishi, J. Buck and L. J. Gudas, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 4879-4884.
- 144 O. Sorg, C. Tran, P. Carraux, C. Barraclough, J. Arrighi, P. Descombes, V. Piguët, J. Saurat and D. Grand, *J. Invest. Dermatol.*, 2007, **127**, S38-S38.
- 145 N. Idres, J. Marill, M. A. Flexor and G. C. Chabot, *J. Biol. Chem.*, 2002, **277**, 31491-31498.
- 146 A. B. Barua and N. Sidell, *J. Nutr.*, 2004, **134**, 286S-289S.
- 147 J. Marill, C. C. Capron, N. Idres and G. G. Chabot, *Biochem. Pharmacol.*, 2002, **63**, 933-943.
- 148 R. D. Bruno and V. C. O. Njar, *Bioorg. Med. Chem.*, 2007, **15**, 5047-5060.
- 149 M. Leid, P. Kastner and P. Chambon, *Trends Biochem. Sci.*, 1992, **17**, 427-433.
- 150 V. Giguere, *Endocr. Rev.*, 1994, **15**, 61-79.
- 151 J. C. Saari, in *The Retinoids: Biology, Chemistry and Medicine*, ed. M. B. Sporn, A. B. Roberts and D. S. Goodman, Raven Press, New York, 2nd edn., 1994, 351-385.
- 152 V. Giguere, E. S. Ong, P. Segui and R. M. Evans, *Nature*, 1987, **330**, 624-629.
- 153 M. Petkovich, N. J. Brand, A. Krust and P. Chambon, *Nature*, 1987, **330**, 444-450.
- 154 D. Benbrook, E. Lernhardt and M. Pfahl, *Nature*, 1988, **333**, 669-672.
- 155 N. Brand, M. Petkovich, A. Krust, P. Chambon, H. Dethé, A. Marchio, P. Tiollais and A. Dejean, *Nature*, 1988, **332**, 850-853.
- 156 A. Krust, P. Kastner, M. Petkovich, A. Zelent and P. Chambon, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 5310-5314.
- 157 A. Zelent, A. Krust, M. Petkovich, P. Kastner and P. Chambon, *Nature*, 1989, **339**, 714-717.
- 158 D. J. Mangelsdorf, U. Borgmeyer, R. A. Heyman, J. Y. Zhou, E. S. Ong, A. E. Oro, A. Kakizuka and R. M. Evans, *Genes Dev.*, 1992, **6**, 329-344.
- 159 M. Leid, P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader and P. Chambon, *Cell*, 1992, **68**, 377-395.
- 160 M. S. Marks, P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem and K. Ozato, *EMBO J.*, 1992, **11**, 1419-1435.
- 161 A. A. Levin, L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzenisen, M. Rosenberger, A. Lovey and J. F. Grippo, *Nature*, 1992, **355**, 359-361.
- 162 R. A. Heyman, D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans and C. Thaller, *Cell*, 1992, **68**, 397-406.
- 163 G. Allenby, M. T. Bocquel, M. Saunders, S. Kazmer, J. Speck, M. Rosenberger, A. Lovey, P. Kastner, J. F. Grippo, P. Chambon and A. A. Levin, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 30-34.
- 164 A. D. Weston, B. Blumberg and T. M. Underhill, *J. Cell Biol.*, 2003, **161**, 223-228.
- 165 C. K. Glass and M. G. Rosenfeld, *Genes Dev.*, 2000, **14**, 121-141.
- 166 F. J. Dilworth and P. Chambon, *Oncogene*, 2001, **20**, 3047-3054.
- 167 L. Altucci and H. Gronemeyer, *Nat. Rev. Cancer*, 2001, **1**, 181-193.
- 168 P. Germain, J. Iyer, C. Zechel and H. Gronemeyer, *Nature*, 2002, **415**, 187-192.
- 169 L. N. Wei, *Annu. Rev. Pharmacol. Toxicol.*, 2003, **43**, 47-72.
- 170 M. L. Privalsky, *Annu. Rev. Physiol.*, 2004, **66**, 315-360.
- 171 J. Bastien and C. Rochette-Egly, *Gene*, 2004, **328**, 1-16.
- 172 K. W. Nettles and G. L. Greene, *Annu. Rev. Physiol.*, 2005, **67**, 309-333.
- 173 J. P. Renaud, N. Rochel, M. Ruff, V. Vivat, P. Chambon, H. Gronemeyer and D. Moras, *Nature*, 1995, **378**, 681-689.
- 174 C. N. Cavasotto, G. Liu, S. Y. James, P. D. Hobbs, V. J. Peterson, A. A. Bhattacharya, S. K. Kolluri, X. K. Zhang, M. Leid, R. Abagyan, R. C. Liddington and M. I. Dawson, *J. Med. Chem.*, 2004, **47**, 4360-4372.
- 175 E. S. Klein, J. W. Wang, B. Khalifa, S. A. Gavigan and R. A. S. Chandraratna, *J. Biol. Chem.*, 2000, **275**, 19401-19408.
- 176 P. Germain, L. Altucci, W. Bourguet, C. Rochette-Egly and H. Gronemeyer, *Pure Appl. Chem.*, 2003, **75**, 1619-1664.
- 177 R. M. Evans, *Science*, 1988, **240**, 889-895.
- 178 J. E. Balmer and R. Blomhoff, *J. Lipid Res.*, 2002, **43**, 1773-1808.
- 179 A. Chawla, J. J. Repa, R. M. Evans and D. J. Mangelsdorf, *Science*, 2001, **294**, 1866-1870.
- 180 S. Nagpal, S. Friant, H. Nakshatri and P. Chambon, *EMBO J.*, 1993, **12**, 2349-2360.
- 181 B. Durand, M. Saunders, P. Leroy, M. Leid and P. Chambon, *Cell*, 1992, **71**, 73-85.
- 182 C. K. Glass, *Endocr. Rev.*, 1994, **15**, 391-407.
- 183 P. Kastner, M. Mark, N. Ghyselinck, W. Krezel, V. Dupe, J. M. Grondona and P. Chambon, *Development*, 1997, **124**, 313-326.
- 184 M. Mark, N. B. Ghyselinck, O. Wendling, V. Dupe, B. Mascrez, P. Kastner and P. Chambon, *Proc. Nutr. Soc.*, 1999, **58**, 609-613.

- 185 X. K. Zhang, J. Lehmann, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran and M. Pfahl, *Nature*, 1992, **358**, 587-591.
- 186 S. A. Kliewer, K. Umesono, D. J. Mangelsdorf and R. M. Evans, *Nature*, 1992, **355**, 446-449.
- 187 X. K. Zhang, B. Hoffmann, P. B. V. Tran, G. Graupner and M. Pfahl, *Nature*, 1992, **355**, 441-446.
- 188 F. Flamant, J. D. Baxter, D. Forrest, S. Refetoff, H. Samuels, T. S. Scanlan, B. Vennstrom and J. Samarut, *Pharmacol. Rev.*, 2006, **58**, 705-711.
- 189 D. D. Moore, S. Kato, W. Xie, D. J. Mangelsdorf, D. R. Schmidt, R. Xiao and S. A. Kliewer, *Pharmacol. Rev.*, 2006, **58**, 742-759.
- 190 S. A. Kliewer, K. Umesono, D. J. Noonan, R. A. Heyman and R. M. Evans, *Nature*, 1992, **358**, 771-774.
- 191 O. Bardot, T. C. Aldridge, N. Latruffe and S. Green, *Biochem. Biophys. Res. Commun.*, 1993, **192**, 37-45.
- 192 S. Kersten, D. Kelleher, P. Chambon, H. Gronemeyer and N. Noy, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 8645-8649.
- 193 S. Kersten, L. Pan, P. Chambon, H. Gronemeyer and N. Noy, *Biochemistry*, 1995, **34**, 13717-13721.
- 194 Z. P. Chen, J. Iyer, W. Bourguet, P. Held, C. Mioskowski, L. Lebeau, N. Noy, P. Chambon and H. Gronemeyer, *J. Mol. Biol.*, 1998, **275**, 55-65.
- 195 P. Kastner, M. Mark and P. Chambon, *Cell*, 1995, **83**, 859-869.
- 196 R. Taneja, P. Bouillet, J. F. Boylan, M. P. Gaub, B. Roy, L. J. Gudas and P. Chambon, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7854-7858.
- 197 D. Lohnes, M. Mark, C. Mendelsohn, P. Dollé, D. Decimo, M. Lemeur, A. Dierich, P. Gorry and P. Chambon, *J. Steroid Biochem. Mol. Biol.*, 1995, **53**, 475-486.
- 198 H. Chiba, J. Clifford, D. Metzger and P. Chambon, *Mol. Cell. Biol.*, 1997, **17**, 3013-3020.
- 199 D. Y. Roy, R. Taneja and P. Chambon, *Mol. Cell. Biol.*, 1995, **15**, 6481-6487.
- 200 J. Y. Chen, J. Clifford, C. Zusi, J. Starrett, D. Tortolani, J. Ostrowski, P. R. Reczek, P. Chambon and H. Gronemeyer, *Nature*, 1996, **382**, 819-822.
- 201 J. D. Love, J. T. Gooch, S. Benko, C. Li, L. Nagy, V. K. K. Chatterjee, R. M. Evans and J. W. R. Schwabe, *J. Biol. Chem.*, 2002, **277**, 11385-11391.
- 202 M. R. Forman, S. D. Hursting, A. Umar and J. C. Barrett, *Annu. Rev. Nutr.*, 2004, **24**, 223-254.
- 203 C. Calleja, N. Messaddeq, B. Chapellier, H. Y. Yang, W. Krezel, M. Li, D. Metzger, B. Mascrez, K. Ohta, H. Kagechika, Y. Endo, M. Mark, N. B. Ghyselinck and P. Chambon, *Genes Dev.*, 2006, **20**, 1525-1538.
- 204 V. Horn, S. Minucci, V. V. Ogryzko, E. D. Adamson, B. H. Howard, A. A. Levin and K. Ozato, *FASEB J.*, 1996, **10**, 1071-1077.
- 205 S. Minucci, M. Leid, R. Toyama, J. P. Saint-Jeannet, V. J. Peterson, V. Horn, J. E. Ishmael, N. Bhattacharyya, A. Dey, I. B. Dawid and K. Ozato, *Mol. Cell. Biol.*, 1997, **17**, 644-655.
- 206 D. L. Crowe, *Curr. Cancer Drug Targets*, 2002, **2**, 77-86.
- 207 P. Dollé, E. Ruberte, P. Kastner, M. Petkovich, C. M. Stoner, L. J. Gudas and P. Chambon, *Nature*, 1989, **342**, 702-705.
- 208 D. J. Mangelsdorf, K. Umesono and R. M. Evans, in *The Retinoids: Biology, Chemistry and Medicine*, ed. M. B. Sporn, A. B. Roberts and D. S. Goodman, Raven Press, Ltd., New York, 2nd edn., 1994.
- 209 P. Chambon, *FASEB J.*, 1996, **10**, 940-954.
- 210 X. J. Peng, T. Maruo, Y. X. Cao, V. Punj, R. Mehta, T. K. Das Gupta and K. Christov, *Cancer Res.*, 2004, **64**, 8911-8918.
- 211 G. Benoit, A. Cooney, V. Giguere, H. Ingraham, M. Lazar, G. Muscat, T. Perlmann, J. P. Renaud, J. Schwabe, F. Sladek, M. J. Tsai and V. Laudet, *Pharmacol. Rev.*, 2006, **58**, 798-836.
- 212 S. Kitareewan, M. J. Spinella, J. Allopenna, P. R. Reczek and E. Dmitrovsky, *Oncogene*, 1999, **18**, 5747-5755.
- 213 M. I. Dawson, *Curr. Med. Chem.: Anti-Cancer Agents*, 2004, **4**, 199-230.
- 214 H. Jiang, J. D. Penner, R. L. Beard, R. A. S. Chandraratna and D. M. Kochhar, *Biochem. Pharmacol.*, 1995, **50**, 669-676.
- 215 K. T. Liby, M. M. Yore and M. B. Sporn, *Nat. Rev. Cancer*, 2007, **7**, 357-369.
- 216 J. M. Lehmann, L. Jong, A. Fanjul, J. F. Cameron, X. P. Lu, P. Haefner, M. I. Dawson and M. Pfahl, *Science*, 1992, **258**, 1944-1946.
- 217 M. F. Boehm, L. Zhang, L. Zhi, M. R. McClurg, E. Berger, M. Wagoner, D. E. Mais, C. M. Suto, P. J. A. Davies, R. A. Heyman and A. M. Nadzan, *J. Med. Chem.*, 1995, **38**, 3146-3155.
- 218 R. Mukherjee, P. J. A. Davies, D. L. Crombie, E. D. Bischoff, R. M. Cesario, L. Jow, L. G. Hamann, M. F. Boehm, C. E. Mondon, A. M. Nadzan, J. R. Paterniti and R. A. Heyman, *Nature*, 1997, **386**, 407-410.
- 219 J. R. Rigas and K. H. Dragnev, *Oncologist*, 2005, **10**, 22-33.
- 220 F. C. Zusi, M. V. Lorenzi and V. Vivat-Hannah, *Drug Discov. Today*, 2002, **7**, 1165-1174.
- 221 A. R. de Lera, W. Bourguet, L. Altucci and H. Gronemeyer, *Nat. Rev. Drug Discovery*, 2007, **6**, 811-820.
- 222 R. Kojima, T. Fujimori, N. Kiyota, Y. Toriya, T. Fukuda, T. Ohashi, T. Sato, Y. Yoshizawa, K. Takeyama, H. Mano, S. Masushige and S. Kato, *J. Biol. Chem.*, 1994, **269**, 32700-32707.
- 223 G. Wolf, *Nutr. Rev.*, 2006, **64**, 532-538.

- 224 J. Urbach and R. R. Rando, *Biochem. J.*, 1994, **299**, 459-465.
- 225 T. E. Gundersen, *J. Neurobiol.*, 2006, **66**, 631-644.
- 226 P. K. Lemotte, S. Keidel and C. M. Apfel, *Eur. J. Biochem.*, 1996, **236**, 328-333.
- 227 A. M. de Urquiza, S. Y. Liu, M. Sjoberg, R. H. Zetterstrom, W. Griffiths, J. Sjoval and T. Perlmann, *Science*, 2000, **290**, 2140-2144.
- 228 J. T. Goldstein, A. Dobrzyn, M. Clagett-Dame, J. W. Pike and H. F. DeLuca, *Arch. Biochem. Biophys.*, 2003, **420**, 185-193.
- 229 M. H. Jin, S. H. Li, W. N. Moghrabi, H. Sun and G. H. Travis, *Cell*, 2005, **122**, 449-459.
- 230 G. Moiseyev, Y. Chen, Y. Takahashi, B. X. Wu and J. X. Ma, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 12413-12418.
- 231 D. J. Mangelsdorf, E. S. Ong, J. A. Dyck and R. M. Evans, *Nature*, 1990, **345**, 224-229.
- 232 E. A. Allegretto, M. R. McClurg, S. B. Lazarchik, D. L. Clemm, S. A. Kemer, M. G. Elgort, M. F. Boehm, S. K. White, J. W. Pike and R. A. Heyman, *J. Biol. Chem.*, 1993, **268**, 26625-26633.
- 233 Z. El Akawi and J. L. Napoli, *Biochemistry*, 1994, **33**, 1938-1943.
- 234 A. Nagao and J. A. Olson, *FASEB J.*, 1994, **8**, 968-973.
- 235 J. L. Napoli, M. Boerman, X. Chai, Y. Zhai and P. D. Fiorella, *J. Steroid Biochem. Mol. Biol.*, 1995, **53**, 497-502.
- 236 J. Paik, S. Vogel, R. Piantedosi, A. Sykes, W. S. Blaner and K. Swisshelm, *Biochemistry*, 2000, **39**, 8073-8084.
- 237 J. A. Fontana and A. K. Rishi, *Leukemia*, 2002, **16**, 463-472.
- 238 C. J. Gambone, J. M. Hutcheson, J. L. Gabriel, R. L. Beard, R. A. S. Chandraratna, K. J. Soprano and D. R. Soprano, *Mol. Pharmacol.*, 2002, **61**, 334-342.
- 239 J. V. Schmidt and C. A. Bradfield, *Annu. Rev. Cell Dev. Biol.*, 1996, **12**, 55-89.
- 240 K. A. Murphy, L. Quadro and L. A. White, in *Vitamin A*, 2007, vol. 75, 33-67.
- 241 T. V. Beischlag, J. L. Morales, B. D. Hollingshead and G. H. Perdew, *Crit. Rev. Eukaryot. Gene Expr.*, 2008, **18**, 207-250.
- 242 R. Cincinelli, S. Dallavalle, L. Merlini, S. Penco, C. Pisano, P. Carminati, G. Giannini, L. Vesci, C. Gaetano, B. Illy, V. Zuco, R. Supino and F. Zunino, *J. Med. Chem.*, 2003, **46**, 909-912.
- 243 S. Dallavalle and F. Zunino, *Expert Opin. Ther. Pat.*, 2005, **15**, 1625-1635.
- 244 E. Parrella, M. Gianni, M. Fratelli, M. M. Barzago, I. Raska, L. Diomedede, M. Kurosaki, C. Pisano, P. Carminati, L. Merlini, S. Dallavalle, M. Tavecchio, C. Rochette-Egly, M. Terao and E. Garattini, *Mol. Pharmacol.*, 2006, **70**, 909-924.
- 245 A. L. Sabichi, D. T. Hendricks, M. A. Bober and M. J. Birrer, *J. Natl. Cancer Inst.*, 1998, **90**, 597-605.
- 246 P. L. Bu and Y. J. Y. Wan, *BMC Cancer*, 2007, **7**, Article 236.
- 247 A. N. Fanjul, D. Delia, M. A. Pierotti, D. Rideout, J. Qiu and M. Pfahl, *J. Biol. Chem.*, 1996, **271**, 22441-22446.
- 248 M. S. Sheikh, Z. M. Shao, X. S. Li, J. V. Ordonez, B. A. Conley, S. L. Wu, M. I. Dawson, Q. X. Han, W. R. Chao, T. Quick, R. M. Niles and J. A. Fontana, *Carcinogenesis*, 1995, **16**, 2477-2486.
- 249 D. Delia, A. Aiello, L. Meroni, M. Nicolini, J. C. Reed and M. A. Pierotti, *Carcinogenesis*, 1997, **18**, 943-948.
- 250 J. L. Clifford, D. G. Menter, M. Wang, R. Lotan and S. M. Lippman, *Cancer Res.*, 1999, **59**, 14-18.
- 251 W. F. Holmes, M. I. Dawson, D. R. Soprano and K. J. Soprano, *J. Cell. Physiol.*, 2000, **185**, 61-67.
- 252 A. L. Sabichi, H. Xu, S. Fischer, C. C. Zou, X. L. Yang, V. E. Steele, G. J. Kelloff, R. Lotan and J. L. Clifford, *Clin. Cancer Res.*, 2003, **9**, 4606-4613.
- 253 N. Hail, H. J. Kim and R. Lotan, *Apoptosis*, 2006, **11**, 1677-1694.
- 254 H. Xu, S. Cheepala, E. McCauley, K. Coombes, L. C. Xiao, S. M. Fischer and J. L. Clifford, *Clin. Cancer Res.*, 2006, **12**, 969-979.
- 255 P. E. Lovat, M. Ranalli, M. Annichiarico-Petruzzelli, F. Bernassola, M. Piacentini, A. J. Malcolm, A. D. J. Pearson, G. Melino and C. P. F. Redfern, *Exp. Cell Res.*, 2000, **260**, 50-60.
- 256 C. P. Reynolds, K. K. Matthay, J. G. Villablanca and B. J. Maurer, *Cancer Lett.*, 2003, **197**, 185-192.
- 257 M. Corazzari, P. E. Lovat, S. Oliverio, F. Di Sano, R. P. Donnorso, C. P. F. Redfern and M. Piacentini, *Biochem. Biophys. Res. Commun.*, 2005, **331**, 810-815.
- 258 F. Villarroya, R. Iglesias and M. Giralt, *Curr. Med. Chem.*, 2004, **11**, 795-805.
- 259 D. Dong and N. Noy, *Biochemistry*, 1998, **37**, 10691-10700.
- 260 A. Arechalde and J. H. Saurat, *BioDrugs*, 2000, **13**, 327-333.
- 261 M. Tsukada, M. Schroder, T. C. Roos, R. A. S. Chandraratna, U. Reichert, H. F. Merk, C. E. Orfanos and C. C. Zouboulis, *J. Invest. Dermatol.*, 2000, **115**, 321-327.
- 262 M. Ramos-e-Silva, D. M. Hexsel, M. S. Rutowitsch and M. Zechmeister, *Clin. Dermatol.*, 2001, **19**, 460-466.
- 263 P. C. M. Van De Kerkhof, M. M. Kleinpenning, E. De Jong, M. J. P. Gerritsen, R. J. Van Dooren-Greebe and H. A. C. Alkemade, *J. Dermatol. Treat.*, 2006, **17**, 198-204.
- 264 B. Shroot and S. Michel, *J. Amer. Acad. Dermatol.*, 1997, **36**, S96-S103.

- 265 Datapharm Communications, <http://www.medicines.org.uk>.
- 266 O. Sorg, C. Antille, G. Kaya and J. J. Saurat, *Dermatol. Ther.*, 2006, **19**, 289-296.
- 267 I. Miwako and H. Kagechika, *Drugs Today*, 2007, **43**, 563-568.
- 268 A. Zelent, F. Guidez, A. Melnick, S. Waxman and J. D. Licht, *Oncogene*, 2001, **20**, 7186-7203.
- 269 D. R. Soprano, P. Qin and K. J. Soprano, *Annu. Rev. Nutr.*, 2004, **24**, 201-221.
- 270 L. Altucci, A. Rossin, W. Raffelsberger, A. Reitmair, C. Chomienne and H. Gronemeyer, *Nat. Med.*, 2001, **7**, 680-686.
- 271 L. Altucci, E. Wilhelm and H. Gronemeyer, *Int. J. Biochem. Cell Biol.*, 2004, **36**, 178-182.
- 272 J. Brtko, *Biomed. Pap. (Olomouc)*, 2007, **151**, 187-194.
- 273 M. Osanai and M. Petkovich, *Mol. Pharmacol.*, 2005, **67**, 1808-1817.
- 274 L. Delva, M. Cornic, N. Balitrand, F. Guidez, J. M. Miclea, A. Delmer, F. Teillet, P. Fenaux, S. Castaigne, L. Degos and C. Chomienne, *Blood*, 1993, **82**, 2175-2181.
- 275 T. Tobita, A. Takeshita, K. Kitamura, K. Ohnishi, M. Yanagi, A. Hiraoka, T. Karasuno, M. Takeuchi, S. Miyawaki, R. Ueda, T. Naoe and R. Ohno, *Blood*, 1997, **90**, 967-973.
- 276 R. E. Gallagher, *Leukemia*, 2002, **16**, 1940-1958.
- 277 H. Kagechika and K. Shudo, *J. Med. Chem.*, 2005, **48**, 5875-5883.
- 278 R. M. Niles, *Nutrition*, 2000, **16**, 1084-1089.
- 279 J. R. Park, A. Eggert and H. Caron, *Pediatr. Clin. N. Am.*, 2008, **55**, 97-120.
- 280 M. Lansink, A. M. VanBennekum, W. S. Blaner and T. Kooistra, *Eur. J. Biochem.*, 1997, **247**, 596-604.
- 281 J. L. Armstrong, C. P. F. Redfern and G. J. Veal, *Biochem. Pharmacol.*, 2005, **69**, 1299-1306.
- 282 C. L. Zimmerman, S. M. Han and T. S. Wiedmann, *Cancer Chemother. Pharmacol.*, 2001, **47**, 27-33.
- 283 J. O. Sass, E. Masgrau, J. H. Saurat and H. Nau, *Drug Metab. Dispos.*, 1995, **23**, 887-891.
- 284 P. E. Lovat, S. P. Lewis, A. D. J. Pearson, A. J. Malcolm and C. P. F. Redfern, *Neurosci. Lett.*, 1994, **182**, 29-32.
- 285 G. Y. Han, B. S. Chang, M. J. Connor and N. Sidell, *Differentiation*, 1995, **59**, 61-69.
- 286 P. E. Lovat, H. Irving, A. J. Malcolm, A. D. J. Pearson and C. P. F. Redfern, *J. Neuro-Oncol.*, 1997, **31**, 85-91.
- 287 F. Ponthan, P. Borgstrom, M. Hassan, E. Wassberg, C. P. F. Redfern and P. Kogner, *Med. Pediatr. Oncol.*, 2001, **36**, 127-131.
- 288 P. C. Adamson, B. C. Widemann, G. H. Reaman, N. L. Seibel, R. F. Murphy, A. F. Gillespie and F. M. Balis, *Clin. Cancer Res.*, 2001, **7**, 3034-3039.
- 289 A. Divinci, E. Geido, E. Infusini and W. Giaretti, *Int. J. Cancer*, 1994, **59**, 422-426.
- 290 A. Mariotti, E. Marcora, G. Bunone, A. Costa, U. Veronesi, M. A. Pierotti and G. Dellavalle, *J. Natl. Cancer Inst.*, 1994, **86**, 1245-1247.
- 291 R. L. Scher, W. Saito, R. K. Dodge, W. J. Richtsmeier and R. L. Fine, *Otolaryngol. Head Neck Surg.*, 1998, **118**, 464-471.
- 292 D. Delia, A. Aiello, L. Lombardi, P. G. Pellicci, F. Grignani, F. Grignani, F. Formelli, S. Menard, A. Costa, U. Veronesi and M. A. Pierotti, *Cancer Res.*, 1993, **53**, 6036-6041.
- 293 W. Malone, M. Perloff, J. Crowell, C. Sigman and H. Higley, *Expert Opin. Invest. Drugs*, 2003, **12**, 1829-1842.
- 294 ClinicalTrials.gov, <http://www.clinicaltrials.gov/>.
- 295 U.S. Food and Drug Administration, <http://www.fda.gov>.
- 296 S. K. Mohsin, R. M. Elledge, B. Arun, A. Miller, K. Wu, K. Johnson, W. W. Lamph and P. H. Brown, *Breast Cancer Res. Treat.*, 2003, **82**, 1027.
- 297 M. Okuno, S. Kojima, R. Matsushima-Nishiwaki, H. Tsurumi, Y. Muto, S. L. Friedman and H. Moriwaki, *Curr. Cancer Drug Targets*, 2004, **4**, 285-298.
- 298 W. Bollag, *Eur. J. Cancer*, 1972, **8**, 689-693.
- 299 H. Mayer, W. Bollag, R. Hanni and R. Ruegg, *Experientia*, 1978, **34**, 1105-1119.
- 300 S. C. Lippman and R. Lotan, *J. Nutr.*, 2000, **130**, 479S-482S.
- 301 S. J. Freemantle, K. H. Dragnev and E. Dmitrovsky, *J. Natl. Cancer Inst.*, 2006, **98**, 426-427.
- 302 International Agency for Research on Cancer, in *IARC Handbooks of Cancer Prevention, Volume 4: Retinoids*, ed., World Health Organization, 1999.
- 303 U. Veronesi, L. Mariani, A. Decensi, F. Formelli, T. Camerini, R. Miceli, M. G. Di Mauro, A. Costa, E. Marubini, M. B. Sporn and G. De Palo, *Ann. Oncol.*, 2006, **17**, 1065-1071.
- 304 J. Auwerx, E. Baulieu, M. Beato, M. Becker-Andre, P. H. Burbach, G. Camerino, P. Chambon, A. Cooney, A. Dejean, C. Dreyer, R. M. Evans, F. Gannon, V. Giguere, H. Gronemeyer, J. A. Gustafson, V. Laudet, M. A. Lazar, D. J. Mangelsdorf, J. Milbrandt, E. Milgrom, D. D. Moore, B. O'Malley, M. Parker, K. Parker, T. Perlmann, M. Pfahl, M. G. Rosenfeld, H. Samuels, G. Schutz, F. M. Sladek, H. G. Stunnenberg, M. Spedding, C. Thummel, M. J. Tsai, K. Umesono, B. Vennstrom, W. Wahli, C. Weinberger, T. M. Willson and K. Yamamoto, *Cell*, 1999, **97**, 161-163.
- 305 E. Garattini, M. Gianni and M. Terao, *Curr. Pharm. Des.*, 2007, **13**, 1375-1400.

- 306 W. C. Yen, M. R. Corpuz, R. Y. Prudente, T. A. Cooke, R. P. Bissonnette, A. Negro-Vilar and W. W. Lamph, *Clin. Cancer Res.*, 2004, **10**, 8656-8664.
- 307 H. Timmer-Bosscha, E. G. E. de Vries, C. Meijer, J. W. Oosterhuis and N. H. Mulder, *Cancer Chemother. Pharmacol.*, 1998, **41**, 469-476.
- 308 D. Simoni, M. Roberti, F. P. Invidiata, R. Rondanin, R. Baruchello, C. Malagutti, A. Mazzali, M. Rossi, S. Grimaudo, L. Dusonchet, M. Meli, M. V. Raimondi, N. D'Alessandro and M. Tolomeo, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2669-2673.
- 309 A. Khandelwal, L. K. Gediya and V. C. O. Njar, *Br. J. Cancer*, 2008, **98**, 1234-1243.
- 310 M. Schupp, J. C. Curtin, R. J. Kim, A. N. Billin and M. A. Lazar, *Mol. Pharmacol.*, 2007, **71**, 1251-1257.
- 311 E. Patatanian and D. F. Thompson, *J. Clin. Pharm. Ther.*, 2008, **33**, 331-338.
- 312 A. Shalita, J. S. Weiss, D. K. Chalker, C. N. Ellis, A. Greenspan, H. I. Katz, I. Kantor, L. E. Millikan, T. Swinehart, L. Swinyer, C. Whitmore, M. Baker and J. Czemielewski, *J. Amer. Acad. Dermatol.*, 1996, **34**, 482-485.
- 313 W. J. Cunliffe, M. Poncet, C. Loesche and M. Verschoore, *Br. J. Dermatol.*, 1998, **139**, 48-56.
- 314 G. Veal, S. Rowbotham and A. Boddy, *Therapie*, 2007, **62**, 91-93.
- 315 P. McCaffery, J. H. Zhang and J. E. Crandall, *J. Neurobiol.*, 2006, **66**, 780-791.
- 316 J. E. Strahan and S. Raimer, *Int. J. Dermatol.*, 2006, **45**, 789-799.
- 317 T. Friedman, Y. Wohl, H. Y. Knobler, G. Lubin, S. Brenner, Y. Levi and Y. Barak, *Eur. Neuropsychopharmacol.*, 2006, **16**, 413-416.
- 318 K. C. O'Reilly, J. Shumake, F. Gonzalez-Lima, M. A. Lane and S. J. Bailey, *Neuropsychopharmacology*, 2006, **31**, 1919-1927.
- 319 S. A. Ferguson, F. J. Cisneros, J. P. Hanig and K. J. Berry, *Neurotoxicol. Teratol.*, 2007, **29**, 642-651.
- 320 B. Szabo, *Med. Hypotheses*, 2007, **69**, 1281-1283.
- 321 M. A. De Marchi, R. C. Maranhao, L. I. V. Brandizzi and D. R. S. Souza, *Arch. Dermatol. Res.*, 2006, **297**, 403-408.
- 322 J. D. Bremner, N. Fani, A. Ashraf, J. R. Votaw, M. E. Brummer, T. Cummins, V. Vaccarino, M. M. Goodman, L. Reed, S. Siddiq and C. B. Nemeroff, *Am. J. Psychiatry*, 2005, **162**, 983-991.
- 323 T. Pham and R. H. Scofield, *Endocr. Pract.*, 2007, **13**, 776-779.
- 324 J. Crandall, Y. Sakai, J. H. Zhang, O. Koul, Y. Mineur, W. E. Crusio and P. McCaffery, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 5111-5116.
- 325 J. Muindi, S. R. Frankel, W. H. Miller, A. Jakubowski, D. A. Scheinberg, C. W. Young, E. Dmitrovsky and R. P. Warrell, *Blood*, 1992, **79**, 299-303.
- 326 M. B. Regazzi, I. Iacona, C. Gervasutti, M. Lazzarino and S. Toma, *Clin. Pharmacokinet.*, 1997, **32**, 382-402.
- 327 B. Ozpolat, K. Mehta, A. M. Tari and G. Lopez-Berestein, *Am. J. Hematol.*, 2002, **70**, 39-47.
- 328 J. L. Armstrong, M. Ruiz, A. V. Boddy, C. P. F. Redfern, A. D. J. Pearson and G. J. Veal, *Br. J. Cancer*, 2005, **92**, 696-704.
- 329 J. R. Muindi, M. D. Roth, R. A. Wise, J. E. Connett, G. T. O'Connor, J. W. Ramsdell, N. W. Schluger, M. Romkes, R. A. Branch and F. C. Sciurba, *J. Clin. Pharmacol.*, 2008, **48**, 96-107.
- 330 M. Takeuchi, T. Yano, E. Omoto, K. Takahashi, M. Kibata, K. Shudo, R. Ueda, R. Ohno and M. Harada, *Br. J. Haematol.*, 1997, **97**, 137-140.
- 331 P. McCaffery and C. Simons, *Curr. Pharm. Des.*, 2007, **13**, 3020-3037.
- 332 P. Ocaya, A. C. Gidlof, P. S. Olofsson, H. Torma and A. Sirsjo, *Arterioscler., Thromb., Vasc. Biol.*, 2007, **27**, 1542-1548.
- 333 J. L. Armstrong, G. A. Taylor, H. D. Thomas, A. V. Boddy, C. P. F. Redfern and G. J. Veal, *Br. J. Cancer*, 2007, **96**, 1675-1683.
- 334 C. H. Han and T. S. Wiedmann, *Int. J. Pharm.*, 1998, **172**, 241-253.
- 335 S. J. Freemantle, M. J. Spinella and E. Dmitrovsky, *Oncogene*, 2003, **22**, 7305-7315.
- 336 K. A. Redmond, T. S. Nguyen and R. O. Ryan, *Int. J. Pharm.*, 2007, **339**, 246-250.
- 337 R. Dinarvand, E. Rahmani and E. Farbod, *Ir. J. Pharm. Res.*, 2003, 47-50.
- 338 *WO Pat.*, 039 532, 2005.
- 339 *WO Pat.*, 018 530, 2005.
- 340 W. Bollag and E. E. Holdener, *Ann. Oncol.*, 1992, **3**, 513-526.
- 341 M. B. Sporn, N. M. Dunlop, D. L. Newton and W. R. Henderson, *Nature*, 1976, **263**, 110-113.
- 342 H. Kagechika, *Curr. Med. Chem.*, 2002, **9**, 591-608.
- 343 H. Greschik and D. Moras, *Curr. Top. Med. Chem.*, 2003, **3**, 1573-1599.
- 344 D. Douguet, E. Thoreau and G. Grassy, *Quant. Struct-Act. Relat.*, 1999, **18**, 107-123.
- 345 M. Schapira, B. M. Raaka, H. H. Samuels and R. Abagyan, *BMC Struct. Biol.*, 2001, **1**, 1-7.
- 346 F. Sussman and A. R. de Lera, *J. Med. Chem.*, 2005, **48**, 6212-6219.
- 347 J. L. Stebbins, D. W. Jung, M. Leone, X. K. Zhang and M. Pellicchia, *J. Biol. Chem.*, 2006, **281**, 16643-16648.

- 348 P. Germain, S. Kammerer, E. Perez, C. Peluso-Iltis, D. Tortolani, F. C. Zusi, J. Starrett, P. Lapointe, J. P. Daris, A. Marinier, A. R. de Lera, N. Rochel and H. Gronemeyer, *EMBO Rep.*, 2004, **5**, 877-882.
- 349 H. Kagechika, E. Kawachi, Y. Hashimoto, T. Himi and K. Shudo, *J. Med. Chem.*, 1988, **31**, 2182-2192.
- 350 B. P. Klaholz and D. Moras, *Pure Appl. Chem.*, 1998, **70**, 41-47.
- 351 P. Jones, G. B. Villeneuve, C. P. Fei, J. DeMarte, A. J. Haggarty, K. T. Nwe, D. A. Martin, A. M. Lebuis, J. M. Finkelstein, B. J. Gour-Salin, T. H. Chan and B. R. Leyland-Jones, *J. Med. Chem.*, 1998, **41**, 3062-3077.
- 352 V. B. Christie, J. B. Barnard, A. S. Batsanov, C. E. Bridgens, E. B. Cartmell, J. C. Collings, C. P. F. Redfern, T. B. Marder, S. A. Przyborski and A. Whiting, *Org. Biomol. Chem.*, 2008, **6**, 3497-3507.
- 353 G. A. Patani and E. J. LaVoie, *Chem. Rev.*, 1996, **96**, 3147-3176.
- 354 C. A. Lipinski, F. Lombardo, B. W. Dominy and P. J. Feeney, *Adv. Drug Delivery Rev.*, 1997, **23**, 3-25.
- 355 P. Germain, P. Chambon, G. Eichele, R. M. Evans, M. A. Lazar, M. Leid, A. R. De Lera, R. Lotan, D. J. Mangelsdorf and H. Gronemeyer, *Pharmacol. Rev.*, 2006, **58**, 712-725.
- 356 P. Germain, P. Chambon, G. Eichele, R. M. Evans, M. A. Lazar, M. Leid, A. R. de Lera, R. Lotan, D. J. Mangelsdorf and H. Gronemeyer, *Pharmacol. Rev.*, 2006, **58**, 760-772.
- 357 M. I. Dawson, L. Jong, P. D. Hobbs, J. F. Cameron, W. R. Chao, M. Pfahl, M. O. Lee, B. Shroot and M. Pfahl, *J. Med. Chem.*, 1995, **38**, 3368-3383.
- 358 P. F. Egea, A. Mitschler and D. Moras, *Mol. Endocrinol.*, 2002, **16**, 987-997.
- 359 P. F. Egea, A. Mitschler, N. Rochel, M. Ruff, P. Chambon and D. Moras, *EMBO J.*, 2000, **19**, 2592-2601.
- 360 Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank, <http://www.rcsb.org/pdb/home/home.do>.
- 361 Y. Hashimoto and H. Miyachi, *Bioorg. Med. Chem.*, 2005, **13**, 5080-5093.
- 362 P. Loeliger, W. Bollag and H. Mayer, *Eur. J. Med. Chem.*, 1980, **15**, 9-15.
- 363 D. M. Benbrook, S. A. Kamelle, S. B. Guruswamy, S. A. Lightfoot, T. L. Rutledge, N. S. Gould, B. N. Hannafon, S. T. Dunn and K. D. Berlin, *Invest. New Drugs*, 2005, **23**, 417-428.
- 364 E. Garattini and M. Terao, *J. Chemother.*, 2004, **16**, 70-73.
- 365 B. Dominguéz, R. Alvarez and A. R. de Lera, *Org. Prep. Proced. Int.*, 2003, **35**, 239-306.
- 366 D. Cartier, A. Valla, R. Le Guillou, R. Labia and P. Potier, *Eur. J. Org. Chem.*, 2003, 2250-2253.
- 367 S. Frigoli, C. Fuganti, L. Malpezzi and S. Serra, *Org. Process Res. Dev.*, 2005, **9**, 646-650.
- 368 F. Frickel, in *The Retinoids*, ed. M. B. Sporn, A. B. Roberts and D. S. Goodman, Academic Press, New York, 1st edn., 1984, 7-145.
- 369 R. S. H. Liu and A. E. Asato, *Tetrahedron*, 1984, **40**, 1931-1969.
- 370 M. I. Dawson and P. D. Hobbs, in *The Retinoids: Biology, Chemistry and Medicine*, ed. M. B. Sporn, A. B. Roberts and D. S. Goodman, Raven Press, New York, 2nd edn., 1994, 5-178.
- 371 C. Mercier and P. Chabardes, *Pure Appl. Chem.*, 1994, **66**, 1509-1518.
- 372 T. B. Marder, D. C. Roe and D. Milstein, *Organometallics*, 1988, **7**, 1451-1453.
- 373 D. N. Coventry, A. S. Batsanov, A. E. Goeta, J. A. K. Howard, T. B. Marder and R. N. Perutz, *Chem. Commun.*, 2005, **16**, 2172-2174.
- 374 I. A. I. Mkhaliid, R. B. Coapes, S. N. Edes, D. N. Coventry, F. E. S. Souza, R. L. Thomas, J. J. Hall, S. W. Bi, Z. Y. Lin and T. B. Marder, *Dalton Trans.*, 2008, 1055-1064.
- 375 T. Ishiyama, J. Takagi, J. F. Hartwig and N. Miyaura, *Angew. Chem., Int. Ed. Engl.*, 2002, **41**, 3056-3058.
- 376 J. Takagi, K. Sato, J. F. Hartwig, T. Ishiyama and N. Miyaura, *Tetrahedron Lett.*, 2002, **43**, 5649-5651.
- 377 T. Ishiyama, J. Takagi, K. Ishida, N. Miyaura, N. R. Anastasi and J. F. Hartwig, *J. Am. Chem. Soc.*, 2002, **124**, 390-391.
- 378 T. Ishiyama, Y. Nobuta, J. F. Hartwig and N. Miyaura, *Chem. Commun.*, 2003, 2924-2925.
- 379 T. Ishiyama and N. Miyaura, *J. Organomet. Chem.*, 2003, **680**, 3-11.
- 380 T. Ishiyama and N. Miyaura, *Chem. Rec.*, 2004, **3**, 271-280.
- 381 T. B. Marder and N. C. Norman, *Top. Catal.*, 1998, **5**, 63-73.
- 382 T. Ishiyama, M. Murata, T. Ahiko and N. Miyaura, *Org. Synth.*, 2000, 176-185.
- 383 N. Miyaura, in *Cross Coupling Reactions*, ed., Springer-Verlag, 2002.
- 384 N. Miyaura, T. Yanagi and A. Suzuki, *Synth. Commun.*, 1981, **11**, 513-519.
- 385 N. Miyaura and A. Suzuki, *Chem. Rev.*, 1995, **95**, 2457-2483.
- 386 A. Suzuki, *J. Organomet. Chem.*, 1999, **576**, 147-168.
- 387 F. Bellina, A. Carpita and R. Rossi, *Synthesis-Stuttgart*, 2004, 2419-2440.
- 388 A. Torrado, S. Lopez, R. Alvarez and A. R. Delera, *Synthesis-Stuttgart*, 1995, 285-293.
- 389 P. Appukkuttan, E. Van der Eycken and W. Dehaen, *Synlett*, 2003, 1204-1206.
- 390 C. O. Kappe, *Angew. Chem., Int. Ed. Engl.*, 2004, **43**, 6250-6284.
- 391 M. Steele, M. Watkinson and A. Whiting, *J. Chem. Soc.-Perkin Trans. 1*, 2001, 588-598.
- 392 L. Vallarino, *J. Chem. Soc.*, 1957, 2287-2292.
- 393 I. A. Mkhaliid, Ph.D. Thesis, Durham University, 2006.
- 394 H. A. Bruson and J. W. Kroeger, *J. Am. Chem. Soc.*, 1940, **62**, 36-44.

- 395 M. F. Boehm, L. Zhang, B. A. Badea, S. K. White, D. E. Mais, E. Berger, C. M. Suto, M. E. Goldman and R. A. Heyman, *J. Med. Chem.*, 1994, **37**, 2930-2941.
- 396 M. M. Faul, A. M. Ratz, K. A. Sullivan, W. G. Trankle and L. L. Winneroski, *J. Org. Chem.*, 2001, **66**, 5772-5782.
- 397 J. B. Barnard, A. S. Batsanov, C. E. Bridgens, V. B. Christie, J. C. Collings, C. P. F. Redfern, T. B. Marder, S. A. Przyborski and A. Whiting, 2008, In preparation.
- 398 S. Strickland, T. R. Breitman, F. Frickel, A. Nurrenbach, E. Hadicke and M. B. Sporn, *Cancer Res.*, 1983, **43**, 5268-5272.
- 399 R. L. Beard, D. W. Gil, D. K. Marler, E. Henry, D. F. Colon, S. J. Gillett, T. Arefieg, T. S. Breen, H. Krauss, P. J. A. Davies and R. A. S. Chandraratna, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 1447-1452.
- 400 M. I. Dawson, L. Jong, P. D. Hobbs, D. M. Xiao, K. C. Feng, W. R. Chao, C. Pan, J. A. Fontana and X. K. Zhang, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1311-1313.
- 401 A. B. Barua and H. C. Furr, *Mol. Biotechnol.*, 1998, **10**, 167-182.
- 402 H. M. Goss, M. Sci. Project Report, Durham University, 2006.
- 403 C. Delescluse, M. T. Cavey, B. Martin, B. A. Bernard, U. Reichert, J. Maignan, M. Darmon and B. Shroot, *Mol. Pharmacol.*, 1991, **40**, 556-562.
- 404 H. Kagechika, *Yakugaku Zasshi-J. Pharm. Soc. Jpn.*, 1994, **114**, 847-862.
- 405 R. L. Beard, T. T. Duong, M. Teng, E. S. Klein, A. M. Standevan and R. A. S. Chandraratna, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3145-3148.
- 406 M. M. A. Elmazar, U. Reichert, B. Shroot and H. Nau, *Teratology*, 1996, **53**, 158-167.
- 407 M. B. Sporn, G. H. Clamon, N. M. Dunlop, D. L. Newton, J. M. Smith and U. Saffiotti, *Nature*, 1975, **253**, 47-50.
- 408 J. Rohwedel, K. Guan and A. M. Wobus, *Cells Tissues Organs*, 1999, **165**, 190-202.
- 409 Y. Xu, Y. Shi and S. Ding, *Nature*, 2008, **453**, 338-344.
- 410 P. A. Hall and F. M. Watt, *Development*, 1989, **106**, 619-633.
- 411 G. D. Fischbach and R. L. Fischbach, *J. Clin. Invest.*, 2004, **114**, 1364-1370.
- 412 G. C. Parker, M. Anastassova-Kristeva, H. E. Broxmeyer, W. H. Dodge, L. M. Eisenberg, U. M. Gehling, L. M. Guenin, R. Huss, N. I. Moldovan, M. Rao, E. F. Srouf and M. C. Yoder, *Stem Cells Dev.*, 2004, **13**, 579-584.
- 413 G. C. Parker, M. Anastassova-Kristeva, L. M. Eisenberg, M. S. Rao, M. A. Williams, P. R. Sanberg and D. English, *Stem Cells Dev.*, 2005, **14**, 463-469.
- 414 R. McKay, *Science*, 1997, **276**, 66-71.
- 415 F. H. Gage, *Science*, 2000, **287**, 1433-1438.
- 416 National Institutes of Health (U.S.A.), <http://www.nih.gov>.
- 417 G. R. Martin, *Science*, 1980, **209**, 768-776.
- 418 P. W. Andrews, *Biochim. Biophys. Acta*, 1988, **948**, 17-36.
- 419 P. W. Andrews, S. A. Przyborski and J. A. Thomson, in *Stem Cell Biology*, ed. D. R. Marshak, D. G. Gardner and D. Gottlieb, Cold Spring Harbor Laboratory Press, New York, 2001, 231-265.
- 420 J. Fogh and G. Trempe, in *Human Tumor Cells in Vitro*, Plenum Press, New York, 1975.
- 421 P. W. Andrews, *Philos. Trans. R. Soc. Lond. Ser. B*, 2002, **357**, 405-417.
- 422 A. Alonso, B. Breuer, B. Steuer and J. Fischer, *Int. J. Dev. Biol.*, 1991, **35**, 389-397.
- 423 M. W. McBurney, *Int. J. Dev. Biol.*, 1993, **37**, 135-140.
- 424 D. R. Soprano, B. W. Teets and K. J. Soprano, in *Vitamin A*, 2007, vol. 75, 69-95.
- 425 S. A. Przyborski, *Stem Cells*, 2001, **19**, 500-504.
- 426 S. A. Przyborski, V. B. Christie, M. W. Hayman, R. Stewart and G. M. Horrocks, *Stem Cells Dev.*, 2004, **13**, 400-408.
- 427 S. A. Przyborski, I. E. Morton, A. Wood and P. W. Andrews, *Eur. J. Neurosci.*, 2000, **12**, 3521-3528.
- 428 G. A. Horrocks, L. Lauder, R. Stewart and S. Przyborski, *Biochem. Biophys. Res. Commun.*, 2003, **304**, 411-416.
- 429 G. Keller, *Genes Dev.*, 2005, **19**, 1129-1155.
- 430 M. J. Evans and M. H. Kaufman, *Nature*, 1981, **292**, 154-156.
- 431 G. R. Martin, *Proc. Natl. Acad. Sci. U. S. A.*, 1981, **78**, 7634-7638.
- 432 J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall and J. M. Jones, *Science*, 1998, **282**, 1145-1147.
- 433 M. Serra, S. B. Leite, C. Brito, J. Costa, M. J. T. Carrondo and P. M. Alves, *J. Neurosci. Res.*, 2007, **85**, 3557-3566.
- 434 Y. R. Ahuja, V. Vijayalakshmi and K. Polasa, *Toxicology*, 2007, **231**, 1-10.
- 435 S. Adler, C. Pellizzer, L. Hareng, T. Hartung and S. Bremer, *Toxicol. in Vitro*, 2008, **22**, 200-211.
- 436 J. W. Langston, *J. Clin. Invest.*, 2005, **115**, 23-25.
- 437 E. Roche, J. Jones, M. I. Arribas, T. Leon-Quinto and B. Soria, *Bioorg. Med. Chem.*, 2006, **14**, 6466-6474.
- 438 C. M. Zhao, W. Deng and F. H. Gage, *Cell*, 2008, **132**, 645-660.

- 439 M. J. Martin, A. Muotri, F. Gage and A. Varki, *Nat. Med.*, 2005, **11**, 228-232.
- 440 A. Heiskanen, T. Satomaa, S. Tiitinen, A. Laitinen, S. Mannelin, U. Impola, M. Mikkola, C. Olsson, H. Miller-Podraza, M. Blomqvist, A. Olonen, H. Salo, P. Lehenkari, T. Tuuri, T. Otonkoski, J. Natunen, J. Saarinen and J. Laine, *Stem Cells*, 2007, **25**, 197-202.
- 441 M. Richards, C. Y. Fong, W. K. Chan, P. C. Wong and A. Bongso, *Nat. Biotechnol.*, 2002, **20**, 933-936.
- 442 T. E. Ludwig, M. E. Levenstein, J. M. Jones, W. T. Berggren, E. R. Mitchen, J. L. Frane, L. J. Crandall, C. A. Daigh, K. R. Conard, M. S. Piekarczyk, R. A. Llanas and J. A. Thomson, *Nat. Biotechnol.*, 2006, **24**, 185-187.
- 443 H. J. Rippon and A. E. Bishop, *Cell Prolif.*, 2004, **37**, 23-34.
- 444 I. Ginis, Y. Q. Luo, T. Miura, S. Thies, R. Brandenberger, S. Gerecht-Nir, M. Amit, A. Hoke, M. K. Carpenter, J. Itskovitz-Eldor and M. S. Rao, *Dev. Biol.*, 2004, **269**, 360-380.
- 445 S. Koestenbauer, N. H. Zech, H. Juch, P. Vanderzwalmen, L. Schoonjans and G. Dohr, *Am. J. Reprod. Immunol.*, 2006, **55**, 169-180.
- 446 R. Josephson, C. J. Ording, Y. Liu, S. Shin, U. Lakshmiathy, A. Toumadje, B. Love, J. D. Chesnut, P. W. Andrews, M. S. Rao and J. M. Auerbach, *Stem Cells*, 2007, **25**, 437-446.
- 447 J. S. Draper, H. D. Moore, L. N. Ruban, P. J. Gokhale and P. W. Andrews, *Stem Cells Dev.*, 2004, **13**, 325-336.
- 448 R. Jaenisch and R. Young, *Cell*, 2008, **132**, 567-582.
- 449 J. W. Kim, J. L. Chu, X. H. Shen, J. L. Wang and S. H. Orkin, *Cell*, 2008, **132**, 1049-1061.
- 450 K. Takahashi and S. Yamanaka, *Cell*, 2006, **126**, 663-676.
- 451 K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda and S. Yamanaka, *Cell*, 2007, **131**, 861-872.
- 452 J. Y. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II and J. A. Thomson, *Science*, 2007, **318**, 1917-1920.
- 453 I. H. Park, R. Zhao, J. A. West, A. Yabuuchi, H. G. Huo, T. A. Ince, P. H. Lerou, M. W. Lensch and G. Q. Daley, *Nature*, 2008, **451**, 141-146.
- 454 M. Rao and M. L. Condic, *Stem Cells Dev.*, 2008, **17**, 1-10.
- 455 I. Chambers and A. Smith, *Oncogene*, 2004, **23**, 7150-7160.
- 456 S. A. Noggle, D. Weiler and B. G. Condie, *Stem Cells*, 2006, **24**, 1646-1653.
- 457 S. Strickland and V. Mahdavi, *Cell*, 1978, **15**, 393-403.
- 458 H. Jakob, P. Dubois, H. Eisen and F. Jacob, *C. R. Acad. Sci. Hebd. Séances Acad. Sci. D*, 1978, **286**, 109-111.
- 459 P. W. Andrews, E. Nudelman, S. I. Hakomori and B. A. Fenderson, *Differentiation*, 1990, **43**, 131-138.
- 460 M. W. McBurney, E. M. V. Jones-Villeneuve, M. K. S. Edwards and P. J. Anderson, *Nature*, 1982, **299**, 165-167.
- 461 P. W. Andrews, E. Gonczol, S. A. Plotkin, M. Dignazio and J. W. Oosterhuis, *Differentiation*, 1986, **31**, 119-126.
- 462 H. K. Kleinman, L. Luckenbilledds, F. W. Cannon and G. C. Sephel, *Anal. Biochem.*, 1987, **166**, 1-13.
- 463 M. P. Lutolf and J. A. Hubbell, *Nat. Biotechnol.*, 2005, **23**, 47-55.
- 464 J. Lee, M. J. Cuddihy and N. A. Kotov, *Tissue Eng. Part B: Rev.*, 2008, **14**, 61-86.
- 465 K. M. Guan, H. Chang, A. Rolletschek and A. M. Wobus, *Cell Tissue Res.*, 2001, **305**, 171-176.
- 466 S. B. Chen, S. Hilcove and S. Ding, *Mol. Biosyst.*, 2006, **2**, 18-24.
- 467 E. M. V. Jones-Villeneuve, M. W. McBurney, K. A. Rogers and V. I. Kalnins, *J. Cell Biol.*, 1982, **94**, 253-262.
- 468 E. M. V. Jones-Villeneuve, M. A. Rudnicki, J. F. Harris and M. W. McBurney, *Mol. Cell. Biol.*, 1983, **3**, 2271-2279.
- 469 C. Rochette-Egly and P. Chambon, *Histol. Histopathol.*, 2001, **16**, 909-922.
- 470 P. W. Andrews, *Dev. Biol.*, 1984, **103**, 285-293.
- 471 P. W. Andrews, I. Damjanov, D. Simon, G. S. Banting, C. Carlin, N. C. Dracopoli and J. Fogh, *Lab. Invest.*, 1984, **50**, 147-162.
- 472 J. Houldsworth, S. C. Heath, G. J. Bosl, L. Studer and R. S. K. Chaganti, *Cell Growth Differ.*, 2002, **13**, 257-264.
- 473 R. Stewart, V. B. Christie and S. A. Przyborski, *Stem Cells*, 2003, **21**, 248-256.
- 474 R. Stewart, L. Coyne, M. Lako, R. F. Halliwell and S. A. Przyborski, *Stem Cells Dev.*, 2004, **13**, 646-657.
- 475 C. H. Xia, C. Wang, K. J. Zhang, C. Qian and N. H. Jing, *Differentiation*, 2007, **75**, 912-927.
- 476 R. H. Miller, *Brain Res.*, 2006, **1091**, 258-264.
- 477 J. S. Draper, C. Pigott, J. A. Thomson and P. W. Andrews, *J. Anat.*, 2002, **200**, 249-258.
- 478 P. W. Andrews, P. N. Goodfellow, L. H. Shevinsky, D. L. Bronson and B. B. Knowles, *Int. J. Cancer*, 1982, **29**, 523-531.
- 479 R. Kannagi, N. A. Cochran, F. Ishigami, S. Hakomori, P. W. Andrews, B. B. Knowles and D. Solter, *EMBO J.*, 1983, **2**, 2355-2361.
- 480 P. W. Andrews, G. Banting, I. Damjanov, D. Arnaud and P. Avner, *Hybridoma*, 1984, **3**, 347-361.

- 481 P. W. Andrews, L. J. Meyer, K. L. Bednarz and H. Harris, *Hybridoma*, 1984, **3**, 33-39.
- 482 G. S. Eisenbarth, F. S. Walsh and M. Nirenberg, *Proc. Natl. Acad. Sci. U. S. A.*, 1979, **76**, 4913-4917.
- 483 R. W. Curley and J. W. Fowble, *Photochem. Photobiol.*, 1988, **47**, 831-835.
- 484 A. Murayama, T. Suzuki and M. Matsui, *J. Nutr. Sci. Vitaminol.*, 1997, **43**, 167-176.
- 485 P. A. Lehman and A. M. Malany, *J. Invest. Dermatol.*, 1989, **93**, 595-599.
- 486 R. W. Berg and M. W. McBurney, *Dev. Biol.*, 1990, **138**, 123-135.
- 487 G. J. Veal, J. Errington, C. R. Redfern, A. D. J. Pearson and A. V. Boddy, *Biochem. Pharmacol.*, 2002, **63**, 207-215.
- 488 C. Lanvers, G. Hempel, G. Blaschke and J. Boos, *FASEB J.*, 1998, **12**, 1627-1633.
- 489 I. Klaassen, R. H. Brakenhoff, S. J. Smeets, G. B. Snow and B. J. M. Braakhuis, *Biochim. Biophys. Acta*, 1999, **1427**, 265-275.
- 490 G. W. Tang and R. M. Russell, *J. Lipid Res.*, 1990, **31**, 175-182.
- 491 W. S. Blaner, *J. Amer. Acad. Dermatol.*, 2001, **45**, S129-S135.
- 492 *WO Pat.*, 033 881, 1997.
- 493 *WO Pat.*, 056 740, 1999.
- 494 D. Ciubotariu, A. Grozav, V. Gogonea, C. Ciubotariu, M. Medeleanu, D. Dragos, M. Pasere and Z. Simon, *Match-Commun. Math. Comput. Chem.*, 2001, 65-92.
- 495 M. I. Dawson, D. L. Harris, G. Liu, P. D. Hobbs, C. W. Lange, L. Jong, N. Bruey-Sedano, S. Y. James, X. K. Zhang, V. J. Peterson, M. Leid, L. Farhana, A. K. Rishi and J. A. Fontana, *J. Med. Chem.*, 2004, **47**, 3518-3536.
- 496 M. I. Dawson, R. L. S. Chan, K. Derdzinski, P. D. Hobbs, W. R. Chao and L. J. Schiff, *J. Med. Chem.*, 1983, **26**, 1653-1656.
- 497 M. I. Dawson and X. K. Zhang, *Curr. Med. Chem.*, 2002, **9**, 623-637.
- 498 *DE Pat.*, 2 854 354, 1979.
- 499 H. Harant, I. Korschineck, G. Krupitza, B. Fazeny, C. Dittrich and T. W. Grunt, *Br. J. Cancer*, 1993, **68**, 530-536.
- 500 D. M. Benbrook, M. M. Madler, L. W. Spruce, P. J. Birckbichler, E. C. Nelson, S. Subramanian, G. M. Weerasekare, J. B. Gale, M. K. Patterson, B. H. Wang, W. Wang, S. N. Lu, T. C. Rowland, P. DiSivestro, C. Lindamood, D. L. Hill and K. D. Berlin, *J. Med. Chem.*, 1997, **40**, 3567-3583.
- 501 R. Alvarez, M. J. Vega, S. Kammerer, A. Rossin, P. Germain, H. Gronemeyer and A. R. de Lera, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 6117-6122.
- 502 A. M. Standeven, M. Teng and R. A. S. Chandraratna, *Toxicol. Lett.*, 1997, **92**, 231-240.
- 503 J. Schnitzer and M. Schachner, *Cell Tissue Res.*, 1982, **224**, 625-636.
- 504 C. Wiese, A. Rolletschek, G. Kania, P. Blyszczuk, K. V. Tarasov, Y. Tarasova, R. P. Wersto, K. R. Boheler and A. M. Wobus, *Cell. Mol. Life Sci.*, 2004, **61**, 2510-2522.
- 505 H. Baharvand, N. Z. Mehrjardi, M. Hatami, S. Kiani, M. Rao and M. M. Haghghi, *Int. J. Dev. Biol.*, 2007, **51**, 371-378.
- 506 J. M. Maris and K. K. Matthay, *J. Clin. Oncol.*, 1999, **17**, 2264-2279.
- 507 J. L. Biedler, S. Rofflertarlov, M. Schachner and L. S. Freedman, *Cancer Res.*, 1978, **38**, 3751-3757.
- 508 A. M. Brown, F. C. Riddoch, A. Robson, C. P. F. Redfern and T. R. Cheek, *Biochem. J.*, 2005, **388**, 941-948.
- 509 T. M. Jessell, *Nat. Rev. Genet.*, 2000, **1**, 20-29.
- 510 X. J. Li, Z. W. Du, E. D. Zarnowska, M. Pankratz, L. O. Hansen, R. A. Pearce and S. C. Zhang, *Nat. Biotechnol.*, 2005, **23**, 215-221.
- 511 S. S. Liour, S. A. Kraemer, M. B. Dinkins, C. Y. Su, M. Yanagisawa and R. K. Yu, *Glia*, 2006, **53**, 43-56.
- 512 R. Nat, M. Nilbratt, S. Narkilahti, B. Winblad, O. Hovatta and A. Nordberg, *Glia*, 2007, **55**, 385-399.
- 513 S. A. Przyborski, S. A. Przyborski, V. B. Christie, M. W. Hayman, R. Stewart and G. M. Horrocks, *Stem Cells*, 2001, **19**, 500-504.
- 514 A. Vahlquist, *Dermatology*, 1999, **199**, 3-11.
- 515 J. M. Wu, A. M. DiPietrantonio and T. C. Hsieh, *Apoptosis*, 2001, **6**, 377-388.
- 516 T. R. Breitman, S. E. Selonick and S. J. Collins, *Proc. Natl. Acad. Sci. U. S. A.*, 1980, **77**, 2936-2940.
- 517 R. Kageyama and T. Ohtsuka, *Cell Res.*, 1999, **9**, 179-188.
- 518 V. Fox, P. J. Gokhale, J. R. Walsh, M. Matin, M. Jones and P. W. Andrews, *Stem Cells*, 2008, **26**, 715-723.
- 519 T. H. El-Metwally and T. E. Adrian, *Biochem. Biophys. Res. Commun.*, 1999, **257**, 596-603.
- 520 S. Adler, C. Pellizzer, M. Paparella, T. Hartung and S. Bremer, *Toxicol. in Vitro*, 2006, **20**, 265-271.
- 521 R. Lotan, M. I. Dawson, C. C. Zou, L. Jong, D. Lotan and C. P. Zou, *Cancer Res.*, 1995, **55**, 232-236.
- 522 C. C. Willhite, M. I. Dawson and U. Reichert, *Drug Metab. Rev.*, 1996, **28**, 105-119.
- 523 Y. Elkabetz, G. Panagiotakos, G. Al Shamy, N. D. Socci, V. Tabar and L. Studer, *Genes Dev.*, 2008, **22**, 152-165.
- 524 P. G. Wilson and S. S. Stice, *Stem Cell Rev.*, 2006, **2**, 67-77.

- 525 S. C. Zhang, X. J. Li, M. A. Johnson and M. T. Pankratz, *Philos. Trans. R. Soc. Lond. Ser. B*, 2008, **363**, 87-99.
- 526 C. P. F. Redfern, P. E. Lovat, A. J. Malcolm and A. D. J. Pearson, *Eur. J. Cancer*, 1995, **31A**, 486-494.
- 527 J. A. Barltrop, T. C. Owen, A. H. Cory and J. G. Cory, *Bioorg. Med. Chem. Lett.*, 1991, **1**, 611-614.
- 528 A. H. Cory, T. C. Owen, J. A. Barltrop and J. G. Cory, *Cancer Commun.*, 1991, **3**, 207-212.
- 529 T. M. Buttke, J. A. McCubrey and T. C. Owen, *J. Immunol. Methods*, 1993, **157**, 233-240.
- 530 N. J. Marshall, C. J. Goodwin and S. J. Holt, *Growth Regul.*, 1995, **5**, 69-84.
- 531 Y. B. Liu, D. A. Peterson, H. Kimura and D. Schubert, *J. Neurochem.*, 1997, **69**, 581-593.
- 532 I. D. Trayner, A. P. Rayner, G. E. Freeman and F. Farzaneh, *J. Immunol. Methods*, 1995, **186**, 275-284.
- 533 V. M. Y. Lee and P. W. Andrews, *J. Neurosci.*, 1986, **6**, 514-521.
- 534 P. W. Andrews, *Int. J. Cancer*, 1982, **30**, 567-571.
- 535 G. Cauffman, H. Van de Velde, I. Liebaers and A. Van Steirteghem, *Mol. Hum. Reprod.*, 2005, **11**, 173-181.
- 536 C. Apfel, F. Bauer, M. Crettaz, L. Forni, M. Kamber, F. Kaufmann, P. Lemotte, W. Pirson and M. Klaus, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 7129-7133.
- 537 P. Leroy, A. Krust, A. Zelent, C. Mendelsohn, J. M. Garnier, P. Kastner, A. Dierich and P. Chambon, *EMBO J.*, 1991, **10**, 59-69.
- 538 L. J. C. Jonk, M. E. J. Dejonge, F. A. E. Kruyt, C. L. Mummery, P. T. van der Saag and W. Kruijer, *Mech. Dev.*, 1992, **36**, 165-172.
- 539 C. Zechel, *Mol. Endocrinol.*, 2005, **19**, 1629-1645.
- 540 A. Simeone, D. Acampora, L. Arcioni, P. W. Andrews, E. Boncinelli and F. Mavilio, *Nature*, 1990, **346**, 763-766.
- 541 L. Hu and L. J. Gudas, *Mol. Cell. Biol.*, 1990, **10**, 391-396.
- 542 R. Borghi, R. Vene, G. Arena, D. Schubert, A. Albin and F. Tosetti, *J. Neurochem.*, 2003, **84**, 94-104.
- 543 R. Haq, M. Pfahl and F. Chytil, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 8272-8276.
- 544 J. M. Lehmann, X. K. Zhang and M. Pfahl, *Mol. Cell. Biol.*, 1992, **12**, 2976-2985.
- 545 C. Mendelsohn, S. Larkin, M. Mark, M. Lemeur, J. Clifford, A. Zelent and P. Chambon, *Mech. Dev.*, 1994, **45**, 227-241.
- 546 C. Brand, P. Ségard, P. Plouvier, P. Formstecher, M. Danzé and P. Lefebvre, *BMC Pharmacol.*, 2002, **2**, 13-26.
- 547 M. Husmann, J. Lehmann, B. Hoffmann, T. Hermann, M. Tzukerman and M. Pfahl, *Mol. Cell. Biol.*, 1991, **11**, 4097-4103.
- 548 Y. Kamei, T. Kawada, R. Kazuki and E. Sugimoto, *Biochem. J.*, 1993, **293**, 807-812.
- 549 P. Leroy, H. Nakshatri and P. Chambon, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 10138-10142.
- 550 H. de The, M. D. Vivancoruiz, P. Tiollais, H. Stunnenberg and A. Dejean, *Nature*, 1990, **343**, 177-180.
- 551 B. Hoffmann, J. M. Lehmann, X. K. Zhang, T. Hermann, M. Husmann, G. Graupner and M. Pfahl, *Mol. Endocrinol.*, 1990, **4**, 1727-1736.
- 552 Y. F. Guo, P. Xiao, S. F. Lei, F. Y. Deng, G. G. Xiao, Y. Z. Liu, X. D. Chen, L. M. Li, S. Wu, Y. Chen, H. Jiang, L. J. Tan, J. Y. Xie, X. Z. Zhu, S. P. Liang and H. W. Deng, *Acta Biochim. Biophys. Sin.*, 2008, **40**, 426-436.
- 553 A. Angulo, C. Suto, M. F. Boehm, R. A. Heyman and P. Ghazal, *J. Virol.*, 1995, **69**, 3831-3837.
- 554 Q. C. Hewson, P. E. Lovat, A. J. Malcolm, A. D. J. Pearson and C. P. F. Redfern, *Neurosci. Lett.*, 2000, **279**, 113-116.
- 555 M. Ebisawa, K. Ohta, E. Kawachi, H. Fukasawa, Y. Hashimoto and H. Kagechika, *Chem. Pharm. Bull. (Tokyo)*, 2001, **49**, 501-503.
- 556 F. Pendino, C. Dudognon, F. Delhommeau, T. Sahraoui, M. Flexor, A. Bennaceur-Griscelli, M. Lanotte and E. Segal-Bendirdjian, *Oncogene*, 2003, **22**, 9142-9150.
- 557 R. L. Beard, R. A. S. Chandraratna, D. F. Colon, S. J. Gillett, E. Henry, D. K. Marler, T. Song, L. Denys, T. Arefieg, E. Klein, D. W. Gil, L. Wheeler, D. M. Kochhar and P. J. A. Davies, *J. Med. Chem.*, 1995, **38**, 2820-2829.
- 558 V. A. Miller, F. M. Benedetti, J. R. Rigas, A. L. Verret, D. G. Pfister, D. Straus, M. G. Kris, M. Crisp, R. Heyman, G. R. Loewen, J. A. Truglia and R. P. Warrell, *J. Clin. Oncol.*, 1997, **15**, 790-795.
- 559 N. Suh, W. W. Lamph, A. L. Glasebrook, T. A. Grese, A. D. Palkowitz, C. R. Williams, R. Risingsong, M. R. Farris, R. A. Heyman and M. B. Sporn, *Clin. Cancer Res.*, 2002, **8**, 3270-3275.
- 560 E. D. Bischoff, M. M. Gottardis, T. E. Moon, R. A. Heyman and W. W. Lamph, *Cancer Res.*, 1998, **58**, 479-484.
- 561 W. C. Yen, R. Y. Prudente and W. W. Lamph, *Breast Cancer Res. Treat.*, 2004, **88**, 141-148.
- 562 Q. C. Hewson, P. E. Lovat, A. D. J. Pearson and C. P. F. Redfern, *J. Cell. Biochem.*, 2002, **87**, 284-291.
- 563 M. A. Pignatello, F. C. Kauffman and A. A. Levin, *Toxicol. Appl. Pharmacol.*, 2002, **178**, 186-194.
- 564 S. Vogel, C. L. Mendelsohn, J. R. Mertz, R. Piantadosi, C. Waldburger, M. E. Gottesman and W. S. Blaner, *J. Biol. Chem.*, 2001, **276**, 1353-1360.
- 565 D. E. Ong, C. H. Tsai and F. Chytil, *J. Nutr.*, 1976, **106**, 204-211.

- 566 J. C. Saari, L. Bredberg and G. G. Garwin, *J. Biol. Chem.*, 1982, **257**, 133329-133333.
- 567 D. E. Ong and D. L. Page, *Am. J. Clin. Nutr.*, 1986, **44**, 425-430.
- 568 E. Li, L. A. Demmer, D. A. Sweetser, D. E. Ong and J. I. Gordon, *Proc. Natl. Acad. Sci. U. S. A.*, 1986, **83**, 5779-5783.
- 569 E. Li, S. J. Qian, N. S. Winter, A. Davignon, M. S. Levin and J. I. Gordon, *J. Biol. Chem.*, 1991, **266**, 3622-3629.
- 570 P. N. Macdonald and D. E. Ong, *J. Biol. Chem.*, 1987, **262**, 10550-10556.
- 571 K. Fogh, J. J. Voorhees and A. Åström, *Arch. Biochem. Biophys.*, 1993, **300**, 751-755.
- 572 P. D. Fiorella, V. Giguere and J. L. Napoli, *J. Biol. Chem.*, 1993, **268**, 21545-21552.
- 573 A. W. Norris, L. Cheng, V. Giguere, M. Rosenberger and E. Li, *Biochim. Biophys. Acta*, 1994, **1209**, 10-18.
- 574 B. N. Chaudhuri, G. J. Kleywegt, I. Broutin-L'Hermite, T. Bergfors, H. Senn, P. Le Motte, O. Partouche and T. A. Jones, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 1999, **55**, 1850-1857.
- 575 A. C. Chen and L. J. Gudas, *J. Biol. Chem.*, 1996, **271**, 14971-14980.
- 576 M. A. Blaese, L. Santo-Hoeltje and H. P. Rodemann, *Int. J. Radiat. Biol.*, 2003, **79**, 981-991.
- 577 J. Y. Won, E. C. Nam, S. J. Yoo, H. J. Kwon, S. J. Um, H. S. Han, S. H. Kim, Y. Byun and S. Y. Kim, *Metab.-Clin. Exp.*, 2004, **53**, 1007-1012.
- 578 G. J. Kleywegt, T. Bergfors, H. Senn, P. Lemotte, B. Gsell, K. Shudo and T. A. Jones, *Structure*, 1994, **2**, 1241-1258.
- 579 P. C. Adamson, J. F. Boylan, F. M. Balis, R. F. Murphy, K. A. Godwin, L. J. Gudas and D. G. Poplack, *Cancer Res.*, 1993, **53**, 472-476.
- 580 P. Gorry, T. Lufkin, A. Dierich, C. Rochette-Egly, D. Decimo, P. Dollé, M. Mark, B. Durand and P. Chambon, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 9032-9036.
- 581 C. Lampron, C. Rochetteegly, P. Gorry, P. Dollé, M. Mark, T. Lufkin, M. Lemeur and P. Chambon, *Development*, 1995, **121**, 539-548.
- 582 S. Y. Wang and L. J. Gudas, *J. Biol. Chem.*, 1984, **259**, 5899-5906.
- 583 J. Schindler, K. I. Matthaei and M. I. Sherman, *Proc. Natl. Acad. Sci. U. S. A.*, 1981, **78**, 1077-1080.
- 584 K. Tamura, H. Kagechika, Y. Hashimoto, K. Shudo, K. Ohsugi and H. Ide, *Cell Differ. Dev.*, 1990, **32**, 17-26.
- 585 R. G. Mehta, L. J. Schiff, S. J. Moore, A. M. Buckley and M. I. Dawson, *In Vitro Cell. Dev. Biol.*, 1986, **22**, 164-168.
- 586 A. Yen, S. L. Reece and K. L. Albright, *Exp. Cell Res.*, 1984, **152**, 493-499.
- 587 E. Sonneveld, C. E. van den Brink, B. J. M. van der Leede, R. Schulkes, M. Petkovich, B. van der Burg and P. T. van der Saag, *Cell Growth Differ.*, 1998, **9**, 629-637.
- 588 P. B. Danielson, *Curr. Drug Metab.*, 2002, **3**, 561-597.
- 589 X. X. Gu, F. Xu, X. L. Wang, X. Gao and Q. S. Zhao, *Gene Expr. Patterns*, 2005, **5**, 733-739.
- 590 V. C. O. Njar, L. Gediya, P. Purushottamachar, P. Chopra, T. S. Vasaitis, A. Khandelwal, J. Mehta, C. Huynh, A. Belosay and J. Patel, *Bioorg. Med. Chem.*, 2006, **14**, 4323-4340.
- 591 X. X. Gu, F. Xu, W. Song, X. L. Wang, P. Hu, Y. M. Yang, X. Gao and Q. S. Zhao, *Mol. Endocrinol.*, 2006, **20**, 1661-1672.
- 592 S. Reijntjes, E. Gale and M. Maden, *Dev. Dyn.*, 2004, **230**, 509-517.
- 593 C. A. Frolik, A. B. Roberts, T. E. Tavela, P. P. Roller, D. L. Newton and M. B. Sporn, *Biochemistry*, 1979, **18**, 2092-2097.
- 594 J. M. Baron, R. Heise, W. S. Blaner, M. Neis, S. Jousen, A. Dreuw, Y. Marquardt, J. H. Saurat, H. F. Merk, D. R. Bickers and F. K. Jugert, *J. Invest. Dermatol.*, 2005, **125**, 143-153.
- 595 S. Y. Kim, H. Adachi, J. S. Koo and A. M. Jetten, *Mol. Pharmacol.*, 2000, **58**, 483-490.
- 596 Y. Marikar, Z. Q. Wang, E. A. Duell, M. Petkovich, J. J. Voorhees and G. J. Fisher, *J. Invest. Dermatol.*, 1998, **111**, 434-439.
- 597 A. Lampen, S. Meyer and H. Nau, *Drug Metab. Dispos.*, 2001, **29**, 742-747.
- 598 M. G. Villani, V. Appierto, E. Cavadini, M. Valsecchi, S. Sonnino, R. W. Curley and F. Formelli, *Clin. Cancer Res.*, 2004, **10**, 6265-6275.
- 599 J. Van Wauwe, G. Van Nyen, M. C. Coene, P. Stoppie, W. Cools, J. Goossens, P. Borghgraef and P. A. J. Janssen, *J. Pharmacol. Exp. Ther.*, 1992, **261**, 773-779.
- 600 C. J. Verfaillie, M. Borgers and M. A. M. van Steensel, *J. Dtsch. Dermatol. Ges.*, 2008, **6**, 355-364.
- 601 P. Stoppie, M. Borgers, P. Borghgraef, L. Dillen, J. Goossens, G. Sanz, H. Szel, C. Van Hove, G. Van Nyen, G. Nobels, H. Vanden Bossche, M. Venet, G. Willemsens and J. Van Wauwe, *J. Pharmacol. Exp. Ther.*, 2000, **293**, 304-312.
- 602 *WO Pat.*, 9749704, 1996.
- 603 *WO Pat.*, 9932098, 1997.
- 604 H. J. Bovenschen, M. E. Otero, A. M. G. Langewouters, I. Van Vlijmen-Willems, D. W. A. Van Rens, M. M. B. Seyger and P. C. M. Van De Kerkhof, *Br. J. Dermatol.*, 2007, **156**, 263-270.

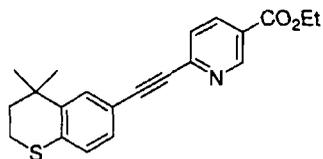
- 605 J. Van Heusden, R. Van Ginckel, H. Bruwiere, P. Moelans, B. Janssen, W. Floren, B. J. van der Leede, J. van Dun, G. Sanz, M. Venet, L. Dillen, C. Van Hove, G. Willemsens, M. Janicot and W. Wouters, *Br. J. Cancer*, 2002, **86**, 605-611.
- 606 J. B. Patel, C. K. Huynh, V. D. Handratta, L. K. Gediya, A. M. H. Brodie, O. G. Goloubeva, O. O. Clement, N. P. Nanne, D. R. Soprano and V. C. O. Njar, *J. Med. Chem.*, 2004, **47**, 6716-6729.
- 607 S. Alvarez, P. Germain, R. Alvarez, F. Rodriguez-Barrios, H. Gronemeyer and A. R. de Lera, *Int. J. Biochem. Cell Biol.*, 2007, **39**, 1406-1415.
- 608 P. Goyette, C. F. Chen, W. Wang, F. Seguin and D. Lohnes, *J. Biol. Chem.*, 2000, **275**, 16497-16505.
- 609 F. A. E. Kruyt, L. J. Vanderveer, S. Mader, C. E. Vandenbrink, A. Feijen, L. J. C. Jonk, W. Kruijer and P. T. Vandersaag, *Differentiation*, 1992, **49**, 27-37.
- 610 F. Zwartkruis, F. Kruyt, P. T. Vandersaag and F. Meijlink, *Exp. Cell Res.*, 1993, **205**, 422-425.
- 611 M. C. Moroni, M. A. Viganò and F. Mavilio, *Mech. Dev.*, 1993, **44**, 139-154.
- 612 M. J. Spinella, S. Kitareewan, B. Mellado, D. Sekula, K. S. Khoo and E. Dmitrovsky, *Oncogene*, 1998, **16**, 3471-3480.
- 613 R. L. Garber, A. Kuroiwa and W. J. Gehring, *EMBO J.*, 1983, **2**, 2027-2036.
- 614 M. P. Scott, A. J. Weiner, T. I. Hazelrigg, B. A. Polisky, V. Pirrotta, F. Scalenghe and T. C. Kaufman, *Cell*, 1983, **35**, 763-776.
- 615 M. P. Scott, J. W. Tamkun and G. W. Hartzell, *Biochim. Biophys. Acta*, 1989, **989**, 25-48.
- 616 P. J. McAlpine and T. B. Shows, *Genomics*, 1990, **7**, 460-460.
- 617 E. Boncinelli, R. Somma, D. Acampora, M. Pannese, M. Desposito, A. Faiella and A. Simeone, *Hum. Reprod.*, 1988, **3**, 880-886.
- 618 D. Acampora, M. Desposito, A. Faiella, M. Pannese, E. Migliaccio, F. Morelli, A. Stornaiuolo, V. Nigro, A. Simeone and E. Boncinelli, *Nucleic Acids Res.*, 1989, **17**, 10385-10402.
- 619 D. Duboule and P. Dollé, *EMBO J.*, 1989, **8**, 1497-1505.
- 620 A. Graham, N. Papalopulu and R. Krumlauf, *Cell*, 1989, **57**, 367-378.
- 621 M. P. Scott, *Cell*, 1992, **71**, 551-553.
- 622 R. S. Mann, *BioEssays*, 1997, **19**, 661-664.
- 623 M. Maconochie, S. Nonchev, A. Morrison and R. Krumlauf, *Annu. Rev. Genet.*, 1996, **30**, 529-556.
- 624 M. Kmita and D. Duboule, *Science*, 2003, **301**, 331-333.
- 625 S. Chambeyron, N. R. Da Silva, K. A. Lawson and W. A. Bickmore, *Development*, 2005, **132**, 2215-2223.
- 626 J. Deschamps and J. van Nes, *Development*, 2005, **132**, 2931-2942.
- 627 B. L. M. Hogan, C. Thaller and G. Eichele, *Nature*, 1992, **359**, 237-241.
- 628 G. S. Daftary and H. S. Taylor, *Endocr. Rev.*, 2006, **27**, 331-355.
- 629 D. J. Wolgemuth, R. R. Behringer, M. P. Mostoller, R. L. Brinster and R. D. Palmiter, *Nature*, 1989, **337**, 464-467.
- 630 A. J. Durston, J. P. M. Timmermans, W. J. Hage, H. F. J. Hendriks, N. J. Devries, M. Heideveld and P. D. Nieuwkoop, *Nature*, 1989, **340**, 140-144.
- 631 O. Chisaka and M. R. Capecchi, *Nature*, 1991, **350**, 473-479.
- 632 O. Chisaka, T. S. Musci and M. R. Capecchi, *Nature*, 1992, **355**, 516-520.
- 633 M. R. Capecchi, *Cold Spring Harbor Symp. Quant. Biol.*, 1997, **62**, 273-281.
- 634 F. R. Goodman and P. J. Scambler, *Clin. Genet.*, 2001, **59**, 1-11.
- 635 G. Breier, M. Bucan, U. Francke, A. M. Colberg-Poley and P. Gruss, *EMBO J.*, 1986, **5**, 2209-2215.
- 636 N. Papalopulu, R. Lovellbadge and R. Krumlauf, *Nucleic Acids Res.*, 1991, **19**, 5497-5506.
- 637 J. Deschamps, R. Delaaf, L. Joosen, F. Meijlink and O. Destree, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 1304-1308.
- 638 J. Deschamps, R. Delaaf, P. Verrijzer, M. Degouw, O. Destree and F. Meijlink, *Differentiation*, 1987, **35**, 21-30.
- 639 G. J. LaRosa and L. J. Gudas, *Mol. Cell. Biol.*, 1988, **8**, 3906-3917.
- 640 F. Mavilio, A. Simeone, E. Boncinelli and P. W. Andrews, *Differentiation*, 1988, **37**, 73-79.
- 641 A. Simeone, D. Acampora, V. Nigro, A. Faiella, M. Desposito, A. Stornaiuolo, F. Mavilio and E. Boncinelli, *Mech. Dev.*, 1991, **33**, 215-228.
- 642 D. Milanovic, P. Maier, F. Lohr, F. Wenz and C. Herskid, *Int. J. Cancer*, 2007, **121**, 1205-1211.
- 643 C. V. E. Wright, K. W. Y. Cho, J. Hardwicke, R. H. Collins and E. M. Derobertis, *Cell*, 1989, **59**, 81-93.
- 644 M. Kessel, R. Balling and P. Gruss, *Cell*, 1990, **61**, 301-308.
- 645 E. Ruberte, P. Dollé, P. Chambon and G. Morriskay, *Development*, 1991, **111**, 45-60.
- 646 J. Rossant, R. Zirngibl, D. Cado, M. Shago and V. Giguere, *Genes Dev.*, 1991, **5**, 1333-1344.
- 647 K. Reynolds, E. Mezey and A. Zimmer, *Mech. Dev.*, 1991, **36**, 15-29.
- 648 M. Maden, D. E. Ong, D. Summerbell and F. Chytil, *Nature*, 1988, **335**, 733-735.
- 649 M. Wagner, C. Thaller, T. Jessell and G. Eichele, *Nature*, 1990, **345**, 819-822.
- 650 D. Zhao, P. McCaffery, K. J. Ivins, R. L. Neve, P. Hogan, W. W. Chin and U. C. Drager, *Eur. J. Biochem.*, 1996, **240**, 15-22.

- 651 K. Niederreither, P. McCaffery, U. C. Drager, P. Chambon and P. Dolle, *Mech. Dev.*, 1997, **62**, 67-78.
- 652 K. Niederreither, J. Vermot, B. Schuhbauer, P. Chambon and P. Dollé, *Development*, 2000, **127**, 75-85.
- 653 T. Oosterveen, F. Meijlink and J. Deschamps, *Gene Expr. Patterns*, 2004, **4**, 243-247.
- 654 A. W. Langston and L. J. Gudas, *Mech. Dev.*, 1992, **38**, 217-228.
- 655 A. W. Langston and L. J. Gudas, *Curr. Opin. Genet. Dev.*, 1994, **4**, 550-555.
- 656 H. Marshall, M. Studer, H. Popperl, S. Aparicio, A. Kuroiwa, S. Brenner and R. Krumlauf, *Nature*, 1994, **370**, 567-571.
- 657 M. Studer, H. Popperl, H. Marshall, A. Kuroiwa and R. Krumlauf, *Science*, 1994, **265**, 1728-1732.
- 658 T. Ogura and R. M. Evans, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 387-391.
- 659 T. Ogura and R. M. Evans, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 392-396.
- 660 H. Popperl and M. S. Featherstone, *Mol. Cell. Biol.*, 1993, **13**, 257-265.
- 661 J. F. Boylan, D. Lohnes, R. Taneja, P. Chambon and L. J. Gudas, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 9601-9605.
- 662 M. A. C. Pratt, A. W. Langston, L. J. Gudas and M. W. McBurney, *Differentiation*, 1993, **53**, 105-113.
- 663 J. F. Boylan, T. Lufkin, C. C. Achkar, R. Taneja, P. Chambon and L. J. Gudas, *Mol. Cell. Biol.*, 1995, **15**, 843-851.
- 664 P. Serpente, S. Tumpel, N. B. Ghyselinck, K. Niederreither, L. M. Wiedemann, P. Dollé, P. Chambon, R. Krumlauf and A. P. Gould, *Development*, 2005, **132**, 503-513.
- 665 A. Stornaiuolo, D. Acampora, M. Pannese, M. Desposito, F. Morelli, E. Migliaccio, M. Rambaldi, A. Faiella, V. Nigro, A. Simeone and E. Boncinelli, *Cell Differ. Dev.*, 1990, **31**, 119-127.
- 666 H. Marshall, A. Morrison, M. Studer, H. Popperl and R. Krumlauf, *FASEB J.*, 1996, **10**, 969-978.
- 667 M. Studer, A. Gavalas, H. Marshall, L. Ariza-McNaughton, F. M. Rijli, P. Chambon and R. Krumlauf, *Development*, 1998, **125**, 1025-1036.
- 668 M. K. Maconochie, S. Nonchev, M. Studer, S. K. Chan, H. Popperl, M. H. Sham, R. S. Mann and R. Krumlauf, *Genes Dev.*, 1997, **11**, 1885-1895.
- 669 M. Gouti and A. Gavalas, *Stem Cells*, 2008, **26**, 1985-1997.
- 670 E. Martinez-Ceballos, P. Chambon and L. J. Gudas, *J. Biol. Chem.*, 2005, **280**, 16484-16498.
- 671 M. Davenne, M. K. Maconochie, R. Neun, A. Pattyn, P. Chambon, R. Krumlauf and F. M. Rijli, *Neuron*, 1999, **22**, 677-691.
- 672 O. O. Gaufo, P. Flodby and M. R. Capecchi, *Development*, 2000, **127**, 5343-5354.
- 673 A. Pattyn, A. Vallstedt, J. M. Dias, O. A. Samad, R. Krumlauf, F. M. Rijli, J. F. Brunet and J. Ericson, *Genes Dev.*, 2003, **17**, 729-737.
- 674 J. M. Goddard, M. Rossel, N. R. Manley and M. R. Capecchi, *Development*, 1996, **122**, 3217-3228.
- 675 D. Huang, S. W. Chen, A. W. Langston and L. J. Gudas, *Development*, 1998, **125**, 3235-3246.
- 676 T. R. J. Lappin, D. G. Grier, A. Thompson and H. L. Halliday, *Ulster Med. J.*, 2006, **75**, 23-31.
- 677 M. Pesce and H. R. Scholer, *Stem Cells*, 2001, **19**, 271-278.
- 678 H. R. Scholer, A. K. Hatzopoulos, R. Balling, N. Suzuki and P. Gruss, *EMBO J.*, 1989, **8**, 2543-2550.
- 679 H. R. Scholer, S. Ruppert, N. Suzuki, K. Chowdhury and P. Gruss, *Nature*, 1990, **344**, 435-439.
- 680 K. Okamoto, H. Okazawa, A. Okuda, M. Sakai, M. Muramatsu and H. Hamada, *Cell*, 1990, **60**, 461-472.
- 681 M. H. Rosner, M. A. Vigano, K. Ozato, P. M. Timmons, F. Poirier, P. W. J. Rigby and L. M. Staudt, *Nature*, 1990, **345**, 686-692.
- 682 Y. Saijoh, H. Fujii, C. Meno, M. Sato, Y. Hirota, S. Nagamatsu, M. Ikeda and H. Hamada, *Genes Cells*, 1996, **1**, 239-252.
- 683 K. Mitsui, Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda and S. Yamanaka, *Cell*, 2003, **113**, 631-642.
- 684 I. Chambers, D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie and A. Smith, *Cell*, 2003, **113**, 643-655.
- 685 A. A. Avilion, S. K. Nicolis, L. H. Pevny, L. Perez, N. Vivian and R. Lovell-Badge, *Genes Dev.*, 2003, **17**, 126-140.
- 686 L. A. Boyer, T. I. Lee, M. F. Cole, S. E. Johnstone, S. S. Levine, J. R. Zucker, M. G. Guenther, R. M. Kumar, H. L. Murray, R. G. Jenner, D. K. Gifford, D. A. Melton, R. Jaenisch and R. A. Young, *Cell*, 2005, **122**, 947-956.
- 687 M. Pesce, X. Y. Wang, D. J. Wolgemuth and H. Scholer, *Mech. Dev.*, 1998, **71**, 89-98.
- 688 L. Hyslop, M. Stojkovic, L. Armstrong, T. Walter, P. Stojkovic, S. Przyborski, M. Herbert, A. Murdoch, T. Strachan and M. Lako, *Stem Cells*, 2005, **23**, 1035-1043.
- 689 Y. H. Loh, Q. Wu, J. L. Chew, V. B. Vega, W. W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K. Y. Wong, K. W. Sung, C. W. H. Lee, X. D. Zhao, K. P. Chiu, L. Lipovich, V. A. Kuznetsov, P. Robson, L. W. Stanton, C. L. Wei, Y. J. Ruan, B. Lim and H. H. Ng, *Nature Genet.*, 2006, **38**, 431-440.
- 690 S. L. Palmieri, W. Peter, H. Hess and H. R. Scholer, *Dev. Biol.*, 1994, **166**, 259-267.
- 691 J. Nichols, B. Zevnik, K. Anastassiadis, H. Niwa, D. Klewe-Nebenius, I. Chambers, H. Scholer and A. Smith, *Cell*, 1998, **95**, 379-391.
- 692 B. Abdelrahman, M. Fiddler, D. Rappolee and E. Pergament, *Hum. Reprod.*, 1995, **10**, 2787-2792.

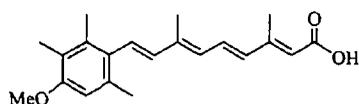
- 693 H. Niwa, J. Miyazaki and A. G. Smith, *Nature Genet.*, 2000, **24**, 372-376.
- 694 A. G. Smith, *Annu. Rev. Cell Dev. Biol.*, 2001, **17**, 435-462.
- 695 L. H. J. Looijenga, H. Stoop, H. de Leeuw, C. A. D. Brazao, A. J. M. Gillis, K. E. P. van Roozendaal, E. J. J. van Zoelen, R. F. A. Weber, K. P. Wolffenbittel, H. van Dekken, F. Honecker, C. Bokemeyer, E. J. Perlman, D. T. Schneider, J. Kononen, G. Sauter and J. W. Oosterhuis, *Cancer Res.*, 2003, **63**, 2244-2250.
- 696 J. Schoorlemmer, A. Vanpuijenbroek, M. Vandeneijnden, L. Jonk, C. Pals and W. Kruijer, *Mol. Cell. Biol.*, 1994, **14**, 1122-1136.
- 697 J. Schoorlemmer, L. Jonk, S. B. Shen, A. vanPuijenbroek, A. Feijen and W. Kruijer, *Mol. Biol. Rep.*, 1995, **21**, 129-140.
- 698 B. E. Reubinoff, M. F. Pera, C. Y. Fong, A. Trounson and A. Bongso, *Nat. Biotechnol.*, 2000, **18**, 399-404.
- 699 M. M. Matin, J. R. Walsh, P. J. Gokhale, J. S. Draper, A. R. Bahrami, I. Morton, H. D. Moore and P. W. Andrews, *Stem Cells*, 2004, **22**, 659-668.
- 700 S. Adler, C. Pellizzer, L. Hareng, T. Hartung and S. Bremer, *Toxicol. in Vitro*, 2008, **22**, 200-211.
- 701 J. Takeda, S. Seino and G. I. Bell, *Nucleic Acids Res.*, 1992, **20**, 4613-4620.
- 702 G. Cauffman, I. Liebaers, A. Van Steirteghem and H. V. de Velde, *Stem Cells*, 2006, **24**, 2685-2691.
- 703 Y. Atlasi, S. J. Mowla, S. A. M. Ziaee, P. J. Gokhale and P. W. Andrews, *Stem Cells*, 2008, **26**, 3068-3074.
- 704 G. L. Suo, J. Han, X. Wang, J. Y. Zhang, Y. N. Zhao, Y. H. Zhao and J. W. Dai, *Biochem. Biophys. Res. Commun.*, 2005, **337**, 1047-1051.
- 705 S. Zangrossi, M. Marabese, M. Broggin, R. Giordano, M. D'Erasmus, E. Montelatici, D. Intini, A. Neri, M. Pesce, P. Rebullà and L. Lazzari, *Stem Cells*, 2007, **25**, 1675-1680.
- 706 V. Kotoula, S. I. Papamichos and A. F. Lambropoulos, *Stem Cells*, 2008, **26**, 290-291.
- 707 T. Cantz, G. Key, M. Bleidissel, L. Gentile, D. W. Han, A. Brenne and H. R. Scholer, *Stem Cells*, 2008, **26**, 692-697.
- 708 M. H. Tai, C. C. Chang, L. K. Olson and J. E. Trosko, *Carcinogenesis*, 2005, **26**, 495-502.
- 709 M. Monk and C. Holding, *Oncogene*, 2001, **20**, 8085-8091.
- 710 V. Botquin, H. Hess, G. Fuhrmann, C. Anastasiadis, M. K. Gross, G. Vriend and H. R. Scholer, *Genes Dev.*, 1998, **12**, 2073-2090.
- 711 G. Fuhrmann, A. C. K. Chung, K. J. Jackson, G. Hummelke, A. Baniahmad, J. Sutter, I. Sylvester, H. R. Scholer and A. J. Cooney, *Dev. Cell*, 2001, **1**, 377-387.
- 712 C. J. Giuliano, J. S. Kerley-Hamilton, T. Bee, S. J. Freemantle, R. Manickaratnam, E. Dmitrovsky and M. J. Spinella, *Biochim. Biophys. Acta*, 2005, **1731**, 48-56.
- 713 V. B. Christie and D. J. Maltman, personal communication.
- 714 W. C. Smith, H. Nakshatri, P. Leroy, J. Rees and P. Chambon, *EMBO J.*, 1991, **10**, 2223-2230.
- 715 T. M. Macgregor, N. G. Copeland, N. A. Jenkins and V. Giguere, *J. Biol. Chem.*, 1992, **267**, 7777-7783.
- 716 S. Vogel, R. Piantedosi, J. Frank, A. Lalazar, D. C. Rockey, S. L. Friedman and W. S. Blaner, *J. Lipid Res.*, 2000, **41**, 882-893.
- 717 L. N. Wei, W. S. Blaner, D. S. Goodman and M. C. Nguyenhuu, *Mol. Endocrinol.*, 1989, **3**, 454-463.
- 718 G. Siegenthaler, I. Tomatis, D. Chatellardgruaz, S. Jaconi, U. Eriksson and J. H. Saurat, *Biochem. J.*, 1992, **287**, 383-389.
- 719 M. A. Lane, J. Xu, E. W. Wilen, R. Sylvester, F. Derguini and L. J. Gudas, *Mol. Cell. Endocrinol.*, 2008, **280**, 63-74.
- 720 C. M. Stoner and L. J. Gudas, *Cancer Res.*, 1989, **49**, 1497-1504.
- 721 A. L. Means, J. R. Thompson and L. J. Gudas, *Cell Growth Differ.*, 2000, **11**, 71-82.
- 722 J. T. Elder, A. Kaplan, M. A. Cromie, S. Kang and J. J. Voorhees, *J. Invest. Dermatol.*, 1996, **106**, 517-521.
- 723 A. Åström, U. Pettersson, P. Chambon and J. J. Voorhees, *J. Biol. Chem.*, 1994, **269**, 22334-22339.
- 724 S. J. Freemantle, J. S. Kerley, S. L. Olsen, R. H. Gross and M. J. Spinella, *Oncogene*, 2002, **21**, 2880-2889.
- 725 V. Giguere, S. Lyn, P. Yip, C. H. Siu and S. Amin, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 6233-6237.
- 726 A. Åström, A. Tavakkol, U. Pettersson, M. Cromie, J. T. Elder and J. J. Voorhees, *J. Biol. Chem.*, 1991, **266**, 17662-17666.
- 727 R. Ruhl, C. Plum, M. M. A. Elmazar and H. Nau, *Toxicol. Sci.*, 2001, **63**, 82-89.
- 728 A. M. Jetten, K. Anderson, M. A. Deas, H. Kagechika, R. Lotan, J. I. Rearick and K. Shudo, *Cancer Res.*, 1987, **47**, 3523-3527.
- 729 C. E. M. Griffiths, J. T. Elder, B. A. Bernard, P. Rossio, M. A. Cromie, L. J. Finkel, B. Shroot and J. J. Voorhees, *J. Invest. Dermatol.*, 1993, **101**, 325-328.
- 730 E. Sonneveld, C. E. van den Brink, L. G. J. Tertoolen, P. van der Burg and P. T. van der Saag, *Dev. Biol.*, 1999, **213**, 390-404.

- 731 L. Luu, H. Ramshaw, A. Tahayato, A. Stuart, G. Jones, J. White and M. Petkovich, *Adv. Enzyme Regul.*, 2001, **41**, 159-175.
- 732 S. Y. Kim, S. J. Yoo, H. J. Kwon, S. H. Kim, Y. Byun and K. S. Lee, *Metab.-Clin. Exp.*, 2002, **51**, 477-481.
- 733 D. Downie, M. C. E. McFadyen, P. H. Rooney, M. E. Cruickshank, D. E. Parkin, I. D. Miller, C. Telfer, W. T. Melvin and G. I. Murray, *Clin. Cancer Res.*, 2005, **11**, 7369-7375.
- 734 D. Liang, M. Zhang, J. Bao, L. Zhang, X. Xu, X. Gao and Q. Zhao, *Gene Expr. Patterns*, 2008, **8**, 248-253.
- 735 J. Marill, N. Idres, C. C. Capron, E. Nguyen and G. G. Chabot, *Curr. Drug Metab.*, 2003, **4**, 1-10.
- 736 P. Dollé, E. Ruberte, P. Kastner, M. Petkovich, C. M. Stoner, L. J. Gudas and P. Chambon, *Nature*, 1989, **342**, 702-705.
- 737 B. A. Bernard, J. M. Bernardon, C. Delescluse, B. Martin, M. C. Lenoir, J. Maignan, B. Charpentier, W. R. Pilgrim, U. Reichert and B. Shroot, *Biochem. Biophys. Res. Commun.*, 1992, **186**, 977-983.
- 738 M. Suzui, M. Shimizu, M. Masuda, J. T. E. Lim, N. Yoshimi and I. B. Weinstein, *Mol. Cancer Ther.*, 2004, **3**, 309-316.
- 739 G. Lemaire, P. Balaguer, S. Michel and R. Rahmani, *Toxicol. Appl. Pharmacol.*, 2005, **202**, 38-49.
- 740 R. M. Nilis, *Acta Pharmacol. Sin.*, 2007, **28**, 1383-1391.
- 741 R. M. Nilis, *Mutat. Res.-Fundam. Mol. Mech. Mutagen.*, 2004, **555**, 81-96.
- 742 V. Giguere, *Endocr. Rev.*, 1994, **15**, 61-79.
- 743 S. Pozzi, S. Rossetti, G. Bistulfi and N. Sacchi, *Oncogene*, 2006, **25**, 1400-1407.
- 744 J. L. Clifford, M. Petkovich, P. Chambon and R. Lotan, *Mol. Endocrinol.*, 1990, **4**, 1546-1555.
- 745 S. Chen, J. Ostrowski, G. Whiting, T. Roalsvig, L. Hammer, S. J. Currier, J. Honeyman, B. Kwasniewski, K. L. Yu, R. Sterzycki, C. U. Kim, J. Starrett, M. Mansuri and P. R. Reczek, *J. Invest. Dermatol.*, 1995, **104**, 779-783.
- 746 J. L. Plassat, L. Penna, P. Chambon and C. Rochette-Egly, *J. Cell Sci.*, 2000, **113**, 2887-2895.
- 747 Y. Wei, T. Harris and G. Childs, *Differentiation*, 2002, **70**, 204-219.
- 748 P. W. Andrews, I. Damjanov, D. Simon and M. Dignazio, *Differentiation*, 1985, **29**, 127-135.
- 749 A. B. Barua and J. A. Olson, *J. Chromatogr. B: Biomed. Sci. Appl.*, 1998, **707**, 69-79.
- 750 M. F. Boehm, M. R. McClurg, C. Pathirana, D. Mangelsdorf, S. K. White, J. Hebert, D. Winn, M. E. Goldman and R. A. Heyman, *J. Med. Chem.*, 1994, **37**, 408-414.
- 751 *US Pat.*, 2 897 237, 1959.
- 752 T. F. Wood, M. S. Carpenter, W. M. Easter and J. Angiolini, *J. Org. Chem.*, 1963, **28**, 2248-2255.
- 753 R. R. Webb, M. C. Venuti and C. Eigenbrot, *J. Org. Chem.*, 1991, **56**, 4706-4713.
- 754 *US Pat.*, 3 499 751, 1970.
- 755 G. Garipova, A. Gautier and S. R. Piettre, *Tetrahedron*, 2005, **61**, 4755-4759.
- 756 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.
- 757 U. K. Laemmli, *Nature*, 1970, **227**, 680-685.
- 758 F. Pattyn, F. Speleman, A. De Paepe and J. Vandesompele, *Nucleic Acids Res.*, 2003, **31**, 122-123.
- 759 F. Pattyn, P. Robbrecht, A. De Paepe, F. Speleman and J. Vandesompele, *Nucleic Acids Res.*, 2006, **34**, D684-D688.
- 760 S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman, *J. Mol. Biol.*, 1990, **215**, 403-410.
- 761 M. Zaitseva, B. J. Vollenhoven and P. A. W. Rogers, *Mol. Hum. Reprod.*, 2007, **13**, 577-585.
- 762 I. Martinez, J. Wang, K. F. Hobson, R. L. Ferris and S. A. Khan, *Eur. J. Cancer*, 2007, **43**, 415-432.
- 763 G. R. Chichili, D. Nohr, M. Schaffer, J. von Lintig and H. K. Biesalski, *Invest. Ophthalmol. Vis. Sci.*, 2005, **46**, 3562-3569.
- 764 Y. Kimura, T. Suzuki, C. Kaneko, A. D. Darnel, T. Moriya, S. Suzuki, M. Handa, M. Ebina, T. Nukiwa and H. Sasano, *Clin. Sci. (Lond.)*, 2002, **103**, 613-621.
- 765 E. Willems, I. Mateizel, C. Kemp, G. Cauffman, K. Sermon and L. Leyns, *Int. J. Dev. Biol.*, 2006, **50**, 627-635.

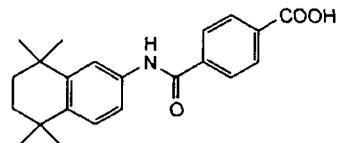




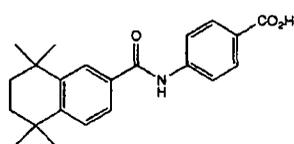
M Tazarotene
(AGN 190168)



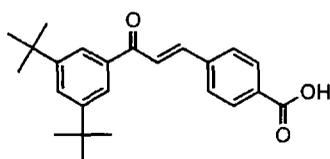
N acitretin
(Neotigason®)



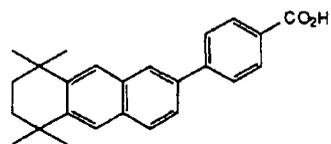
O Tamibarotene
(Am80)



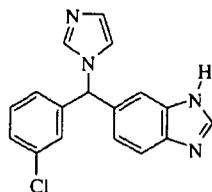
P Am580



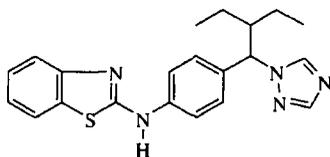
Q Ch55



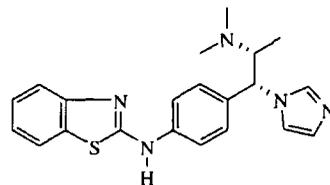
R CD367, TTAB



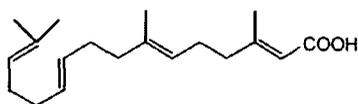
S Liazal®



T Rambazole®



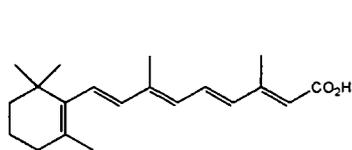
U R116010



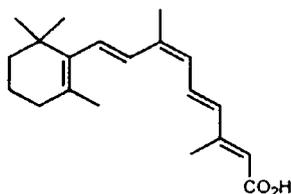
V acyclic retinoid

Appendix 1: Structures of natural and synthetic retinoids and RAMBAs discussed in text

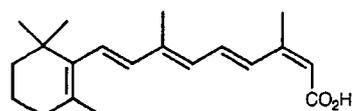
(numbering distinct from synthetic scheme in Chapter 2)



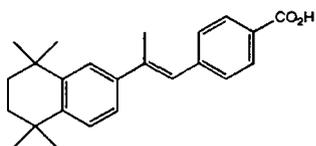
A ATRA



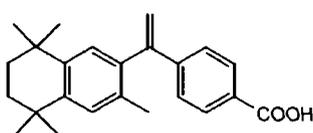
B 9-*cis*-retinoic acid



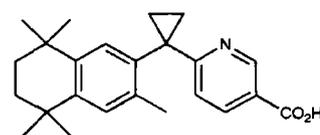
C 13-*cis*-retinoic acid



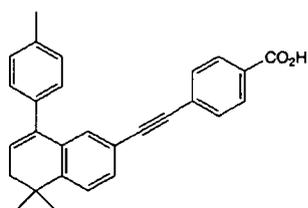
D TTNPB



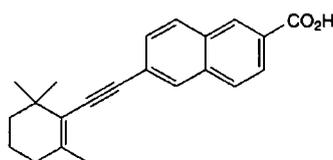
E Targretin[®]
(LGD1069, bexarotene)



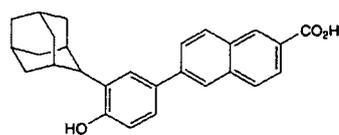
F LG100268



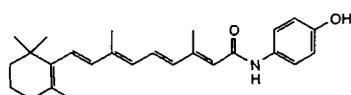
G AGN 193109



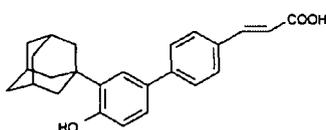
H AGN 190730



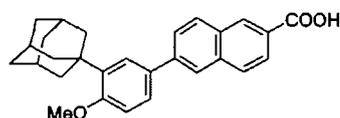
I CD437
(AHPN, AGN 192837)



J Fenretinide
(4-HPR)



K ST1926



L Adapalene
(CD271)

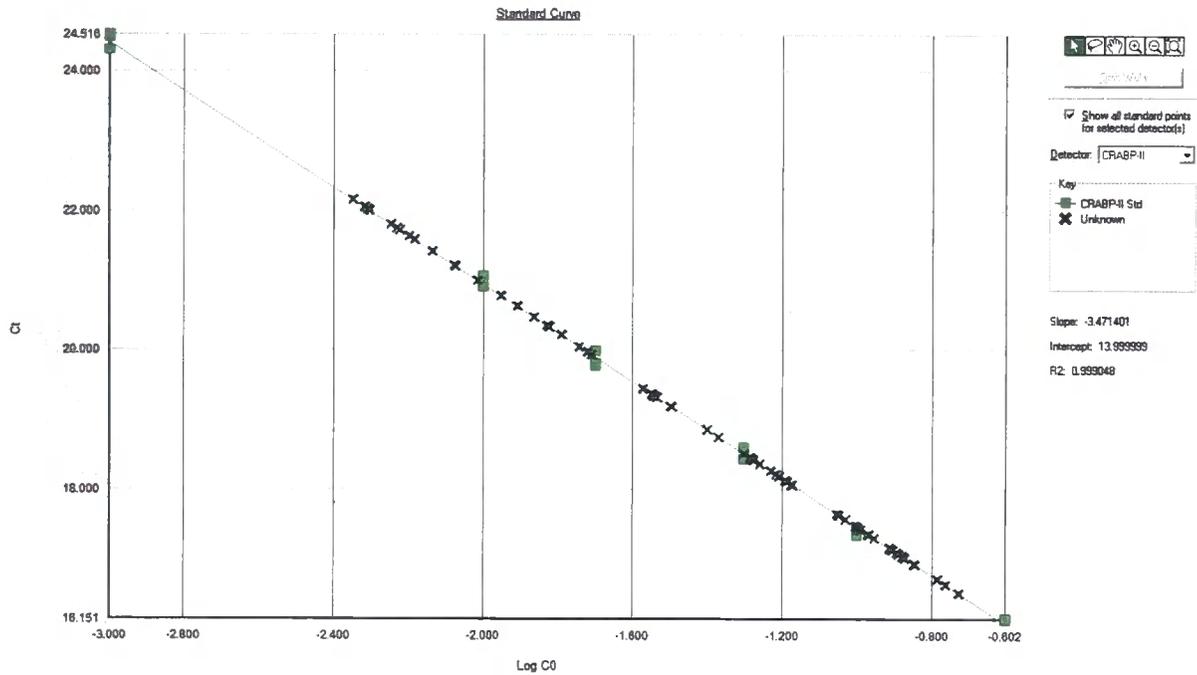
Appendix 2: Crystallographic data

Crystal structure and refinement data for compounds **4**, **5**, **14** and **17**.

Molecule	4 – CEB17	5 – CEB18	14	17
Identification code	05srv167	06srv233	05srv133	05srv196
Empirical formula	C ₂₃ H ₂₆ O ₂	C ₂₅ H ₂₆ O ₂	C ₁₇ H ₂₄ O	C ₁₄ H ₁₉ Br
Formula weight	334.44	358.46	244.36	267.20
Temperature / K	120(2)	120(2)	120(2)	120(2)
Crystal system	triclinic	triclinic	monoclinic	orthorhombic
Space group	P-1	P-1	P2(1)/c	P2 ₁ 2 ₁ 2 ₁ (No. 19)
Unit Cell dimensions:				
a / Å	5.9101(18)	5.9191(10)	10.0785(12)	7.760(1)
b / Å	8.622(3)	8.5375(15)	13.9074(17)	10.508(1)
c / Å	17.961(6)	19.455(3)	10.5905(13)	15.461(1)
α / °	84.739(6)	96.334(16)	90	90
β / °	89.610(6)	92.672(14)	95.808(2)	90
γ / °	84.674(7)	103.035(13)	90	90
Volume / Å ³	907.4(5)	949.4(3)	1476.8(3)	1260.7(2)
Z	2	2	4	4
Density (calculated) / Mg/m ³	1.224	1.254	1.099	1.408
Absorption coefficient / mm ⁻¹	0.076	0.078	0.066	3.228
Crystal size / mm ³	0.28 x 0.12 x 0.05	0.2 x 0.1 x 0.02	0.45 x 0.40 x 0.35	0.35 x 0.31 x 0.29
Theta range for data collection / °	2.55 to 30.51	2.56 to 24.25	2.03 to 30.24	2.3 to 30.0
Reflections collected	10605	1049	10343	23007
Independent reflections	5376 [R(int) = 0.0834]	3293	4006 [R(int) = 0.0241]	3666 [R(int) = 0.0210]
Absorption correction	none	none	none	semi-empirical from equivalents
Data / restraints / parameters	5376 / 0 / 232	3293 / 0 / 235	4006 / 0 / 259	3666 / 0 / 176
Final R indices [I > 2σ(I)]	R1 = 0.0875 wR2 = 0.2535	R1 = 0.0778	R1 = 0.0451 wR2 = 0.1154	R1 = 0.0170 wR2 = 0.0402
R indices (all data)	R1 = 0.1445 wR2 = 0.2722	R1 = 0.1316	R1 = 0.0616 wR2 = 0.1249	R1 = 0.0201 wR2 = 0.0413

Appendix 3: Typical standard curve generated during rt qPCR analysis.

Six dilutions of an appropriate standard were prepared and analysed in triplicate for each gene on each PCR plate. Overall, greater than 99% of samples with detectable levels of gene expression were within the corresponding standard range (>90% of all samples).



Standard curve generated for one plate of the *CRABP-II* rt qPCR analysis. The fluorescence of all samples in this analysis was within the standard range.