Evaluation and comparison of natural and synthetic retinoids on model of neural development

Buyers Christie, Victoria

Evaluation and Comparison of Natural and Synthetic Retinoids on Models of Neural Development.

Victoria Buyers Christie

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Durham University

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Abstract

Vitamin A and its derivatives, collectively termed retinoids, are a group of natural and synthetic molecules that are structurally and/or functionally analogous. Retinoids are essential for many biologically important processes during mammalian embryogenesis and adult homeostasis. Consequently, retinoids are often used to modulate cell proliferation and differentiation of cultured stem and progenitor cells in the laboratory, and possess the potential for use in numerous clinical applications. For example, retinoids are already applied as treatments of dermatological disorders, and as part of chemotherapeutic programmes for certain types of cancer.

The importance of optimal retinoid function in embryonic neural development is well known, and is now being realised in certain adult neural progenitor cell populations. It has been shown that *all-trans*-retinoic acid (ATRA), which activates all retinoic acid receptor subtypes, is the main active natural retinoid, inducing neural differentiation in several cell model systems, including embryonic stem cells and adult neural progenitors. The *in vitro* study of ATRA, however, is complicated by its photo-isomerisation and degradation when used under standard laboratory conditions. This instability has obvious disadvantages, for example, in stem cell biology where the ability to control the development of cells and tissues in a predictable way is paramount. Human stem cells present the opportunity to produce tissues that can be used for basic research into health and disease, development of pharmaceuticals, toxicological tests and ultimately cell replacement therapy. Biologists require molecules that induce reliable and reproducible biological activity resulting in consistent modes of cell differentiation. To address this issue, a small library of synthetic retinoids have been designed and prepared and their biological activity investigated. This thesis focused on the synthesised ATRA retinoid analogues, EC19 and EC23 and aimed to evaluate their biological activity in relation to ATRA on a number of different embryonic and adult model systems. Unlike ATRA, these compounds do not isomerise in response to light or heat. Data show that both compounds are effective at inducing differentiation. In model embryonic stem and adult progenitor cell culture systems both neural and non-neural cell sub-types are induced after EC19 or EC23 treatment. Specifically, EC23 elicited similar cellular responses to ATRA when tested *in vitro,* inducing significant neural differentiation in the model cell systems and appears to do so via specific retinoic acid functional pathways. EC19, however, induced different proportions of the cell-types observed after ATRA and EC23 media supplementation, inducing differentiation towards predominantly non-neural, epithelial-like phenotypes in stem cell models, and enhanced glial differentiation in adult progenitor models. Interestingly, EC19 also appeared to enhance the proliferation of a neuroblastoma model, in stark contrast to both ATRA and EC23 which induced a degree of neural differentiation.

Both synthetic retinoids induce an up-regulation of cellular proteins involved with retinoic acid metabolism and signalling, suggesting that they elicit their effects directly via these pathways. Therefore EC19 and EC23 represent promising candidates as alternatives to ATRA for directing cell differentiation and investigating the molecular pathways involved in neural cell development and retinoid modes of action *in vitro*. It is anticipated that this work will enhance research into retinoid mediated neural differentiation. Finally, stable, synthetic modulators of cell differentiation offer distinct advantages over existing technology and will be of significant commercial value.

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Declaration:

I confirm that no part of the material offered has previously been submitted by me for a degree in this or any other University. Material generated through joint work has been acknowledged and the appropriate publications cited. In all other cases material from the work of others has been acknowledged and quotations and paraphrases suitably indicated.

Statement of Copyright:

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Publications arising from this thesis:

- **Christie V.B.** Marder T.B. Whiting A. Przyborski S.A.
  The role of retinoids in the adult nervous system and their therapeutic potential.
  Mini Rev. Med. Chem. 2008 Jun;8(6):601-8.

- **Christie V.B.** Barnard J.H. Batsanov A.S. Bridgens C.E. Cartmell E.B. Collings J.C. Maltman D.J. Marder T.B. Redfern C.P.F. Whiting A. Przyborski S.A.
  Synthesis and Evaluation of Synthetic Retinoid Derivatives as Inducers of Stem Cell Differentiation

  Org. Bio. Chm. 2008 Aug;(6):3497-3507

- Maltman D.J. **Christie V.B.** Collings J.C. Fenk S. Marder T.B. Whiting A. Przyborski S.A.
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  Submitted to Molecular Biosystems Oct 2008

• List of Abbreviations:

13-cisRA	13-cis-retinoic acid
9-cisRA	9-cis-retinoic acid
AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein E
ATRA	*All-trans*-retinoic acid
bFGF	Basic fibroblast growth factor
bp	Base pairs
BMPs	Bone morphogenetic proteins
BrdU	Bromodeoxyuridine
CNS	Central nervous system
CO_2	Carbon dioxide
CRABP-I	Cellular retinoic acid binding protein I
CRABP-II	Cellular retinoic acid binding protein II
CRBP-I	Cellular retinol binding protein I
CRBP-II	Cellular retinol binding protein II
DBD	DNA binding domain
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
EC cells	Embryonal carcinoma cells
EGF	Epidermal growth factor
ES cells	Embryonic stem cells
FCS	Foetal calf serum
GFAP	Glial fribrillary acidic protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPLC	High performance liquid chromatography
IUPAC-IUB	International Union of Pure and Applied Chemistry-International Union of Biochemistry
kDa	KiloDalton

LBD	Ligand binding domain
LTD	Long term depression
LTP	Long term potentiation
M	Molar
MALDI- MS	Matrix assisted laser desorption ionisation – mass spectrometry
MALDI-TOF	Matrix assisted laser desorption ionisation – time of flight
MEF	Mouse embryonic feeder layer
MOWSE	MOlecular Weight Search
NCoR	Nuclear receptor corepressor
NEAA	Non-essential amino acids
NF-200	Neurofilament – 200
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
Pmol	Picomole
PVDF	Polyvinyldiene fluoride
RALDH	Retinal dehydrogenase
RAMBAs	Retinoic acid metabolism blocking agents
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
ROLDH	Retinol dehydrogenase
rpm	Rotations per minute
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
RXR	Retinoid X receptor
SDM	Standard deviation of the mean
SDS-Page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF	Surface enhanced laser desorption ionisation-time of flight
SMRT	Silencing mediator of RAR and thyroid hormone receptor
SSEA-1	Stage specific embryonic antigen-1
SSEA-3	Stage specific embryonic antigen-3
SSEA-4	Stage specific embryonic antigen-4
TMSA	trimethylsilylacetylene

TTR	Transthyretin
VAD	Vitamin A deficiency
WHO	World Health Organisation

1 Chapter 1: Introduction

1.1 Literature review

In 1960, the International Union of Pure and Applied Chemistry – International Union of Biochemistry (IUPAC-IUB) recommended the nomenclature of the three parent vitamin A compounds to be retinol, retinal and retinoic acid. By 1982, IUPAC-IUB had defined the term vitamin A as 'The generic descriptor for retinoids exhibiting qualitatively the biological activity of retinol,' and a retinoid as 'a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner, formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion'. This nomenclature is still in part used today and incorporates both natural and synthetic analogues of retinol. The correct structure for retinoic acid (*all-trans*) (ATRA) was proposed in 1931 [1].

Natural retinoids are essential for all chordates, being involved in a wide variety of processes during embryogenesis and adulthood. In humans, vitamin A deficiency (VAD) is characterised by xerophthalmia (dry eyes), night blindness and impaired immune responses which, in clinical cases, can prove fatal. VAD is largely associated with developing countries where vitamin A fortified or rich foods are in poor supply. According to a report by the World Health Organisation (WHO) in 1995 [2], VAD is a public health problem in over 120 countries. Over 1 million childhood deaths are associated with VAD annually and 3 million people are said to be clinically vitamin A deficient. However, excessive dietary or therapeutic intake of vitamin A (Hypervitaminosis A) can also give rise to a range of problems associated with skin, nervous system, circulation, bone formation and immune system and malformations of the foetus [3]. Both of these conditions indicate the importance of the close regulation of retinoic acid up-take and metabolism.

As retinoids are able to be transported to the central nervous system (CNS), an excess or deficiency can prove detrimental to normal mental development, being linked to various disorders such as retardation and depression. This literature review summarises work into retinoid function and biochemical pathways, and focuses on the roles of retinoic acid and

its isomers in normal and abnormal neural regulation and differentiation. It also covers the instability of ATRA when used under standard laboratory conditions and the potential use of synthetic retinoid compounds to overcome this issue. This review also covers the potential therapeutic applications of synthetic retinoid compounds in treatment of numerous neurological disorders.

1.2 Retinoid Function:

One of the earliest discoveries of a specific retinoid function was in 1968 [4], when it was discovered that 11-cis-retinal was the chromophore component of the photoreceptors in the visual cycle. Since then, however, retinoids have been found to play key essential roles in numerous biological processes, including vision [4], reproduction [5], immune competence [6], cellular regulation and differentiation in post-natal and adult organisms [7, 8] and embryonic growth and development [9].

The importance of vitamin A during embryonic development was exemplified in studies of VAD and hypervitaminosis A during pregnancy. Both conditions resulted in embryonic/foetal malformations ranging from disorders of the skin, nervous system, and immune system (for a detailed review see Blomhoff and Blomhoff 2006 [10]). This need for a very defined and regulated concentration of retinoids during embryogenesis fitted with the discovery that ATRA was a potent morphogen during embryonic development [11], involved in limb formation, body axis orientation and dorsal/ventral patterning of the CNS in vertebrates, which will be discussed in more detail below.

The ability of certain tissues, and indeed whole organs, to regenerate under defined conditions is well-known and retinoic acid has been shown to play a key part. The specific pathways and genes involved have been shown to be the same as those activated during the initial development of the tissue in question [12]. Therefore, any pathway which involved retinoic acid during development, would involve retinoic acid during adult regeneration, if it occurred. The ability of tissues to regenerate is classically illustrated in experiments of limb regeneration in urodele amphibians. During amputation studies in axolotls, it was shown that application of retinoic acid to the wound site induced a

concentration-dependent increase in the amount of tissue regenerated after exposure [13, 14]. For example, at low concentrations of retinoic acid, an extra complete ulna and radius was regenerated, and at high retinoic acid concentrations a complete extra limb was regenerated from the amputation site. This ability to regenerate tissue is not exclusive to amphibians, but has been reported in many species including mammals, for example lung tissue regeneration after retinoic acid treatment of adult rat models of lung disease [15, 16]. As retinoic acid is involved in many developmental processes, including lung and CNS development, retinoic acid induced regeneration has obvious therapeutic potential as a 'self-repair' treatment for many different medical conditions. Unfortunately, in the case of spinal chord injury, the adult mammalian CNS has lost its ability to regenerate itself after damage or disease. However, as retinoic acid and the machinery required for gene activation are all present during the tissue's development, it can be assumed that adult regeneration does not occur as a result of one or more of these components either being inhibited or absent in the adult system [17]. This feature has therefore become the focus for numerous research studies into spinal chord regeneration and neurodegenerative disorder treatment.

1.3 Vitamin A Intake and Storage:

Vitamin A cannot be synthesised by any animal species, and is obtained through diet, either in the form of pro-vitamin A carotenoids (such as β-carotene) from plants or pre-formed vitamin A, usually in the form of retinyl esters, from eating animal products (stores of ingested vitamin A). Once digested, all of these compounds are re-esterified, via enzymatic action, in the intestinal lumen and mucosa, and then packaged into chylomicrons and transported to the liver. The liver is the major site for storage and processing of these compounds within the body [18]. Much of the retinol is stored as retinyl palmitate, which is formed after reversible enzymatic activity. When required, retinol is secreted from the liver and transported around the vascular system bound to retinol binding protein (RBP) [19].

Circulating retinol is aided across the cytoplasmic membrane by the retinol binding protein receptor [20]. Once retinol has entered the cell cytosol it can either be stored locally

for future use (as retinyl esters) or be converted into forms that activate certain cellular biochemical pathways leading to a modulation of gene transcription (**Fig. 1.1a**).

The predominant active retinoid in the body is ATRA. ATRA is created from retinol via a two step reaction. Retinol is oxidised to retinal, catalysed by retinol dehydrogenases (ROLDH), then retinal is oxidised to ATRA catalysed by retinal dehydrogenases (RALDH)[18], (see **Fig. 1.1b**). The storage or breakdown of retinol occurs within the cytosol due to the presence of proteins which bind it with high affinity, called cellular retinol binding protein I (CRBP-I) and cellular retinol binding protein II (CRBP-II). CRBP-I is ubiquitously expressed throughout the organism, and appears to facilitate cellular uptake and metabolism of retinol into either its storage or active forms. CRBP-II is exclusively found in the villi of enterocytes where it is involved in the conversion of retinol into retinyl esters for chylomicron export to the liver [21]. Within some cells there are also cellular retinoic acid binding proteins I and II (CRABP-I and CRABP-II). CRABP-I has been shown to play a key role in ATRA homeostasis, however, it was also shown to not be directly involved in activating the retinoic acid receptor signalling pathway [22], and later it was discovered that CRABP-I was involved in the storage and metabolism of free ATRA, thereby inhibiting the biological activity of the metabolite [23]. Generally, it has been found that cells containing an excess of CRABP-I do not respond as readily to ATRA. The role of CRABP-II is less well understood and has only recently been validated. It has been shown that CRABP-II aids the transport of ATRA into the nucleus and interacts with receptor heterodimers [24], channelling ATRA to the receptors thereby sensitising cells to ATRA [25]. The tissue specific expression of these important retinoid regulators indicates their complex nature of action and involvement in many biological processes.

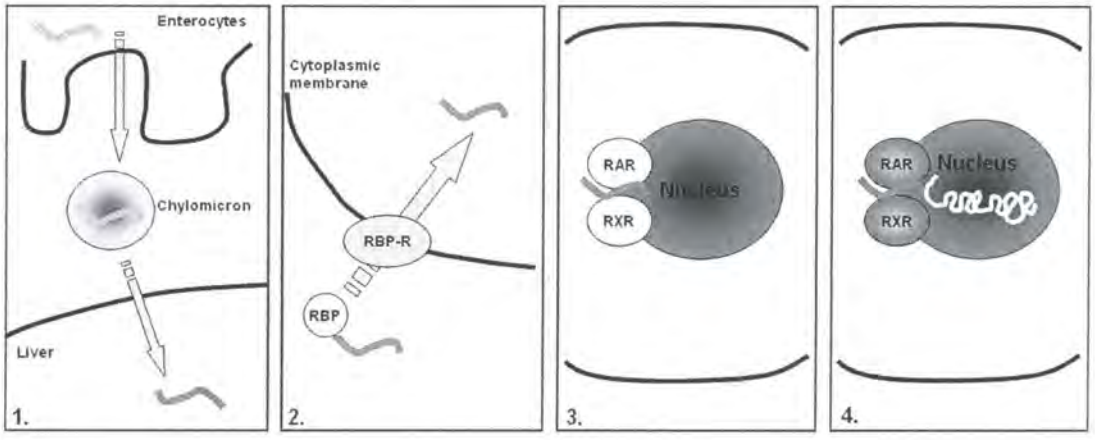


Figure 1.1A: Overview of retinoid action. **1:** Ingested retinoids are converted to all-trans-retinol in the intestinal lumen and mucosa by enzymatic action. All-trans-retinol is then packaged into chylomicrons and transported to the liver, which is the major site for storage and processing of this compound. Much of the retinol is stored as retinyl palmitate, which is formed after reversible enzymatic activity. **2:** All-trans-retinol is transported around the vascular system bound to retinol-binding protein (RBP), and accesses target cells via the retinol binding protein receptor (RBP-R). **3:** The retinoid binds to the nuclear retinoic acid receptors (RAR & RXR) which act as signal transducers. **4:** Activated ligand/receptor complexes modulate gene expression by binding to specific retinoic acid response elements (RARE) located in the regulatory regions of target genes. Diagram taken from Christie et al 2008[123].

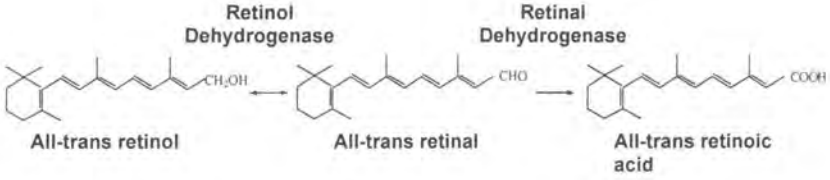


Figure 1.1B: Retinol is converted to the active form 'all-trans retinoic acid' via a two step reaction. Retinol is first reversibly converted to retinal, catalysed by retinol dehydrogenase. Secondly, retinal is irreversibly converted to all-trans retinoic acid, catalysed by retinal dehydrogenase. Diagram taken from Christie et al 2008[123].

1.4 Retinoic Acid Receptors:

Retinoids elicit biological activity via retinoic acid receptors (RAR) which are located in the nuclear envelope. The molecular action of retinoic acid and RARs was first hypothesised during studies of steroid and thyroid hormone action [26], and has since been validated by the identification of the DNA- and ligand-binding domains within the receptors [27] alongside the identification of the receptors crystal structures [28]. It was proposed that retinol entered the cell, was converted to ATRA via enzymatic action and was transported to the nucleus by CRABP-II, where it bound with high affinity to retinoic

acid receptors via a ligand binding domain. This interaction would lead to a conformation change to the receptor-ligand complex, enabling the binding of a specific retinoic acid response element (RARE) located upstream of the promoter region of a target gene via the DNA-binding domain (**Fig. 1.2**). The first nuclear retinoid receptor was independently discovered by two groups in 1987, now known to be RAR-α1 [29, 30]. RARs can be divided into three sub-groups, RAR-α, RAR-β and RAR-γ, which in turn can be sub-divided into multiple receptor isoforms (i.e. RAR-β1, RAR-β2 and RAR-β3). This heterogeneity is due to the fact that a separate gene that has multiple promoters encodes each subtype, and the products are also able to undergo differential splicing. The complexity of retinoid action is further complicated with the discovery of other nuclear receptors that also exhibit affinity for retinoids. These were termed retinoid X receptors (RXRs) [31]. RXRs could also be divided into three isoforms RXR-α, RXR-β, and RXR-γ. RARs always bind to DNA as heterodimers, pairing up with an RXR. The RXRs are able to act as homodimers or heterodimers, pairing up with themselves, RARs or other receptor compounds [32]. The RARs have the highest affinity for ATRA whereas the RXRs have higher affinity for other retinoid isoforms such as 9-cis-retinoic acid (9-cisRA) [33]. The tissue expression of these numerous RAR and RXR subtypes and isoforms varies greatly throughout the organism, again an indication of the complex and varied role the retinoids play in adult and embryonic cell systems. For example, during neural development, RAR-γ is found exclusively in the open neural folds, wheras RAR-β is found in the neural tube only after it has closed [34].

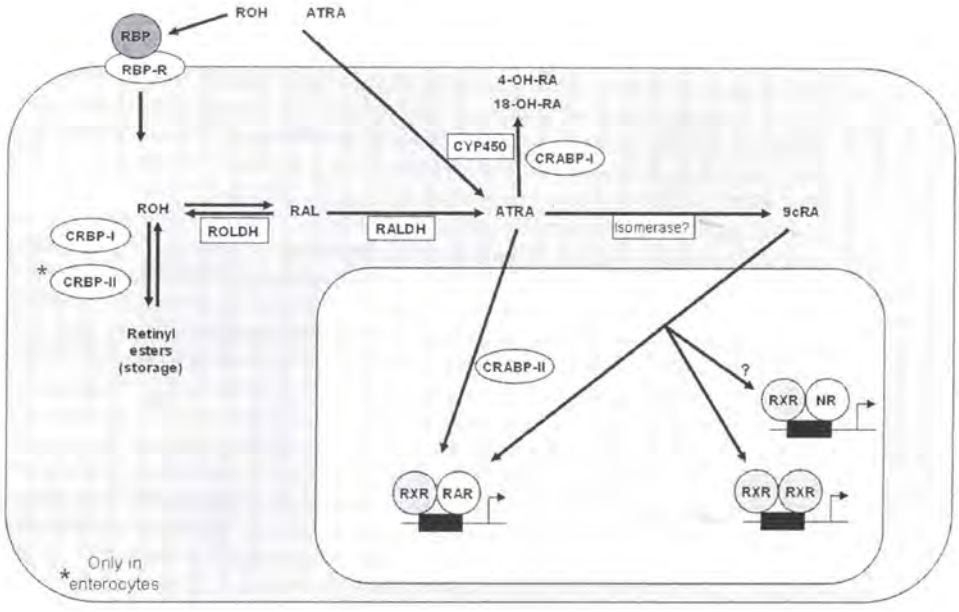


Figure 1.2: General overview of retinoid action. Retinol enters the cell via the retinol binding protein receptor and once in the cell is bound to cellular retinol binding proteins (CRBP I & II). These proteins aid either the breakdown of retinol for storage into retinyl esters, or the conversion to all-trans retinoic acid (ATRA). ATRA can be metabolised to more polar molecules catalysed by the CYP450 enzymes and cellular retinoic acid binding protein I (CRABP-I). Cellular retinoic acid binding protein II (CRABP-II) is thought to be involved in the delivery of ATRA to the nuclear envelope where it binds to the retinoic acid receptors (RARs). ATRA has high affinity for the RARs whereas other natural retinoids such as 9-cis retinoic acid (9-cisRA) has high affinity for the retinoid X receptors (RXRs). Once bound to the receptors the ligand/ receptor complex undergoes a conformational change rendering it active, and allows the binding of retinoic acid response elements (RAREs) of target genes. Diagram taken from Christie et al 2008[123].

Retinoic acid receptors are transcription factors that have a modular structure commonly associated with nuclear receptors, which contains six conserved regions, named A-F respectively (**Fig.1.3**). The N-terminal A/B region is the most variable in size between the RARs and the RXRs. The A/B region always consists of a ligand independent transcriptional activation function (AF-1) which contains several phosphorylation sites including those activated by MAP kinases [35]. The C region is the most highly conserved between all RARs and RXRs (~66 amino acids long) and harbours the DNA binding domain (DBD). The domain is made up of two zinc-finger motifs which mediate sequence specific DNA recognition of target genes, two α-helices and a COOH-terminal extension [36, 37]. At the base of the first zinc finger is located a three amino acid section termed the P-box, which plays a key role in determining the specificity of DNA

recognition for the receptor [38]. This C-region also contains a small protein dimerisation surface (D-box), which allows the RAR to interact with an RXR when bound to particular types of DNA elements. The D region is a short linker region which allows the correct orientation of the DBD and the ligand binding domain (LBD) within DNA-bound dimers. The second most conserved region is region E (~200 amino acids long) which is multi-functional. Region E is made up of the LBD, containing the ligand-binding pocket, the main dimerisation surface (termed I-box), the ligand-dependent transcriptional activation function (AF-2), and also contains an area involved in transcriptional silencing in the absence of a ligand (NCoR box) [39]. Region E is the main area of transcriptional regulation [40]. Finally the C-terminal F-region has been shown to be present in RARs but absent in RXRs and its function is as yet unknown.

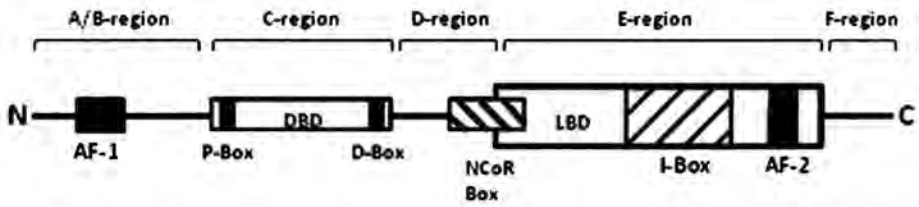


Figure 1.3 Schematic of RAR functional domains. Region A/B contains the ligand-independent activation function (AF-1) and is closest to the N terminal. Region C is the most highly conserved between the receptor subtypes and contains the DNA-binding domain (DBD), along with the P- and D-Box which are involved in DNA recognition and protein dimerisation respectively. The D region is the hinge, allowing correct orientation of the binding domains after the receptor dimers have bound DNA. Region E contains the ligand binding domain (LBD), the main dimerisation surface (I-Box), the ligand-dependent transcriptional activation function (AF-2), and the NCoR Box which is involved in transcriptional silencing in the absence of ligand. F-region is only present in RARs and its function remains unknown.

There are three main types of classical RARE, all of which contain two direct repeats of a core hexameric motif, separated by a specific number of base pairs (bp) [41]. The most common RARE is the direct repeated sequence separated by a 5 bp section, termed DR5. RAR/RXR heterodimers are also able to bind to the direct repeated sequence separated by a 2 bp section (DR2) and a 1 bp section (DR1). RXR/RXR homodimers are only able to bind to DR5 and DR1 classical RAREs [42]. **Table 1.1** lists some of the classical RARE and their associated direct repeated sequence. The polarity of the receptor dimers when they bind to either DR5 or DR2 is the RXR binds to the 5' hexameric motif and RAR binding to the 3' motif, whereas polarity is reversed in DR1 binding, introducing an inhibitory effect in these receptor subtypes [43].

DR	Gene	Sequence	Reference
DR5	hRARβ2	AGGGTTCACCGAAAGTTCACT	De The *et. al.* [44]
DR5	hCYP26	TTAGTTCACCCAAAGTTCATC	Loudig *et. al.* [45]
DR5	hHoxa-1	CAGGTTCACCGAAAGTTCAAG	Dupe *et. al.* [46]
DR5	hHox-d-4	TAAGGTGAAATGCAGGTCACA	Morrison *et. al.* [47]
DR2	mCRBP-I	GTAGGTCAAAAGGTCAGA	Smith *et. al.* [48]
DR2	mCRABP-II	CCAGTTTCACCAGGTCAGG	Durand *et. al.* [49]
DR1	rCRBP-II	ACAGGTCACAGGTCACA	Mangelsdorf *et.al.* [50]

Table 1.1 List of some of the classical RAREs. A RARE is a direct repeat of the hexameric motif spaced by 5 (DR5), 2 (DR2) or 1 (DR1) base pairs. Abreviations: RAR, retinoic acid receptor, CYP: Cytochrome, CRBP: Cellular retinol binding protein, CRABP: Cellular retinoic acid binding protein, h: Human, m: Mouse, r: Rat

It is known that the receptor dimers are able to bind to the target DNA in the absence of any ligand, however the complexes are inactive. This was explained with the discovery of additional proteins that interact with RARs and either inhibit or enhance its transcriptional activity in a ligand-dependent fashion [51] (**Fig. 1.4**). In the absence of ligand, corepressor proteins such as nuclear receptor corepressor (NCoR)-1 and NCoR-2/SMRT (silencing mediator of RAR and thyroid hormone receptor) would be bound to the RAR/RXR heterodimer through the NCoR box. These corepressors recruit other factors including histone deacetylases (HDACs) which are involved in maintaining chromatin in its condensed state, leading to inhibition of transcription[52, 53]. After ligand binding, the corepressor is released and coactivators such as CBP/p300, p300/CBP-associated factor and p160 family members (including RIP140 and SRC) are recruited, binding through a motif containing the sequence LXXLL, where L represents lysine and X represents any amino acid [54]. In turn, these coactivators recruit further proteins which contain histone acetyltransferase (HAT) activity to aid in chromatin decompaction leading to gene transcription [55]. Many of these coactivators interact with the receptor through its AF-2 domain in region E.

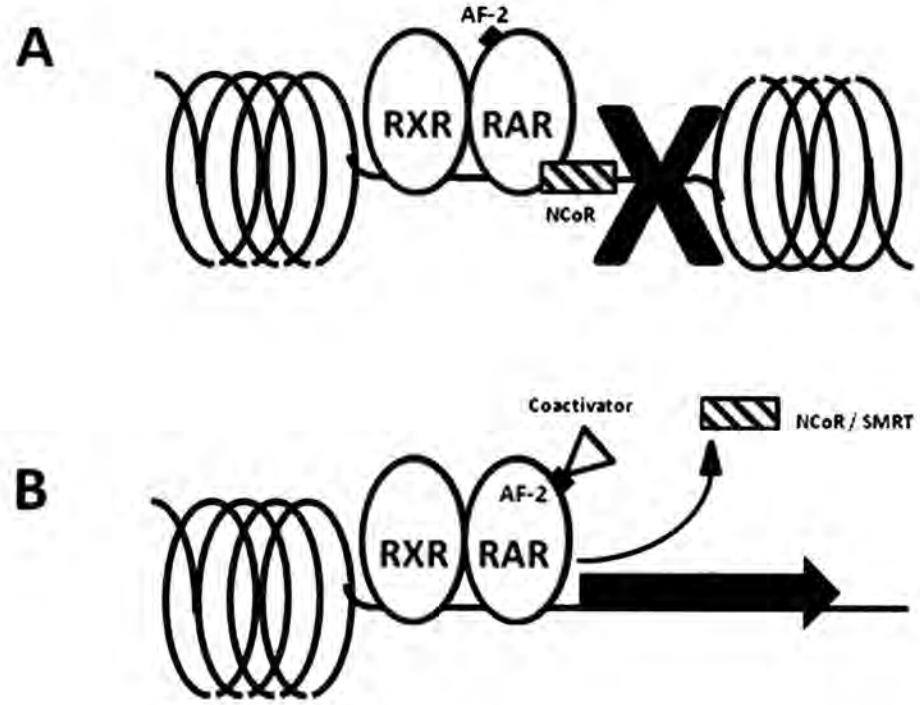


Figure 1.4 Involvement of co-regulatory proteins which either inhibit or enhance receptor activity. (A) In the absence of ligand corepressor proteins (NCoR or SMRT) are bound to the RAR/RXR dimers via the NCoR box domain, leading to inhibition of transcription. (B) Upon ligand binding, the corepressor proteins are released leading to a conformational change exposing the AF-2 site allowing coactivator recruitment, which in turn leads to chromatin decompaction allowing gene transcription.

1.5 Retinoids and CNS Development in the Embryo:

Experiments on retinoid function within the embryonic CNS are extensive (for a recent review see Clagett-Dame and DeLuca, 2002 [56]). ATRA is widely known to have potent teratogenetic effects, and as outlined above, experiments detailing the effects of VAD during pregnancy noted a wide range of birth defects, including several that occurred within the CNS and spinal chord, for example exencephaly and various degrees of spina bifida [57]. Furthermore, when retinoic acid was removed from either quail or rat embryos to generate retinoic acid-deficient embryos, the CNS showed several severe abnormalities, including no neurite outgrowth in the spinal chord [58, 59]. Some of these types of neural tube defects were also witnessed if an excess of retinoic acid was administered [60]. These *in vivo* experiments and those which revealed ATRA's ability to induce neural differentiation of embryonal carcinoma cultures *in vitro,* prompted the

hypothesis that retinoic acid must play an important role in the regulation and development of the developing CNS in the embryo [61]. It was shown that cultures of embryonal carcinoma cells induced to differentiate with ATRA exhibited an increase in expression of the homeobox cluster of genes termed HOX. Predominantly an increase in the 4.2 kB transcript of HOX 5.1 was observed. This particular HOX gene is almost exclusively found in the CNS of the developing human embryo. Therefore this finding supported the notion that ATRA was inducing neural derivatives from embryonal carcinoma stem cells, and is important in CNS development *in vivo* [62]. Backing up these findings were experiments using microinjection of dominant-negative retinoid receptors into *Xenopus* embryos [63, 64]. These were administered to block retinoic acid receptor pathway signalling. It was reported that this inhibition lead to an expansion of anterior structures located in the CNS, and a decrease in posterior markers. The differentiation of sensory, motor, and interneurons was also inhibited. Finally, one of the first genes to be classified as a retinoid target gene was RAR-β2 [44], and it was found to be regulated in neural cells and tissues [65]. RAR-β mRNA expression is limited to the posterior hindbrain and spinal chord during early CNS development, however following exposure of the embryo to retinoic acid, the expression profile is expanded to the whole neuroectoderm [66], outlining the importance of this receptor sub-type during neurogenesis.

CNS development is initiated during gastrulation, briefly; the embryo divides into three distinct layers, namely the endoderm, mesoderm and ectoderm. The ectoderm gives rise to the outer epithelium of the body and the neural tube and neural crest. Neural tube and neural crest development is instigated by the release of specific growth factors, such as chordin, follistatin and noggin from the Spemann organiser [67]. These molecules bind to bone morphogenetic proteins (BMPs) inhibiting their function which, when active, bind to nuclear receptors and activate genes specific for epidermal differentiation. The inhibition of these BMPs blocks epidermal differentiation and promotes neural differentiation; this process is termed the 'default model' of neurogenesis [68]. Initially, as the neural plate forms, after signalling from the organiser, it forms cells which are associated with the forebrain only. It is only after the mesodermal transformation signal that this anterior neural tissue is converted into the more posterior cell types representing the midbrain, hindbrain and spinal chord respectively, which can be identified by the expression of specific groups of markers [57]. It was hypothesised that at least one of the

transformational signals released from the paraxial mesoderm must be ATRA, which was known to induce neuronal phenotypes.

Multiple experiments have been carried out verifying the presence of retinoids in the developing CNS of embryos. High-performance liquid chromatography (HPLC) was carried out on mouse embryo extracts at day 10.5 and day 13 and proved the presence of ATRA in the CNS. Endogenous ATRA was mainly found in the spinal cord, with much lower and decreasing amounts found in the hindbrain, midbrain and forebrain respectively [69]. However, ATRA cannot be synthesised by the neural tube itself at these time points, as it contains no RALDH to catalyse its formation from retinal. RALDH activity is, however, found in the neighbouring paraxial mesoderm, and may enter the neural tube via passive diffusion. It is thought that ATRA may act in a paracrine fashion, patterning the CNS axis via pathways activated at specific positions along the ATRA concentration gradient [57].

Throughout spinal chord development, ATRA expression becomes less uniform and localises where the limb buds form and where motor neurons are concentrated that service the limbs [70]. It has been discovered that the general positional orientation of the spinal chord is instigated in a concentration dependent manner by FGF and then ATRA refines the general patterning by inducing the expression of specific HOX genes associated with motor neurons, namely the HOXC genes [71]. These HOXC genes form expression 'hotspots' which become the sites that go on to form the limbs.

1.6 Retinoids and the Adult CNS:

Much of the early research into retinoid modes of action focussed on the role of retinoids during embryonic neural development. This was due to the fact that ATRA is known to induce and regulate differentiation in many cell types, not a role which was thought to be required in the adult neural system. However, since the discovery that discrete areas of proliferative cells are present in the adult brain, the study of retinoids has revealed an important regulatory role of these compounds within the adult CNS. The ability of retinol to cross the blood/brain barrier was demonstrated in studies which localised CRBP and

RBP binding sites in the blood capillary endothelium and in the epithelial cells of the choroid plexus respectively [72]. The presence of several members of the RALDH enzyme family within the adult brain itself also indicated its ability to synthesis the active retinoid ATRA [73, 74]. It was demonstrated that specific areas of the brain synthesised ATRA, namely the basal ganglia, olfactory bulbs, hippocampus and auditory afferents, all areas that are known to undergo active remodelling of neural connections throughout adulthood. *In-situ* hybridisation studies showed a close correlation between this apparent ability to synthesise ATRA and the presence of CRBP-I, the compound that facilitates the conversion of retinol into active ATRA [75]. Further studies used radio-labelled retinol administered to VAD rats. Ten hours post injection, brain areas were harvested and radioactivity detected. The highest amount of activity was found in the hippocampus-cortex with the lowest being detected in the cerebellum. The radio-label tag was found to be connected to ATRA not retinol, again indicating the ability of cells in the adult CNS to convert retinol to ATRA [76]. The high levels of ATRA and associated proteins in the hippocampus, specifically the dentate gyrus, strongly implied the involvement of these molecules in region-specific neurogenesis. It was revealed that ATRA contributed significantly to neuronal differentiation within the dentate gyrus, being involved at a very early stage throughout this process [77]. Areas which are non-responsive, therefore sensitive to ATRA in the brain, have been shown to express specific enzymes, such as the cytochrome P450 enzymes P450RA-1 and P450RA-2, which inactivate ATRA by metabolizing the compound to more polar metabolites, including 4-oxo-, 4-hydroxy-, (4-OH-) and 18-hydroxy- (18-OH-) ATRA. These enzymes have been localised to the pons and the cerebellum, areas known to be unresponsive to ATRA stimulation [78, 79]. These experiments all indicate that ATRA is an important regulatory molecule within the adult CNS and interference of these pathways can result in neurological disorders. More evidence indicating the regulatory role of retinoids in the adult CNS is discussed below.

1.7 Retinoids and the Hippocampus (Involvement in Learning and Memory).

As mentioned above, the hippocampus is an area of the adult brain with known neuronal plasticity. This remodelling of neural connections is the underlying process involved in episodic, declarative and spatial learning and memory establishment. Retinoic acid has

been found to play a regulatory role in the long term potentiation (LTP) and long term depression (LTD) processes involved in this neuronal plasticity within the hippocampus [80]. Investigations into VAD revealed a significant deficit in spatial learning and memory in rats deprived of vitamin A. As little as 12 weeks of dietary deficiency was enough to induce these deficits, which upon re-introduction of vitamin A into the diet were reversed [81]. Other studies have shown a more permanent reduction in the expression of RAR-β and RARβ/γ receptor subunits after 31 weeks deficiency [82]. These studies all support the theory that retinoids are involved in the signalling pathways leading to higher cognitive functions.

1.8 Retinoids in Neurological Disorders (Schizophrenia, Alzheimer's and Motor Neuron Disease).

The retinoic pathway is becoming a key focus in many studies looking at neurological disorders. As with the embryonic regulation of the retinoid signalling pathway, any deviation from optimal levels appears to induce neurological dysfunctions. Recent papers have linked the addition of retinoic acid to the alleviation of oxidative stress-induced neuronal death in specific areas of the adult brain, namely those involved in cognition and conditions such as depression, Alzheimer's and Parkinson's disease [83-86]. These data and those discussed below, indicate the importance of this particular pathway in optimal brain management, and hint at future therapeutic remedies in the control or treatment of this group of disorders.

1.8.1 Schizophrenia.

One such area where retinoic acid dysregulation appears to be involved in the diseased state is schizophrenia. The learning and memory pathways of the hippocampus are selectively impaired in schizophrenia, so it would stand to reason that retinoic acid pathway abnormalities could be involved in this disease. Many reports eluded to retinoid dysfunction as one of a number of causative factors for schizophrenia (for a review see Goodman, 1998 [87]). More recent experiments show concrete evidence of this effect. A decrease in RXR signalling is seen in some schizophrenia patients [88] and the administration of natural RXR agonists such as Docosahexaenoic acid (DHA) can

overcome some symptoms [89]. Comparisons of normal and schizophrenia brain section expression of RARs indicated a two-fold increase of RAR-α in the dentate gyrus of the schizophrenia sections [90]. Proteomic evaluation of body fluids from schizophrenia patients versus mentally healthy controls indicated a significant down-regulation of transthyretin (TTR) and apolipoprotein E (ApoE) in the cerebrospinal fluid. Both proteins are associated with the transport of retinol around the body and brain, therefore an important step leading to correct retinoid functioning [91]. Potentially, data presented in these studies could signify that an insufficient amount of retinoids are being transported around the brain leading to the abnormal expression levels of retinoid signalling components. It was also noted that, after 2 months treatment with antipsychotic drugs, the plasma expression of TTR tetramer was significantly increased in those patients that were responding to the treatment, indicating that these aberrant expression levels could be rescued [91]. It can be concluded that the synthesis of specific retinoid compounds/retinoid transporter molecules could be advantageous in the treatment of this disorder and this is discussed in more detail below.

1.8.2 Alzheimer's Disease.

Retinoids have also been implicated in Alzheimer's disease (AD), another disease associated with cognitive abnormalities. Although, to date, no specific genes have been linked to AD, genome scans have indicated strong gene linkages, and at these specific loci important genes related to the retinoid pathway are always found [92]. As in the schizophrenia research, some of these retinoid genes have been identified as playing a role in the transport of retinoids [92]. Again, if these transporters are not functioning adequately, or are found in decreased levels, this has the potential to have serious implications for optimal retinoid functioning. Disruption of the retinoid signalling pathway also appears to increase the amount of amyloid β protein in the brain, one of the major components involved in amyloid plaque formation, a major characteristic of AD. In one year old rats that have been VAD since weaning, positive expression for amyloid β protein is seen in the cerebral cortex, compared to negative results in corresponding control samples [93]. In similar *in vivo* deficiency models, an increase in amyloid precursor protein is seen with a decrease in the expression of the retinoid receptor RAR-β [94], which can be rescued upon re-introduction of vitamin A into the diet. This is consistent with *in vitro* observations that a high level of retinoic acid is able to protect against the death of

hippocampal neurons mediated by amyloid β, another characteristic of AD [95]. All of these studies again indicate the therapeutic potential of retinoids in the control or possible treatment of this disease, however it must be noted that the effects witnessed after retinoid supplementation may not necessarily be mediated via receptor activation, and could be non-receptor mediated.

1.8.3 Motor Neuron Disease.

There are many other neurological disorders which the symptoms are associated with a dysregulation of the retinoid signalling pathway. Another example is motor neuron disease. In this disease, an accumulation of neurofilament and vacuolations in motor neurons followed by motor neuron cell death is characteristic, alongside an increase in astrocytosis. In VAD rat models, these characteristics were noted in samples of motor neurons from the spinal cord, along with a decrease in expression of RAR-α [96]. This study also examined expression patterns in human patients of amyotrophic lateral sclerosis (ALS). They showed a decrease in the total number of RAR-α positive neurons and a decrease in RALDH-2 positive neurons compared to control samples, and in the motor neurons that were left, there was a decrease in expression of both of these transcripts. Other studies examined whether there was a difference in serum levels of retinol between ALS patients and age-matched healthy controls. Using HPLC, the authors concluded that there was no significant difference between the healthy and diseased groups [97]. When combined, these studies lead to the conclusions that motor neuron disease is in part a consequence of retinoid signalling malfunctions and not in general due to a deficiency of vitamin A. Again, it appears that the transport of the retinoid is defective, leading to an aberrant expression of the receptors involved. It is yet to be established whether motor neuron cell death can be rescued via some form of retinoid therapy.

1.9 Novel Retinoid Compounds as a Therapy for Neurological Diseases and Cancer.

As alluded to above, there is evidence that in some neurological disorders, at least in *in vitro* experiments, symptoms associated with retinoid dysfunction can be overcome with

retinoid therapy. The natural retinoids ATRA, (**Fig. 1.5a**), 13-cis-retinoic acid (13-cisRA), (**Fig. 1.5b**) and 9-cisRA, (**Fig. 1.5c**) are already used in some therapy regimes for some cancers and for some dermatological diseases such as acne. For example, ATRA is used with relative success in the treatment of acute promyelocytic leukaemia due to its ability to induce differentiation of the aberrant cell population [98]. However, due to its rapid metabolism and catabolism by the cytochrome P450 enzymes within the body, and serious side-effects after long-term treatment, it is a far from ideal compound. Accutane (containing 13-cisRA) is used for the treatment of acne. 13-cisRA has been shown to remain in the circulation much longer than ATRA in human subjects, with an elimination half-life of 20hrs compared to just 0.9hrs for ATRA [99]. This makes it a more attractive therapeutic compound than ATRA, however, its use is the subject of serious controversy (summarised in Strahan and Raimer, 2006 [100] and references therein). Some scientific groups have proposed a link between the chronic use of this compound and depression and suicidal tendencies, due to a decrease in hippocampal cell formation (see, for example, Crandall *et al.* 2004 [101]). However, other groups disagree with these findings (see, for example, Jick *et al.* 2000 [102]).

Both of these compounds are pan-RAR activators, and therefore, have the potential to effect numerous different biological processes which could explain the associated debilitating side effects. For example, activation of the RAR-γ receptor sub-types is associated with retinoid skin toxicity side-effects [103]. Much research has focussed on the synthesis of specific and selective RAR agonists and antagonists to use in the treatment of many neurological disorders. The rationale is that these more selective activators of retinoid pathways could overcome the side effects associated with the use of their natural pan-RAR activating counterparts. Whilst there is reference to the chemical production of many thousands of different synthetic retinoids in the literature, there are comparatively few data concerning an in-depth characterisation of their biological activity, let alone their effect on neural cells. One promising study which does show good evidence of biological activity was reported in 2005 [104]. Simoni *et al.* synthesised a number of terphenyl compounds, synthetic retro-retinoid analogues, to try to establish their apoptotic qualities. They discovered that these compounds actually protected promyelocytic HL-60 cells against apoptosis. As many neurological diseases are associated with apoptosis of neurons, i.e. AD, Parkinson's disease, Huntington's disease

and motor neuron disease to name a few, the group became interested in this quality. However, they found that the compounds only had modest protective apoptotic activity in neuronal cell lines, but concluded that further study into this group of compounds could be very worthwhile for treatment of neurological disorders.

Another synthetic retinoid which has promising therapeutic potential for a number of different neurological disorders is Am80 (tamibarotene, (**Fig. 1.5d**) [105]). This synthetic retinoid has been shown to rescue scopolamine induced memory-loss in rat model systems [106]: the most effective result was observed when the retinoid was administered in tandem with the rexinoid HX630. In this model system no adverse side effects was seen. These data point to the use of these compounds to treat neurological disorders associated with learning deficits such as AD. Human clinical trials of Am80 have already been carried out in other fields, investigating Am80 as a possible treatment for APL. As mentioned above, ATRA is the main drug of choice for this disease; however, in patients who relapse from complete remission after ATRA therapy, a second course of this drug is relatively ineffective. In one study, patients who had relapsed were treated with Am80 and 58% achieved complete remission. As Am80 is an agonist for only RAR-α and RAR-β, milder side effects were observed with this compound [107]. Again, this established specificity can be seen as advantageous in the treatment of many neurological disorders.

More specific agonists of the RARs have also been developed. Using their novel cell-based assay R-SAT, Piu *et al.* identified a variety of selective RAR compounds [108], the most interesting being a potent RAR-β2 agonist, (**Fig. 1.5e**), that distinguishes between the different RAR-β sub-types [109]. This particular sub-type has been shown to be very important in the neurite out-growth of the embryonic spinal cord, and is absent in adult spinal cord sections. When it was transfected into neurons of the spinal cord from adult rats and mice, neurite outgrowth was observed *in vitro* [110]. This receptor sub-type has also been associated with the dopamine signalling pathway [111]. Dysregulation of this pathway is seen in some neurological disorders, for example Parkinson's disease and schizophrenia; therefore, this agonist may have potential utility as a treatment for these types of neurological disorders.

The synthesis of molecules with higher cytotoxic activity on cancer cells and increased water solubility improves their ability to act as chemotherapeutic and chemopreventative agents. Novel synthesis approaches and biological evaluations of the products are producing compounds which appear to be exciting new anti-cancer drugs [112].

Other areas of retinoid research have focussed on the development of compounds which will enhance the therapeutic potential of already available retinoid compounds. For example, in order to overcome the rapid breakdown and sensitizing of ATRA by the body after use as a therapeutic treatment, retinoic acid metabolism blocking agents (RAMBAs) have been developed. These compounds elicit a response by inhibiting the P450 enzymes, namely CYP enzymes, which catabolises ATRA (for a recent review see Njar *et al.* 2006 [113]). The most studied RAMBA is Liarozole, (**Fig. 1.5f**), developed by the Janssen Research Foundation [114]. In 2004, Liarozole was granted orphan drug designation by the U.S. Food and Drug Administration for the treatment of congenital ichthyosis (a severe dermatological disorder) and has also been granted orphan drug status by the European Commission. This is a good example of how manipulation of the retinoic acid signalling pathway can be key to the treatment of certain related diseases.

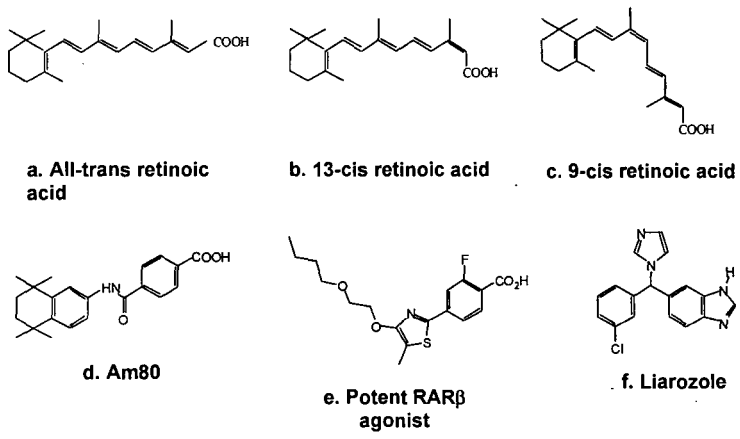


Figure 1.5: Chemical structures of retinoids (a-e) and a retinoic acid metabolism blocking agents (RAMBAs) compound (f) which are either already being used in therapy regimes for neurological disorders (a, b, c, d & f) or show promising potential as therapeutic agents (e). a, b, & c are naturally occurring retinoids and d, e, & f are synthetic compounds. Diagram taken from Christie et al 2008[123].

1.10 Aim of Thesis:

Instability of ATRA *in vitro* and the Need for Stable Alternatives

As it has been outlined above, ATRA is a very important molecule *in vivo,* being involved in numerous regulatory processes throughout both embryonic and adult neural development. Due to its prominent involvement during these *in vivo* processes, ATRA has been used extensively to promote neurogenesis *in vitro* in many different cell model systems. It has been this *in vitro* use of ATRA which has lead to many of the findings discussed above. However, the *in vitro* use of ATRA is complicated by its structural instability, leading to a tendency to isomerise and degrade when exposed to light or heat [115-118]. The five conjugated double bonds which give ATRA its excellent chomophore properties, efficiently absorbing light in the region of 300-400 nm, are what also make this compound susceptible to photoisomerisation. This leads to degradation of ATRA into a mixture of several different retinoic acid isomers and degradation products. Previous studies have shown that during the preparation and maintenance of cell cultures with ATRA, the concentration levels have been shown to decrease markedly over time, and this appears to be both a consequence of its degradation and metabolism [119]. Therefore, compound stability is an important feature when reagents are being routinely used for cell culture in *in vitro* applications, one which is often overlooked by cell-biologists. There is an unavoidable variation in actual ATRA concentration being applied throughout investigations, dependant on how often or how long ATRA has been thawed out and/or exposed to light when in use. As explained earlier, very defined concentrations of ATRA are required to induce specific neuronal phenotypes, therefore any discrepancy in concentration accuracy can potentially elicit dramatically different biological results. It is also known that some of the degradation products of isomerised ATRA also elicit differing biological activities, adding to the problems associated with *in vitro* ATRA use. For example these different isomers effect the ability of embryonic stem cells to differentiate into defined neuronal products [119, 120]. These problems have prompted efforts to create more stable synthetic ATRA analogues which are able to elicit similar biological effects. In essence, retinoids contain three structurally important regions, a hydrophobic section, a variable linker, and an acidic function respectively. Extensive chemical modifications to these three separate regions are possible, some of which are able to instil extra chemical stability. For example, the incorporation of an aromatic ring

to replace part of the polyene chain increases stability, whilst the compound still retains the ability to induce differentiation of embryonal carcinoma cells. This new group of retinoids is termed arotinoids [121]. As with the creation of synthetic retinoids for therapeutic use, many retinoid libraries have been formed and published, with their specific receptor binding affinities calculated, however, very few of these retinoid analogues have undergone sufficient biological analysis and screening to be verified biologically (For example [122]). These libraries of synthetic retinoids make a solid starting point in the hunt for a more stable ATRA alternative which would have the potential to replace ATRA in *in vitro* investigations. This approach is likely to yield successful candidates. Therefore, to try and address this issue, the aim of this thesis is to focus on the synthesis and subsequent biological analysis of two arotinoid compounds, termed EC19 and EC23, which are hypothesised to have inferred stability compared to ATRA.

1.11 Synthesis of retinoid analogues EC19 and EC23

To address the stability issues outlined above with the *in vitro* use of ATRA, in collaboration with scientists from the Chemistry Department, Durham University, this thesis aims to investigate the biological activity of synthetic retinoids on a variety of cell model systems. Specifically looking at their ability to induce neurogenesis compared to the natural retinoid ATRA. As outlined above ATRA is a very important regulatory molecule during neurogenesis in both embryonic neural development and adult neural re-modelling and development. Using available literature, and chemical modelling programmes, two isometric analogues of ATRA were synthesised with the aim of improving retinoid stability when handled under standard laboratory conditions. One compound analysed was a known synthetic retinoid which has been shown to activate all of the retinoic acid receptors in previous binding studies [122]. The other compound was novel, and its structure was based on that of the previous compound. These two synthetic retinoids were then put forward for biological analysis on a range of cell-based model systems to test their biological reactivity, and these were compared against the natural retinoid ATRA [123]. Models of embryonic stem cells and embryonic and adult neural progenitor cells were used as ATRA is known to influence the differentiation of all of these different cell types *in vitro*. It was therefore important to asses the ability of the

synthetic retinoids to induce differentiation in all of these cell types too. Differentiation was assessed using a number of different cellular and molecular techniques including evaluation of cell viability, cell markers of differentiation and 'stemness', followed by proteomic and molecular analysis of markers of the retinoic acid receptor pathway. A model of a neural childhood tumour, which responds in part to ATRA treatment, was also evaluated as it was also important to address whether the synthetic compounds may have enhanced therapeutic potential. To evaluate these properties analysis of cell cycle markers and proliferation analysis was carried out.

To create the new synthetic retinoids many modifications to the ATRA common retinoid structure were made. As mentioned above, to instil stability, an aromatic ring was incorporated, replacing some of the long polyene carbon chain. A triple carbon bond was also added to incorporate stability along the carbon linker region, and finally in place of the trimethylcyclohexenyl ring and part of the polyene chain a 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronapthalene unit was incorporated. It was anticipated that an acetylene moiety would induce the desired linear structure, and also act as a non-isomerisable linker unit. Due to this non-isomerisable quality, it was decided that two isomers of this compound would be synthesised and analysed, whereby the position of the carboxylic acid function was placed in either the *para-* or *meta-* position. The *para-* isomer was termed EC23, and the *meta-* isomer was termed EC19, and their structure and chemical alignment are shown in **Figure 1.6.**

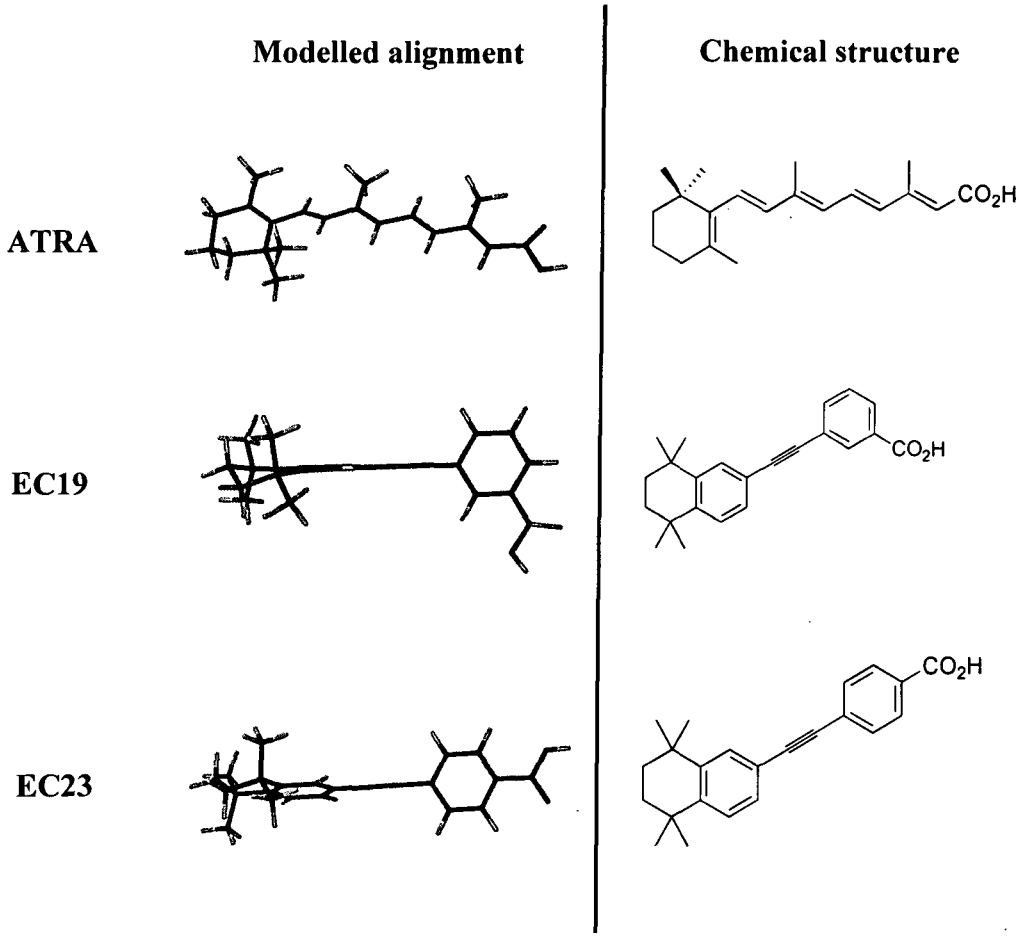


Figure 1.6: Structure and chemical alignment of ATRA and the synthetic retinoids EC19 and EC23. It is noted that the chemical alignment of EC23 is most similar to the natural retinoid ATRA. Both EC19 and EC23 have a triple carbon bond inserted into the polyene chain. This is hypothesised to instil stability to these synthetic analogues.

To synthesise the retinoid analogues EC19 and EC23, in brief, tetramethyltetrahydronaphthalene units underwent Sonagashira cross-coupling with trimethylsilylacetylene (TMSA), this reaction was enhanced with the addition of an iodide group to the starting molecule (71% yield), and allowed for lower levels of palladium to be used as the catalyst for the Sonagashira cross-coupling, i.e. with iodide the coupling with TMSA under Sonogashira conditions at room temperature, only required 1% Pd catalyst, resulting in an 88% yield of the product. Subsequent deprotection of the linker region was carried out with hydroxide, giving the terminal alkyne product. The terminal alkyne was then subjected to a further Sonogashira coupling with both *para*- and *meta*-iodobenzoate methyl esters to provide smooth conversion to each of the isomeric esters respectively in 87% and 59% yield. Finally, saponification of

the methyl esters produced the carboxylic acids EC23 and EC19 in 72 and 78% yields, for the *para*- and *meta*-isomers respectively (Scheme outlined in **Fig. 1.7**).

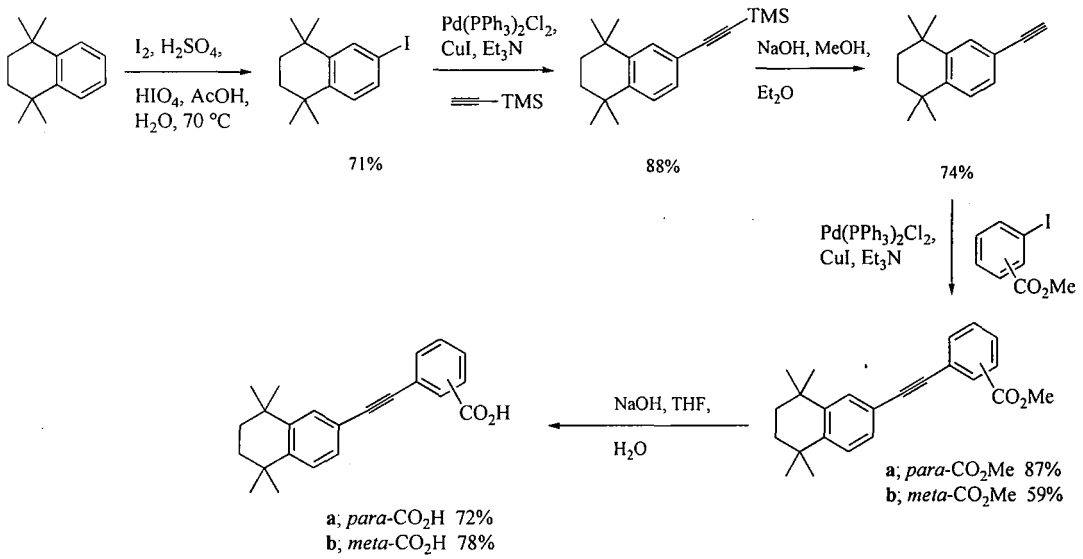


Figure 1.7: Scheme of synthesis of synthetic retinoids EC19 and EC23. The starting material underwent a Sonagashira coupling with TMSA, followed by a deprotection step followed by a final Shonagashira coupling to obtain the terminal alkyne in both the *para*- and *meta*- isoforms. Subsequent saponification of the methyl esters resulted in the carboxylic acid isomers. Diagram adapted from Christie et al 2008[123].

1.12 Conclusions

Retinoids have been demonstrated to be powerful regulators of neural differentiation and regulation both within embryonic development and adult brain functioning. They elicit biological activity by binding to specific receptors located within the nuclear envelope. Activation of these receptors results in a mediation of specific gene expression. A dysfunction in this pathway leads to many abnormalities in embryonic development and is shown to be associated with numerous neurological disorders within the adult. The natural retinoids are involved in the treatment programmes for many different disorders; however, their utility is limited due to the rapid breakdown of the compounds within the body and debilitating side effects associated with their long-term use. To overcome these problems, novel retinoid compounds have been synthesised which are much more receptor-specific. The limited biological activity data available suggests that these compounds are as potent as their natural counterparts, but it remains to be seen whether they over-come the side effects.

It appears that the dysregulation of the retinoid pathway in the neurological disorders discussed above often results from a defect in the transport of retinol into the cells in question. It would be of interest to investigate synthesising more efficient novel retinoid transporter molecules, which could lead to an increase in retinoid availability. This approach could provide an improved method of treatment/management for these debilitating diseases.

The natural retinoid ATRA is used extensively for *in vitro* work, for example as an agent to induce neurogenesis in many different cell-based model systems. However, due to its chemical structure ATRA is very unstable when exposed to light or heat, isomerising and degrading readily. This has obvious problematic implications associated with its *in vitro* use. One area of science which is trying to over-come these issues is the synthesis of retinoid analogues which elicit the same biological responses to ATRA. In collaboration with the Chemistry Department, Durham University, this thesis aims to evaluate the biological activity of two synthetic retinoids EC19 and EC23. Their ability to induce neuronal differentiation will be studied and compared to the natural retinoid ATRA. It

was hoped that these synthetic analogues would elicit similar effects to ATRA in the range of cell model systems tested, but that they would be able to withstand a much higher level of light and heat exposure, making them an attractive alternative for *in vitro* investigations into retinoid biology.

2 Chapter 2: Materials and Methods:

2.1 Cell Culture:

All cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C in a Sanyo CO_2 incubator. Cell cultures were handled using aseptic techniques in a class II bio-safety flowhood (BioMat). Cultures incubated with retinoid compounds were done so in reduced light conditions. All tissue culture plastic-ware was purchased from Nunc unless otherwise stated. Phase contrast images of growing cultures were obtained using a light microscope (Nikon Diaphot 300) and photomicrographs captured using digital photography (Nikon). Media recipes and specific culture techniques are found in individual Chapters.

2.2 Retinoid Stocks:

Stock solutions of synthetic retinoid compounds EC19 and EC23 (Durham University Chemistry Department), ATRA and 13-cis-Retinoic acid (Sigma), were prepared in dimethylsuloxide (DMSO) (Sigma) to concentrations of 10mM. Aliquotted stock solutions were stored at -80°C in the dark.

EC19 and EC23 molecules obtained from the Chemistry department both had molecular weights of 332, therefore stock solutions were worked out as follows:

1M = 332g/litre

10mM = 3.32g/litre

3.32mg/ml

33.2mg/10ml DMSO

2.3 MTS Cell Viability Assay:

The MTS cell viability assay worked via a colorimetric change that can be quantified on a spectrometer. MTS is a yellow tetrazolium-based dye that is reduced by live (and not dead) cells to a purple formazan compound. Cells were cultured on 24-well plates for MTS cytotoxicity assay analysis. 50,000 cells/well were seeded and either cultured in proliferation media (control), or differentiation media containing one of the retinoids being investigated. Cells were cultured for up to 14 days and at 3, 7 and 14 day time

points the assay was carried out. 200µl of MTS reagent (CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay, Promega) was added to 1ml media/well. A control well containing no cells was also set up in the same way. The solutions were triturated then incubated for 4 hours at 37°C. The resulting colorimetric change was recorded as an absorbance, read at 490nm on a spectrometer (Thermospectronic, Fisher Scientific). All results were carried out in at least triplicate and were standardized against the no-cell control to account for background absorbance.

2.4 Flow Cytometry:

Flow cytometric analysis was carried out on either live cells using cell surface markers, or fixed cells using intra-cellular markers. A single suspension of cells or their differentiated derivatives was formed by the addition of 1ml 0.25% trypsin/EDTA solution (Cambrex), and cell numbers were determined using a haemocytometer (500,000/antibody). For live staining cells were taken straight through the staining protocol outlined below. To fix cells they were incubated in 0.02% paraformaldehyde (PFA) solution (PFA in PBS (Sigma)), for 24hrs, then washed in PBS. Prior to staining, fixed cells were permeablised by incubation with 2% Triton-X-100 (in PBS, Sigma) at 37°C for 30 minutes.

Staining protocol: Suspended cells were pelleted and re-suspended in wash buffer (PBS containing 0.1% bovine serum albumin (Sigma)). To an appropriate number of wells in a 96 well plate $0.5x10^6$ cells were added in a 200µL volume. Plates were lightly centrifuged and the supernatant removed. Each cell pellet was re-suspended in 50µl of primary antibody solution followed by incubation for 1 hour at 4°C, after which the antibody was removed by 3 washes in wash buffer. Cells were then suspended in 50µl of FITC-conjugated secondary antibody (Cappell, 1:100) and incubated in the dark for 30 minutes, at 4 °C. Secondary antibody was removed by a further 3 washes in wash buffer. Finally, cell pellets were re-suspended in 500µl of wash buffer and samples were analysed in the flow cytometer (BD Biosystems FACSFlow). Thresholds determining the number of positively expressing cells were set against the negative control antibody P3X (used neat, gift from Prof. P. Andrews, University of Sheffield). Cytometric analysis was carried out on three individual cultures per treatment of cultured cells.

2.5 Cell cycle analysis:

Cell cycle analysis was carried out using the CycleTEST™ PLUS DNA Reagent kit from Becton Dickinson. Protocols were carried out as directed in the instruction manual. In brief, a single cell suspension was gained after incubation with 0.25% trypsin/EDTA solution and washed 3X in the provided buffer solution. Cell number was then adjusted to 500,000 cells/test in buffer solution. Samples were centrifuged at 400g for 5 minutes at room temperature (RT) and the supernatant is removed. 250µL of solution A (trypsin solution to lyse cytoplasm) was added to samples and mixed by gentle tapping then left for 10 minutes at RT. 200µL solution B (trypsin inhibitor plus RNase buffer) was added to each sample and incubated for a further 10 minutes. Finally, 200µL of cold solution C (propidium iodide stain) was added, and resulting solution was mixed then incubated on ice in the dark for 10 minutes. Samples were then filtered through 50µm nylon mesh and run using the appropriate settings on a flow cytometer (BD Biosystems FACSFlow). Cytometric analysis was carried out on three separate experimental culture samples for reproducibility.

2.6 Immunocytochemistry:

Cells were cultured on 12-well or 24-well plates for immunocytochemical analysis. Undifferentiated cultures were fixed PFA (Sigma) when confluent as outlined below, and differentiated cultures were fixed after the specified time point of growth in the presence of the respective retinoid compounds. Cell fixing was performed by incubation with ice-cold 4% PFA in PBS (Sigma) for 30 minutes at RT, followed by 3 washes in PBS, and storage in the final wash solution at 4°C until required. Cell membranes were permeablised by treatment with 1% Triton-X-100 (Sigma) in PBS for 10 minutes at RT. Non-specific binding of antibodies was blocked using a solution of 1% goat serum (Sigma) in PBS containing 0.2% Tween-20 (Sigma). Fixed cells were incubated with the blocking solution on a bench-top shaker (Fisher Scientific) for 30 minutes at RT. Dilution of primary antibodies was carried out using blocking solution, and incubated with the cells for 1 hour at RT (see individual chapters for specific antibody information). Cells were then washed 3 times for 15 minutes in PBS followed by one hour's incubation in the dark at RT with appropriately diluted FITC or Cy3-conjugated secondary antibody (Alexafluor 488 1:600 or JacksonLabs 1:600). A further 2 washes of PBS was carried out in the dark, then cells were incubated with 1µg/ml Hoechst 33342 nuclear staining dye

(Molecular Probes, diluted in PBS). Cultures were then washed an extra 2 times in PBS, left in the final wash and imaged straight away. Fixed and immunostained cultures were examined using a fluorescent microscope under restricted light conditions (Nikon Diaphot 300). DAPI, and the corresponding fluorescent photomicrographs were collated and stored using the appropriate filter sets and an adapted digital camera (Nikon). Imaged wells were then stored for further analysis with preservative mountant. Cultures were covered with a glass cover-slip (13 mm, VWR) over a small drop of Vectashield mountant, (Vector Labs) and stored at 4°C. Triplicate experiments were carried out for reproducibility.

2.7 Phalloidin immunostaining:

Cells were PFA fixed as outlined above, then permeablised and blocked also according to the methods outlined above, and then cultures were incubated with TRITC-phalloidin (Sigma) for 30 mins at RT in the dark. For a 12-well plate 2 units of active solution in 200µl PBS was required (1 unit = 5µl). Cells were washed 6X for 10 mins in PBS, then mounted ready for visualisation as above.

2.8 Bromodeoxyuridine (BrdU) Immunostaining:

Stock BrdU Solution: Stock solutions were made by dissolving 10mM BrdU (Sigma) in PBS. Solutions were sterilised by filtration (f/s) through a 0.2um filter and were store at -20°C.

BrdU labelling solution: BrdU stock solution was diluted 1/100 in culture medium to yield a 100µM solution (10X). The 10X solution was added to cultures to give a final concentration of 10 µM.

DNA denaturing solutions:

2N HCl: 83.33ml conc. HCl (pure HCl is 11.6M)
Bring up to 500ml in dH20

Borate buffer: Borax solution from Sigma is used to neutralise the acid solution.

BrdU Pulsing: 24hrs before time point (i.e. on day 2 before 3 day time point), 10µM BrdU solution was added to cells and left for 24hrs (i.e. 200uL of BrdU labelling solution is added to 2ml media). The BrdU WAS NOT added with fresh media as the renewal of FCS stimulates cell proliferation.

Immunocytochemistry: Cells were cultured on 12-well or 24-well plates for immunocytochemical analysis. Undifferentiated and differentiated cultures were fixed at 3 and 7 day time points in 4% paraformaldehyde (PFA) (Sigma) which was found to be optimal for each of the antigens detected. Cells were incubated with ice-cold PFA (4% in PBS) for 30 mins at RT, then washed 3X in PBS and stored in the final wash solution at 4°C until required. Cells were permeablised by the incubation of cultures with 1% Triton-X-100 (Sigma) in PBS for 10 mins at RT. Cultures were then denatured to expose DNA for BrdU detection by incubation with 2N HCl for 30mins at RT. The acid was then neutralised with a wash of Borax solution (Sigma).

Cultures were then stained us outlined in the above protocol. Primary antibody BrdU (Molecular Probes, 1:30) was diluted in the blocking solution as appropriate and incubated with the cells for 1 hour at RT.

2.9 Protein Extraction:

Protein samples were prepared from undifferentiated cells and their 7 day retinoid-induced derivatives in igepal lysis buffer (1% igepal (Sigma), 50mM Tris-HCl [pH 8.0], 150mM NaCl, 1mM $MgCl_2$, and protease inhibitors (Roche Diagnostics)). After a 30 minute incubation period the resulting protein solutions were clarified by centrifugation at 15000 rpm for 4 minutes, and the supernatant transferred to a fresh tube. Protein concentrations were determined using the standard Bradford based assay and concentrations were adjusted to contain 20µg protein.

2.10 Fractionation of Protein Lysates:

Strong anion exchange fraction was carried out on EC cell lysates using Vivapure Q Mini H strong anion exchange (SAX) spin columns (Sartorius). A single replicate sample aliquot for control untreated and each retinoid treatment containing 200 µg protein was diluted to 50 µl with cell lysis buffer, then diluted further to 400 µl with 25 mM Tris-HCl, pH 9.0. Columns were prepared by adding 400 µl 25 mM Tris-HCl, pH 9.0 followed by centrifugation for 5 minutes at 4,000 *g*. Sample solution was applied followed by centrifugation and the flow-through retained. Two successive washes of 200 µl 25 mM Tris-HCl, pH 9.0 which were pooled and retained. Step elutions were performed using a series of 200 µl volumes of increasing concentrations of NaCl

dissolved in 25 mM Tris-HCl, pH 9.0 (0.1, 0.25, 0.5, 0.75, 1.0 and 1,5 M NaCl). Each eluate was retained in a separate tube.

2.11 SDS-Polyacrylamide Gels Electrophoresis:

Total cell lysate protein samples were prepared for SDS-PAGE using sample loading buffer and reducing agent (NuPage, Invitrogen) and separated using NuPage Novex 4-12 % Bis-Tris pre-cast gels (Invitrogen). Gels were run at 200V for 1hr in the XCell SureLock Mini-Cell tank system (Invitrogen). For total protein visualisation gels were incubated at room temperature in three successive solutions of Coomassie brilliant blue R-250: (i) 10% acetic acid, 25% isopropanol, 2% Coomassie R-250; (ii) 10% acetic acid, 10% isopropanol, 0.25% Coomassie R-250; (iii) 10% acetic acid, 0.25% Coomassie R-250. Preparation of SAX eluates for SDS-PAGE involved overnight precipitation of protein by overnight incubation with four volumes of acetone at room temperature. Precipitates were sedimented by centrifugation and resuspended in 1 x NuPage sample loading buffer and 10% reducing agent (Invitrogen).

2.12 MALDI-TOF Analysis:

Samples were prepared for protein profiling using ZipTip fractionation (C-18, Millipore). For analysis of total lysates aliquots containing 20 µg protein were normalized to a volume of 10 µl using lysis buffer and acidified with TFA (final concentration 1%). ZipTips were prepared with three 10 µl passes of acetonitrile and three 10 µl passes of 0.1% TFA. Sample was applied by pipetting up and down 10 times. The resin was washed by three 10 µl passes of 0.1% TFA before sample elution into a clean tube by pipetting up and down five times with 7 µl 50% acetonitrile, 0.1% TFA. For analysis of SAX eluates, ZipTip desalting was performed directly as outlined.

Mass profiling and biomarker analysis

Aliquots of each ZipTip eluate were mixed with 1 volume of 2% TFA and 1 volume of matrix solution (0.1 M 2,5-dihydroxyacetophenone (Bruker Daltonics) in 75% ethanol and 20 mM dibasic ammonium citrate. Of this mixture 1 µl was spotted onto individual sample target spots on AnchorChip 600 MALDI target plates (Bruker Daltonics). Mass profiling was performed on a Bruker Daltonics Autoflex II MALDI TOF-TOF instrument, operated via Flex Control software. External mass calibration was performed using standard ubiquitin ($[M+H^+]^+$ = 8565.885 m/z) and myoglobin ($[M+H^+]^+$ = 16,952.55 m/z; $[M+2H^+]^{2+}$ = 8476.775 m/z). Linear mode mass spectra were acquired in

the detection range 4-50,000 m/z (510 shots per sample). Raw data were visualised following spectral smoothing and baseline subtraction in FlexAnalysis software (Bruker Daltonics). For biomarker analysis triplicate sample spectra were opened as individual groups in ClinProTools version 2.0 software (Bruker Daltonics). Analysis was performed in the mass range 4,000-20,000 m/z, at a resolution setting of 800. Spectral smoothing was performed using the Savitsky Golay function (width 10 m/z, 1 cycle). Recalibration settings allowed for a 0.2% maximal peak shift, 30% match to calibrant peaks, and exclusion of not recalibratable spectra. Null spectra exclusion was enabled. Peak area calculation was performed using end-point level area calculation, and a signal-to-noise threshold of 5.0 was applied.

2.13 Western Blot:

Total lysates: Protein samples were prepared in sample loading buffer (NuPage, Invitrogen) and run using a NuPage Novex 4-12 % Bis-Tris pre-cast gel (Invitrogen). Samples were run at 200V for 1hr using the XCell SureLock Mini-Cell tank system (Invitrogen). Resolved proteins were immediately blotted (30V, 1hr) onto polyvinylidene difluoride (PVDF) membranes (Amersham Parmacia) using the XCell II Blot Module (Invotrogen).

For immunoblotting, PVDF membranes were blocked in a solution of 5% dried milk powder and 0.2% Tween-20 in TBS (TBSM) for 1hr at RT. After blocking, membranes were incubated for 1hr with the primary antibody, cellular retinoic acid binding protein I (CRABP-I, 1:600, AbCam) diluted in TBSM. Blots were subsequently washed 3 times in TBSM for 10 minutes each, followed by incubation with mouse horseradish peroxidise secondary antibody for a further 1hr (Amersham, 1:1000 in TBSM). A final 3 10-minute washes was performed in TBS before protein-antibody interactions were visualised using chemiluminescence solutions (Santa Cruz). Detection occurred using the Intelligent Dark Box II transilluminator (FujiFilm) and subsequent densitometric analysis of results was performed using FijiImage software (Fuji).

Evidence of equal sample loading was obtained by probing for the ubiquitously expressed protein β-actin (Sigma, 1:1000 in TBSM). In all cases triplicate experiments were carried out for reproducibility.

Fractionated samples: Western analysis was also carried out on protein samples obtained after strong anion exchange fractionation of 50μg total lysate. Methods as described

above, apart from individual fractions were prepared for electrophoresis by acetone precipitation as described in fractionation of protein lysates section.

2.14 Mass Spectrometry Protein Identification:

Gel bands of interest were excised from SDS-PAGE gels, sliced into 1mm cubes and washed with 50 mM NH_4HCO_3 in 50% acetonitrile for 15 minutes. This solution was discarded and gel pieces were covered with a small volume of acetonitrile. Excess acetonitrile was removed and gel pieces were rehydrated by incubation 50 mM NH_4HCO_3 for 15 minutes followed by addition of an equal volume of acetonitrile. The solution was allowed to stand for a further 15 minutes before being discarded. Acetonitrile was added to the gel pieces until they had shrunk and excess acetonitrile discarded. Gel was dried in a vacuum centrifuge followed by incubation in 10 mM DTT in 25 mM NH_4HCO_3 for 45 minutes at 56°C. Samples were cooled and supernatant removed. Alkylation was performed in two 15 minute washes in 55 mM iodioacetamide in 15 mM NH_4HCO_3. Supernatant was removed and gel pieces were dehydrated by washing in acetronitrile followed by vacuum centrifugation. For tryptic digestion samples were covered with trypsin solution (5 ng/µl in 25 mM NH_4HCO_3) and incubated for 30 minutes at 37°C. Sample coverage was maintained by additional 25 mM NH_4HCO_3 if required, followed by overnight incubation at 37°C. Samples were sonicated for 10 minutes and the supernatant was transferred to a clean tube. To the gel pieces an equal volume of 50% acetonitrile, 1% TFA was added. Sonication was repeated followed by further addition 50% acetonitrile, 1% TFA was added. Supernatants were pooled in the clean tube. Peptide samples were prepared for MALDI MS using ZipTips (C-18, Millipore). Samples were eluted in 50% acetonitrile. For MALDI analysis 1 µl sample was mixed with 10 µl matrix solution (0.3 mg/ml α-cyano-4-hydroxycinnamic acid in 2:1 ethanol:acetone), and 1 µl spotted onto AnchorChip 600 µm target plates (Bruker Daltonics).

Mass analysis was performed in a Bruker Daltonics Autoflex II MALDI –TOF/TOF instrument using default settings for peptide analysis in reflectron mode. External mass calibration was performed to within 10 ppm mass accuracy using the Bruker Daltonics Peptide Calibration Standard mixture. Sample spectra were acquired and submitted for

interrogation against human entries in the Swissprot database (100 ppm mass tolerance, variable modification: oxidation of methionine).

Peptides selected for MS/MS fragmentation were re-acquired in LIFT mode. Peptide fragment spectra were accumulated and submitted to the Swissprot database as described above.

2.15 RNA extraction and Real Time PCR:

RNA extraction using Qiagen RNeasy Mini kit: Before 1st time use of kit Buffer RPE must be diluted by adding 4 volumes of ethanol (96-100%).

Prepare DNase 1 stock solution. Dissolve the lyophilized DNase1 in 550 µl of RNase free water provided (to avoid loss of DNase1 inject water into vial with RNase free needle and syringe). Mix gently by inverting the vial, DO NOT VORTEX. Aliquot and store at -20, do not re-freeze thawed aliquots. Can be stored for 6-8weeks at 2-8°C.

Cells grown in a monolayer (no more than 1 x 10^7) are trypsinised (aspirate medium, add 0.25% trypsin solution, deactivate with trypsin inhibitor), solution is transferred to RNase free centrifuge tube and centrifuged at 300g for 5 mins. Supernatant is completely removed.

Cells are disrupted by adding Buffer RLT: Loosen pellet by flicking tube, add 600µl of buffer RLT. Vortex or pipette to mix. Pipette thoroughly to homogenize the lysate (10-20 times). Add 1 volume of 70% ethanol and mix well by pipetting (precipitates may be seen at this stage). Transfer up to 700µl to an RNeasy spin column placed in a 2ml collection tube. Close lid gently and centrifuge for 15 s at 8000g. Discard the flow-through

On column DNase digestion: Add 350µl buffer RW1 to column and centrifuge for 15 s at > 8000g to wash, discard flow-through. Add 10µl DNase1 stock to 70µl buffer RDD. Mix gently by inverting tube and centrifuge briefly to collect solution. Add DNase1 incubation mix directly to the column membrane and incubate on bench top for 15mins. Add 350µl buffer RW1 to column and centrifuge 15 s at >8000g. Discard flow-through.

Wash steps: Add 500µl buffer RPE to column and centrifuge 15 s at >8000g to wash. Discard flow-through. Repeat step but centrifuge for 2 mins.

Collection of RNA: Place the column in a new 1.5ml collection tube. Add 30-50µl RNase-free water directly to spin column membrane. Centrifuge for 1 min at > 8000g to elute RNA. Snap freeze and store at -80.

RNA purification using the Nanodrop: Add 2.1µl RNase free water to sample arm and close arm, click together then reopen and clean with tissue. In window change sample type to RNA 40, lamba to 260, rest leave the same. Add more water to the arm and press blank. Clean arm with tissue. Load 2µl of sample onto arm and close. Click measure clean arm and repeat for remaining samples. Clean with water and leave arm closed. Click Show report. Print data.

Reverse Transcription of RNA with High capacity cDNA r-t kit (Applied Biosystems):

Making the Master Mix:

Allow the components to thaw on ice.

Calculate the volume of components needed to prepare the required number of reactions. Prepare on ice (include additional reactions to account for loss that occurs during reagent transfers)

Component	Volume (uL)/Reaction kit	
	For 1 reaction	X4 (to get 40uL) and X21 (for number of samples plus 1)
10X RT Buffer	2.0	168
25X dNTP Mix (100mM)	0.8	67.2
10X RT Random Primers	2.0	168
MultiScribe Reverse Transcriptase	1.0	84
RNase Inhibitor	1.0	84
Nuclease-free water	3.2	268.8
Total per reaction	10.0	40.0

Place the 2X RT master mix on ice and mix gently.

Preparing the cDNA Reverse Transcription reactions:

Pipette 40uL of the 2X RT master mix into each individual tube.

Pipette 40uL of RNA sample (correct amount of RNA to give 2ug then made up to 40uL with RNase free water) into each tube. Pipette up and down two times to mix.

Briefly centrifuge tubes to spin down contents and eliminate any air bubbles.

Place tubes on ice until ready to load onto thermal cycler.

Programme the thermal cycler using conditions below then run.

	Step 1	Step 2	Step 3	Step 4
Temperature °C	25	37	85	4
Time	10min	120min	5sec	∞

Store samples at -80 until required.

Relative Quantitation using Comparative Ct Real time PCR:

Setting up the Plate:

Want to have between 10-100ng of cDNA per sample. After rt step have 2ug cDNA/80uL, which is 25ng/uL. Will add 2uL of stock giving 50ng cDNA/reaction.

Prepare in eppendorfs a pre-mix for each individual TaqMan Gene Expression Assay (RAR-b, CYP26A1 and GAPDH) containing sufficient reagents for all reactions plus extra for pipetting errors.

Reagent	**1X uL**
TaqMan Gene Expression Assay	1
TaqMan Fast Universal Mastermix	10
Water	7
Total	**18**

Vortex each tube to create a homogenous solution and spin briefly.

Add 18uL of reagent cocktail and 2uL of template cDNA to a 96-well optical plate according to a plate map, also adding a no template control (NTC). Do each sample in triplicate for technical repeats. NB: place plate in cradle so bottom of well isn't contaminated.

Load plate into PCR machine and run a relative quantitation plate assay from software. Run with default universal settings.

Export data into relative quantitation study document to analyse data. Duplicate experiments were analysed for reproducibility.

2.16 Statistical Analysis of Data:

Where appropriate statistical analysis of data was performed using the one-tailed, paired student t-test. Statisitical significance of the finding was determined compared to the undifferentiated control sample unless otherwise stated. Levels of statistical significance are outlined in the individual figure legends. For comparisons between the four sample groups within the proteomics section, statistical significance was determined using ANOVA statistical analysis.

3 Chapter 3: Evaluation of the Biological Activity of Synthetic Retinoids EC19 and EC23 on Embryonic Stem Cell Model Systems.

3.1 Introduction:

This chapter is concerned with the ability of synthetic retinoid compounds EC19 and EC23 to induce neural differentiation in pluripotent stem cell model systems. Their natural counterpart ATRA is a well known modulator of development and cell differentiation and is frequently used to induce neurogenesis in *in-vitro* stem cell model systems (as outlined in Chapter 1). Here we evaluated ATRA vs. synthetic retinoid stem cell neurogenesis ability. Two model systems were analysed, a human embryonal carcinoma (EC) cell line and a human embryonic stem (ES) cell line. The EC cell line is a more robust model to use to follow responses to synthetic retinoid culture and to dissect the retinoic acid receptor pathway. EC cells are a relevant model to establish embryonic cell responses as they have been shown to be the malignant counterpart to ES cells, as outlined below. Once suitable analysis had been obtained, synthetic retinoids which appeared to be suitable alternatives to ATRA were verified in ES cell cultures.

3.1.1 Embryonal carcinoma cells

EC cells are obtained from teratocarcinomas derived from germ cell tumours, most commonly from either dermoid cysts of the ovaries or tumours of the testis. Teratocarcinomas contain both differentiated tissue and undifferentiated epithelial cells, which give rise to the malignant nature of these tumour types [124]. It is these undifferentiated cells that are termed EC cells. The relationship between EC cells and cells found in the embryo was first analysed back in the seventies with the aid of cell surface markers [125]. One of the first of these studies showed that antibodies raised against the F9 murine EC cells were not expressed by any differentiated cells types tested, but were also highly expressed by cells of the inner cell mass of the embryonic blastocyst. Further evidence of the close relationship between EC and ES cells came when studies outlined the ability of murine EC cells to be incorporated into developing murine embryos. Chimeric off-spring were produced containing the EC cells which had differentiated into many different tissue types [126]. It was also shown that the EC component within these chimeric mice was capable of extending to the germ line, bringing the relationship of EC cells to ES cells even closer [127].

One of the first and most well known human EC cell lines is the TERA2 cell line, isolated from a lung metastasis originating from a testicular germ cell tumour [128]. Studies of human EC cells highlighted significant differences to their murine counterpart, namely, the expression of different cell surface markers such as TRA-1-60, stage specific embryonic antigen-3 and -4 (SSEA-3 & -4) but not SSEA-1 [61]. The expression of SSEA-3 and -4 appeared to relate human EC cells not to the cells of the inner cell mass, but to cells from an earlier stage of embryonic development, found in cleavage stage embryos. These cleavage stage embryonic cells and human EC cells both retain the capacity for trophoblastic differentiation, something which murine EC cells rarely do. The original human TERA2 cell line was impure and frequently only contained around 1-2% EC cells, making study of these cells difficult. To overcome this problem numerous cloned lines were derived from the parent culture, all of which showed varying abilities to differentiate upon instruction. One of the most studied and robust cloned lines was created by Andrews et al in 1984 and termed NTERA2 [129]. This clonal line responded to culture with retinoic acid, forming neurons. Another more recent clone which was shown to have enhanced neuronal ability was created in 2001 by immunomagnetic isolation and subsequent single cell selection and culture. This cell line was termed Tera2.sp12 [130], and is the cell line used in this study. Undifferentiated Tera2.sp12 cells retain the cell surface antigens TRA-1-60, SSEA-3 and -4 signifying their EC cell state. They also have been shown to differentiate in response to ATRA, down-regulating these stem cell markers and up-regulating cell surface antigens associated with neural differentiation, such as A2B5 and VINIS-53 [131, 132]. After 21 days of culture in the presence of ATRA, TERA2.sp12 cells differentiate into a heterogeneous population containing many mature functioning neurons [133], making them a useful model system for the *in vitro* study of neurogenesis.

3.1.2 Embryonic stem cells

It was the discovery of EC cells that led to the ground-breaking research of isolating murine ES cells [134, 135], then ultimately to the isolation of human ES cells [136] in 1998. This first human ES cell line was derived from a human blastocyst and shown to have a normal karyotype and express cell surface markers which characterised primate ES cells. The cell line was shown to remain in an undifferentiated, proliferative state *in-vitro* for 4 to 5 months, after which the cells still maintained their full developmental potential by forming trophoblast cells and derivatives of all three of the embryonic germ layers. The cell line was termed H9, and is the cell line used throughout this investigation. As found

in EC cell studies, significant differences between murine and human ES cell cultures was observed, namely the different expression profiles of the SSEA antigens alongside low expression of LIFR in human cells compared to the mouse [137].

Unlike their EC counterparts, ES cells remain a challenge to culture and there use is surrounded by ethical controversy. For the purposes of this investigation initial experiments were carried out on EC cells, and further analysis was completed on ES cells. ES cells require a very closely defined culture medium, which up until recent advances required the co-culture with mouse embryonic feeder layers (MEF) (as practiced in this investigation). Feeder layers release soluble factors and extra-cellular matrix components which remain unidentified, but are essential for ES cell proliferation. MEFs therefore have to be seeded at a sufficient density so that there are enough factors to support ES growth, but not too many cells so they inhibit colony outgrowth. For the H9 line a density of 20,000 cells/cm^2 has been found to be optimal [138]. As in the EC cell system, ATRA is used in many defined differentiation protocols for the induction of neuronal sub-types [139]. In a recent investigation on H9 ES cells, it has been shown that regionally specific neural precursors can be obtained in chemically defined media conditions. The addition of ATRA into the media acted as a caudalising agent, inducing motor neurons [140].

3.1.3 ATRA - Instability.

While ATRA remains a very important molecule associated with, among other things, the correct neurogenesis and orientation of the embryonic central nervous system, its use in *in-vitro* study is hampered by its inherent ability to isomerise and degrade in response to light and heat [119], as outlined in Chapter 1. Even under standard laboratory conditions this instability is observed. As part of this multidisciplinary study of retinoids and further to earlier published studies, chemists at Durham University highlighted the ease at which ATRA degrades, outlining the need for more stable alternatives for *in-vitro* investigations. The susceptibility of ATRA to photoisomerisation and degradation when exposed to light in the 300-400 nm range was first demonstrated (**Fig 3.1.1A**). Proton NMR spectroscopy showed that prolonged exposure to fluorescent light led to progressive degradation of ATRA. After 3 days of exposure approximately 37% of ATRA was present in its original state. ATRA was found to be stable for at least 3 days

when kept in the dark, whereas exposure to fluorescent light for the same time period resulted in substantial isomerisation/degradation **(Fig 3.1.1B)**.

3.1.4 Stability of Synthetic Retinoids EC19 and EC23

As outlined in Chapter 1, to address the issue of instability of ATRA, when used *in-vitro*, synthetic retinoid analogues were created as part of work contributing to this thesis [123] for synthesis methods refer to Chapter 1. It was hypothesised that these retinoids would possess greater physical and chemical stability during routine *in-vitro* use. To test this theory, initially two synthetic arotinoids were chosen for analysis and were termed EC19 and EC23. Their photostability was tested. Synthetic retinoids were exposed to the same wavelength of fluorescent light for 3 days as in the ATRA analysis, 300-400 nm range, and NMR spectra were compared with control samples, which had been incubated in the dark for the same length of time (**Fig 3.1.1C & D**). Both EC19 and EC23 were found to be completely unaltered by the light treatment. These results demonstrate that the introduction of an aromatic ring into the polyene chain, and the incorporation of a triple bond into the linker region results in greatly enhanced photostability under conditions which have dramatic effects on ATRA. Both of these synthetic retinoids were put forward for biological analysis.

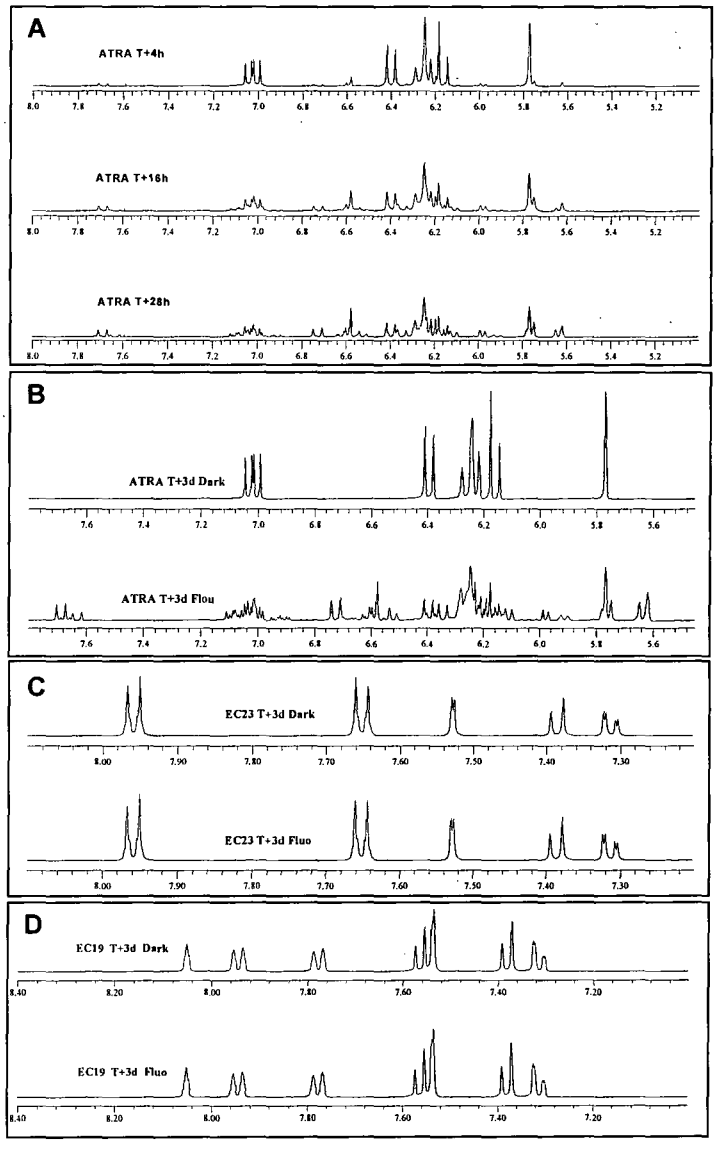


Figure 3.1.1 : Proton NMR at 400 MHz of ATRA, EC23 and EC19 in DMSO-d6 (5.0-8.0 ppm region) **A.** NMR spectra of ATRA after exposure to white fluorescent light at 40 cm distance for 4 h (top), 16 h (middle) and 24 h (bottom) showing increased degradation with increased time. **B.** NMR spectra of ATRA after 3 days in absence of light (top), and 3 days exposure to white fluorescent light at 40 cm distance (bottom) showing degradation only with exposure to light. **C.** NMR spectra of EC23 after 3 days in absence of light (top), and 3 days exposure to white fluorescent light at 40 cm distance (bottom), showing no degradation under exposure to light. **D.** NMR spectra of EC19 after 3 days in absence of light (top), and 3 days exposure to white fluorescent light at 40 cm distance (bottom), showing no degradation under exposure to light.

3.1.5 Aim and Objectives

Aim

The aim of this Chapter was to evaluate the biological activity of the synthetic retinoid analogues EC19 and EC23 on models of embryonic stem cells. ATRA is a well known and studied morphogen, inducing the correct dorsal/ventral and anterior/posterior axis of many different embryonic developmental processes. One such area where ATRA is

influential is the patterning of the CNS. It is already known that ATRA induces neural differentiation in stem cell model systems *in-vitro* and here we aim to study the ability of our more stable synthetic retinoids to do the same.

Objectives

- Analysis of the ability of synthetic retinoids EC19 and EC23 to induce differentiation in the EC cell line TERA2.sp12.
- Quantification of stem cell and neural differentiation markers.
- Proteomic analysis of retinoid induced differentiation products.
- Analysis of the ability of synthetic retinoids EC19 and EC23 to induce differentiation in the ES cell line H9.
- Quantification of neural differentiation markers.
- The only chemical difference between EC19 and EC23 is the position of the carboxyl group either in the *para-* (EC19) or *meta-* (EC23) position. Whether this subtle change had any effect on the biological activity visualised was analysed.

3.2 Methods:

3.2.1 Tissue Culture

3.2.1.1 Tera2.sp12 human embryonal carcinoma cells:

Cells were cultured under standard laboratory conditions as described in Chapter 2. Cells were cultured in Dulbecco's modified eagles medium (DMEM) (Sigma) supplemented with 10% foetal calf serum (FCS) (Gibco), 2mM L-Glutamine and 100 active units each of penicillin and streptomycin (Gibco). Cultures were passaged using acid-washed glass beads (VWR) unless a single cell suspension was required for counting, in which case 0.25% trypsin EDTA (Cambrex) solution was administered. For optimal cell culture, and to inhibit spontaneous differentiation, flasks were maintained at high confluency and split at a ratio of no more than 1:3. Cultures intended for flow cytometric and proteomic analysis were set up in T25 flasks (Nunc) while 12-well and 24-well plates (Nunc) were used for MTS assays and immunocytochemical analysis.

Differentiation protocol:

Single cell suspensions were obtained using 0.25% trypsin EDTA and either 500,000 cells/T25 flask or 1,200,000 cells/plate were seeded and left in proliferative media to settle for 24 hours. Cultures were then switched to differentiation media; standard proliferative media supplemented with either 1μM or 10μM retinoid as stated in results, and incubated as normal. Cultures were then left for the dedicated time period and underwent full media changes every three days. Subsequent analysis of obtained samples was carried out as described in protocols found in Chapter 2.

3.2.1.2 H9 human embryonic stem cells:

Cells were cultured under standard laboratory conditions as described in Chapter 2. Cells were co-cultured with mitotically inactivated mouse embryonic fibroblast feeder layers (MEF) in serum-free conditions. Proliferative media was knockout DMEM (Gibco) supplemented with 20% knockout serum (Gibco), 1% Glutamax (Gibco), 1% non-essential-amino-acids (NEAA) (Gibco), 8ng/ml basic fibroblast growth factor (bFGF) (Gibco) and 1% penicillin and streptomycin (Gibco). Cultures were maintained and expanded in 6-well plates (Iwaki). After initial seeding, ES cells underwent a full media change after day 2 of culture. On day 3, cultures were 'cleaned-up', i.e. underwent removal of all spontaneously differentiated cells which usually occurred around the edge

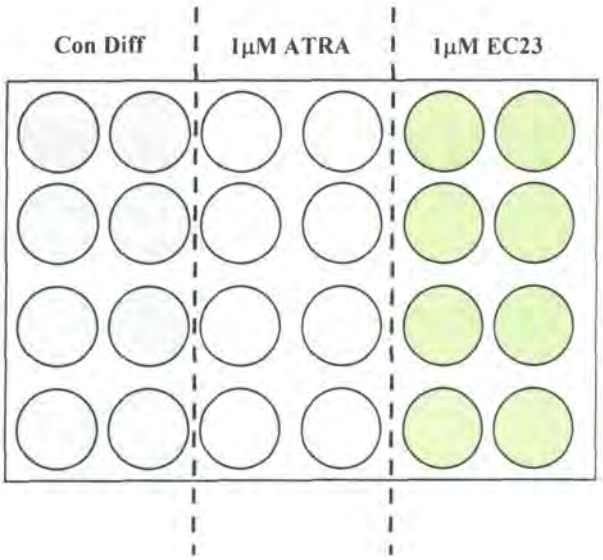
of colonies. This was done by scratching cells off the plate using a 100μL pipette tip visualised down a dissection microscope. Media containing the scratched off cells was removed and refreshed. On day 6 or 7 cultures were passaged. As MEFs only remain optimal for up to 7 days in culture, plates had to be passaged every week into fresh MEF-coated plates. Cultures were split according to ES colony size ranging from a 1:1 split up to 1:3 for confluent cultures. Routine passage of cultures was carried out by mechanically dissociating the ES cell colonies from the tissue culture plastic using pipette tips. Colonies were cross-hatched with a 100μL tip then lifted off using a 1000μL tip and the resulting floating ES colonies were transferred to the fresh MEF plates.

Differentiation protocols:

Neuronal-specific differentiation protocol: Monolayer differentiation without MEFs was followed in this established protocol. First, 12-well plates (Iwaki) were coated overnight with 1% gelatine solution (in PBS) then washed twice with PBS prior to use and not allowed to dry out. Confluent ES cell colonies were cleaned of differentiated cells without disrupting the ES colony, then treated with collagenase solution (1mg/ml in knockout DMEM media) (Sigma) for 15 minutes at 37°C. Detached colonies were transferred into the pre-coated 12-well plates (1:1 plating ratio) and neuronal differentiation was induced by growth in differentiation media (DMEM/F12 (PAA), supplemented with 1X N2 supplement (PAA), 1X B27 supplement (PAA), 1% NEAA (Gibco), 1% L-Glutamine (Sigma) and 1% Penicillin and streptomycin (Sigma)). After 7 days, medium was changed to a neuronal base medium (DMEM/F12:Neuronal base medium (PAA) 1:1, supplemented with 0.5X N2 supplement, 0.5X B27 supplement, 1% NEAA, 1% L-glutamine, 20ng/ml bFGF, 1% penicillin and streptomycin). Cultures at this stage were incubated with and without 1μM retinoids (without served as differentiation control). Cultures were maintained for a further 2 weeks with media changes every 3 days. Subsequent analysis of cultures was carried out as described in protocols found in Chapter 2.

General embryoid body differentiation protocol: This method was a standard generic differentiation protocol incorporating embryoid body (EB) formation. Confluent ES cell colonies were cleaned of differentiated cells without disrupting the ES colony, then treated with collagenase solution (1mg/ml in knockout DMEM media) (Sigma) for 15 minutes at 37°C. Detached colonies were transferred to bacteriological dishes (VWR) to undergo EB aggregate formation in suspension (4 plates:1 dish seeding ratio). Media

used was knockout DMEM supplemented with 10% FCS (Hyclone), 1% NEAA, 1% L-glutamine and 1% penicillin and streptomycin. Dishes were cultured with and without 1μM addition of retinoids (without served as the differentiation control). Media was changed every 3 days by allowing the EBs to settle on the bottom of a 50ml tube and removing old media then refreshing with new. Cultures were also transferred to fresh bacteriological plates during this process. Cultures were maintained for 2 weeks, after which EBs were transferred a 24-well plate (8 wells/dish, i.e. 8 wells/condition studied, see diagram below) pre-coated with 10μg/ml poly-l-ornothine (Sigma) overnight followed by 5μg/ml laminin (Sigma) coating also overnight with subsequent PBS washes, and cells maintained for a further week. Subsequent analysis of cultures was carried out as described in protocols found in Chapter 2.



3.2.2 Flow cytometry

For complete methods refer to Chapter 2, section 2.4.

Stem cell markers SSEA-3 and TRA-1-60 (used neat, generous gift from Prof. P. Andrews, University of Sheffield) and neural differentiation markers A2B5 (1:100, Abcam) and VINIS-53 (used neat, generous gift from Prof. P. Andrews, University of Sheffield) were analysed. The expression of cell surface antigens has been used previously to indicate the status of cellular differentiation by human pluripotent TERA2.SP12 stem cells.

3.2.3 Immunocytochemistry

For complete methods refer to Chapter 2, section 2.6.

Antibodies used:

Antibody	Company	Dilution	Incubation time	Secondary
Nestin	Chemicon	1:100	1hr	Alexafluor 488
TUJ1	Covance	1:500	1hr	Cy3
NF-200	Sigma	1:200	1hr	Alexafluor 488
Cytokeratin-8	Sigma	1:200	1hr	Alexafluor 488
HB9	Abcam	1:100	1hr	Alexafluor 488

3.2.4 Western blot

For complete methods refer to Chapter 2, section 2.13.

Antibody used:

Antibody	Company	Dilution	Incubation time	Secondary
CRABP-1	Abcam	1:200	1hr	Horse-radish peroxidase

3.3 Results:

3.3.1 Morphological Effects of Natural and Synthetic Retinoids on Tera2.sp12 EC Cells

To compare the effect of synthetic retinoid analogues EC19 and EC23 versus known natural retinoid responses on embryonal carcinoma cells, cultures of Tera2.sp12 EC cells were incubated with 1 μM and 10 μM concentrations of each retinoid compound tested. Control cultures consisted of untreated stem cells as well as cultures exposed only to the retinoid solvent DMSO, termed vehicle control.

3.3.1.1 Cell viability assay

Retinoid treated cultures were maintained for up to 21 days. At time points of 3 and 7 days after retinoid addition, cell viability/number was analysed (MTS assay, **Fig. 3.3.1**). ATRA treatment at both concentrations resulted in reduced cell number compared with both control samples, and this effect was concentration dependent. Decreased cell number was also observed in cultures treated with both concentrations of EC23, although the decrease was not as great as in ATRA cultures. There was no difference between cell numbers in control and 1μM EC19 treated cultures, indicating inability of this compound to arrest cell proliferation at this concentration. In particular, cells treated with 1 μM EC19 continued to proliferate rapidly, to the point that, after 7 days the cultures had become over-confluent and all cells had died. 10μM EC19 cultures did show an attenuation of cell number at the 7 day time point when compared with controls, however, this was to a much lesser extent than that seen with ATRA and EC23.

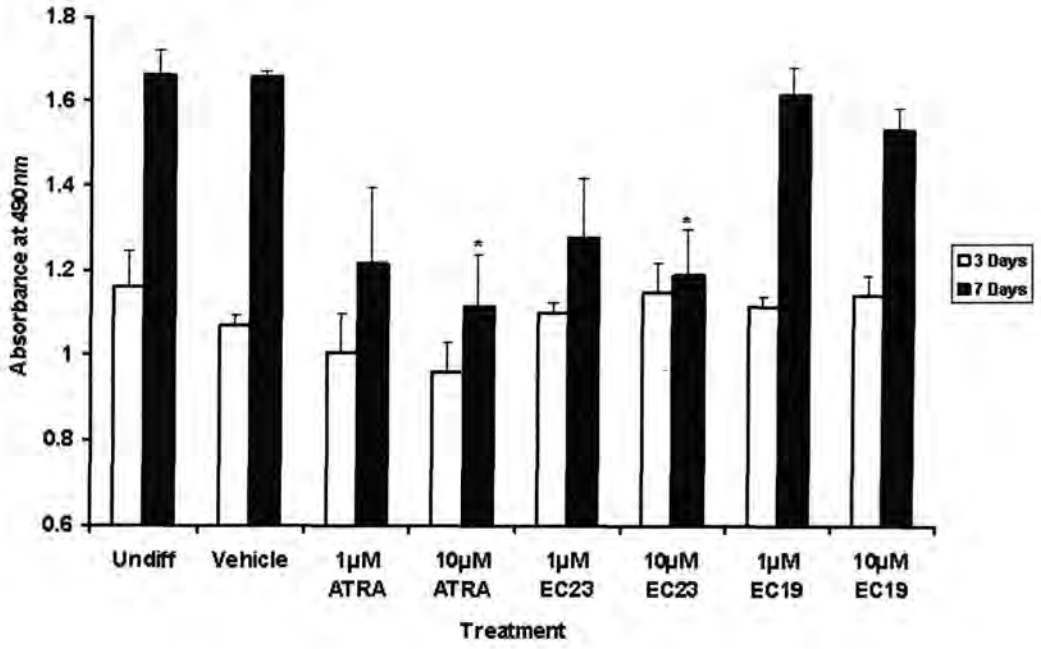


Figure 3.3.1: Cell number analysis using the MTS assay kit. Cultures exposed to either 1μM or 10μM ATRA, EC23 or EC19 were assayed at various time points and compared to control undifferentiated and vehicle exposed (DMSO) cultures. Cell number, expressed as an absorbance value, was attenuated in all ATRA and EC23 cultures compared to the controls, most predominantly in the 10μM ATRA culture. However, 1μM EC19 cultures did not reduce cell number seen compared to control samples, whereas at the 10μM concentration by 7 days cell numbers were attenuated but not to the same extent as in ATRA and EC23 samples. Results are presented with ± standard deviation of the mean (SDM), n=3. * $p \leq 0.05$, student's t-test compared to relevant undifferentiated control.

3.3.1.2 Morphological analysis of induced cell types

Morphological analysis of retinoid treated cultures revealed dramatic effects of all three compounds (**Fig 3.3.2**). Cultures exposed to EC23 appeared to mimic their ATRA counterparts (**Fig 3.3.2B & C**), both displaying a high degree of heterogeneity and containing rosette-like areas typically seen in neural cell culture. On the other hand, treatment with 10 μM EC19 induced different cellular morphologies divided into two cell types. The first were highly proliferative cells which had become highly confluent and begun to layer upon one another. These cells were surrounded by a second large, flat cell type (**Fig 3.3.2D**). The 'plaques' formed by the large flat cells were distributed throughout the culture and were large enough to be seen with the naked eye. These plaques, also observed to a lesser extent in ATRA and especially EC23 cultures, were clearly visible upon immunocytochemical staining with cytokeratin-8, a marker epithelium (**Fig 3.3.3**). Cytokeratin-8 staining reinforced the apparent similarity between

ATRA and EC23 treatment (**Fig 3.3.3A-D**), and highlighted the high plaque content of 10 μM EC19 cultures (**Fig 3.3.3E**).

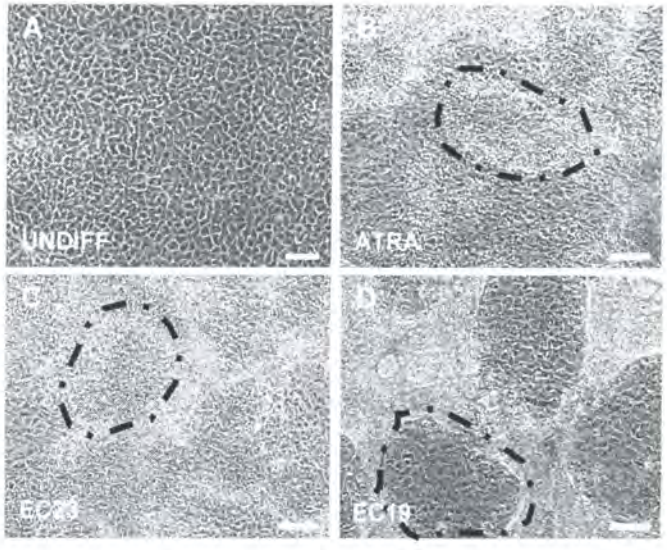


Figure 3.3.2: Structural phenotype of Tera2.sp12 cells treated with retinoids. Morphological analysis of differentiation of Tera2.sp12 embryonal carcinoma stem cells. 21 days of culture with 10μM concentrations of the natural retinoid all-trans-retinoic acid (ATRA) or synthetic retinoids EC23 or EC19 induced significant morphological changes. Undifferentiated Tera2.sp12 cultures **(A)** retained a homogeneous 'cobblestone' appearance throughout the culture. Cultures exposed to ATRA **(B)** exhibited highly heterogeneous morphology, including numerous neural-like rosettes (bordered area). Similarly cultures exposed to EC23 **(C)** exhibited heterogeneous morphology and rosettes (bordered area). Cultures exposed to EC19 **(D)** were distinct in morphology, characterised throughout by areas containing large flat cells (bordered area) surrounded by highly proliferative cells becoming layered on top of one another. Scale bar: 100μm.

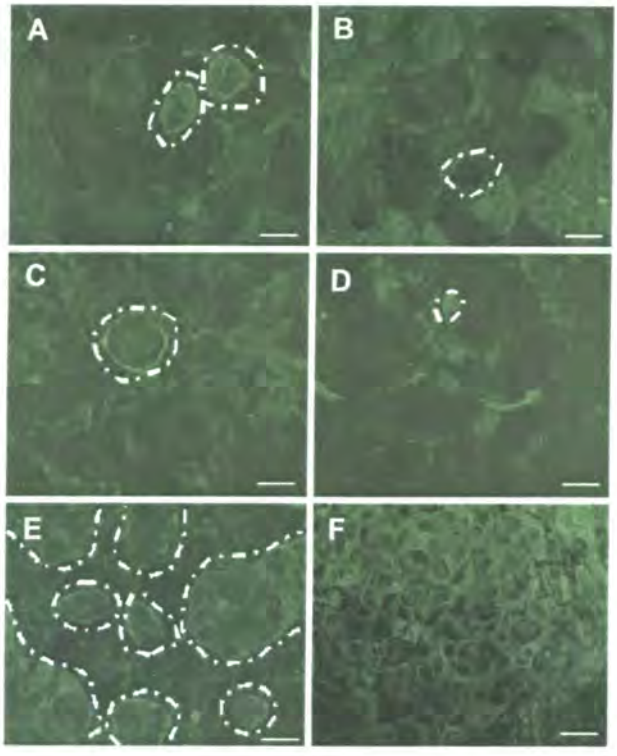


Figure 3.3.3: Cytokeratin-8 expression in cultures of Tera2.sp12 embryonal carcinoma stem cells incubated with **(A)** 1μM or **(B)** 10μM ATRA, **(C)** 1μM or **(D)** 10μM EC23, and **(E)** 10μM EC19. Low power (x 4 magnification) images show the proportion of the culture area made up of cells with the large, flat morphology, seen predominantly in the EC19 culture (bordered areas). Micrograph **(F)** shows a high power (x 40 magnification) image of these large flat cells. Scale bars: A-E 250μm, F 50μm.

The number of plaques and the average area which they occupied in each culture was calculated, using ImageJ software, from 12 random images taken using the 4 X objective (**Fig 3.3.4**). In cultures treated with ATRA, plaque number and average area decreased slightly from 1 μM to 10 μM treatment. A similar, though slightly more pronounced trend was observed in EC23 cultures. Slightly fewer plaques were seen in 1 μM ATRA cultures, and slightly more in the 10 μM ATRA cultures, compared with the respective EC23 counterparts. The area of plaque coverage in 10 μM EC23 cultures is notably the lowest of all treatments. Treatment with 10 μM EC19 resulted in approximately 4 times the number of plaques than in 10 μM ATRA cultures. More strikingly, plaques in the EC19 culture occupied a dramatically greater area of the culture than in the ATRA counterpart.

Morphological analysis indicated that while both synthetic retinoids elicited differentiation of the Tera2.sp12 cell line, EC23 appeared the more functionally related to ATRA at this stage of analysis. Although the different treatments were seen to give rise to similar cell types, the proportions of these derivatives were significantly different in EC19 cultures compared with those of ATRA and EC23 treatments.

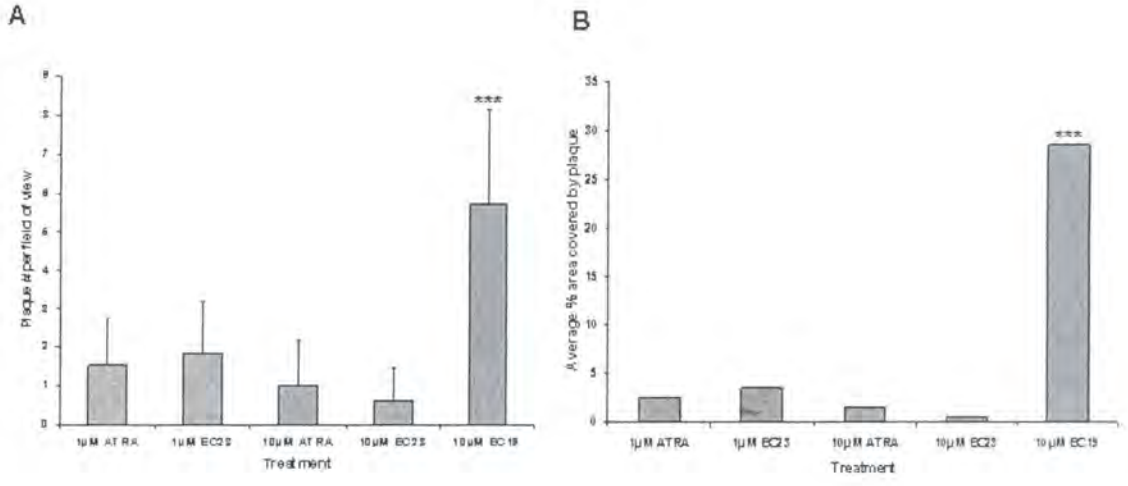


Figure 3.3.4: The number and average area covered by the non-neural, cytokeratin-8 positive large flat cell 'plaques' increases in EC19 cultures. Tera2.sp12 EC cells were incubated with 1µM or 10µM ATRA or EC23 or 10µM EC19 and cultured for 21 days. Cultures were immunocyto-stained for the cyto-skeletal marker cytokeratin-8. This marker was able to distinguish between plaque areas and the other cell types within the cultures. A random collection of 12 images/condition was collected. Using ImageJ the number of plaques/field of view (A) and area of plaques was analysed. The total area made up of plaques was compared to the total area studied and the average % plaque coverage was calculated for each condition (B). As the concentration of ATRA or EC23 increased the number of plaques visualised per field of view and subsequently the average area covered by plaques decreased, being almost totally inhibited in the 10µM EC23 culture. Compared to the control retinoid ATRA, EC19 increased the number of plaques visualised and greatly increased the average area covered by the plaques, indicating this retinoid greatly enhances the differentiation of these EC cells into this particular cell type. Error bars on A: SDM n=12. *** $p \leq 0.0005$, student's t-test compared to 10µM ATRA.

3.3.2 Immunological Analysis of Tera2.sp12 EC Cells Exposed to Natural and Synthetic Retinoids

3.3.2.1 Flow cytometry

Flow cytometry was performed to demonstrate and characterise differentiation of Tera2.sp12 cells in response to treatment with synthetic and natural retinoids. In line with

the known neural effect of ATRA in this cell line, a panel of markers was chosen to specifically follow neural differentiation.

Two stem cell markers were analysed, since a reduction in their expression would signify a departure from stem cell state. These markers were globoseries antigen stage specific embryonic antigen-3 (SSEA-3), and the keratin-sulphate-associated glycoprotein antigen surface marker TRA-1-60. Within 7 days of ATRA or EC23 addition to cultures, the levels of both SSEA-3 and TRA-1-60 had dropped dramatically, indicating exit from the cell cycle and a commitment to differentiation (**Fig. 3.3.5**). Expression of both markers remained low through to the conclusion of the 21 day schedule, although it can be seen that the expression of these markers in 10 μM EC23 is approximately 5% elevated over the 1 μM EC23 and ATRA treatments. This perhaps reflects a proliferating subpopulation, in keeping with the slightly increased cell numbers determined by MTS assay. SSEA-3 and TRA-1-60 expression levels remained comparatively high in EC19 cultures up to 7 days after retinoid addition. Nonetheless, the decrease relative to control cultures was marked indicating that cellular differentiation had been triggered in a proportion of cells. After 7 days 1 μM EC19 cultures were discarded due to overgrowth. However, in 10 μM EC19 cultures, SSEA-3 and TRA-1-60 continued to decline to levels comparable with ATRA and EC23 by the 14 day time point. Therefore it appears as though 10μM EC19 induces the differentiation of Tera2.sp12, albeit at a slower rate than either ATRA or EC23.

The differentiation markers VINIS-53 (an antibody reactive against neuronal cell adhesion molecule - NCAM), and A2B5 (a ganglioseries antigen marking early-stage neural cells), were analysed by flow cytometry (**Fig. 3.3.5**). All retinoid treatments resulted in elevated expression of these markers compared to the time zero control. It is important to note that the percent of cells expressing markers are relative to the threshold fluorescent settings, which was fixed throughout all analysis, including all biological repeats. 10 μM ATRA causes the most rapid increase in VINIS-53 expression, peaking at approximately 25% cells expressing the marker at 7 days. 10 μM EC23 expression increased at a slower rate and peaked at 14 days to a level of more than 30% positive cells (this number subsequently returned to approximately 25 % cells by day 21). Cells treated with 10μM EC19 displayed a marked delay in increase of VINIS-53 expression

compared with the other two retinoids. However, after 14 days the expression profile closely resembled that of the 1 μM EC23 culture, ultimately resulting in almost 20% positive cells. The expression profiles for A2B5 largely mirrored those for VINIS-53, although initial rates of expression increase were much closer between the ATRA and EC23 treatments. Once again, 10 μM EC23 caused the greatest increase occurring at 14 days (~ 40%) before returning to a lower level by day 21, and this was mimicked by 1 μM ATRA. Of particular note is the exact correlation between the 1 μM EC23 and 10 μM ATRA profiles of A2B5 expression. As with VINIS-53, cells treated with 10μM EC19 showed delayed expression of A2B5 which ultimately reached numbers comparable with the other treatments. Taken together these findings support a close relationship between ATRA and EC23. Furthermore, it appears as though both synthetic retinoids are able to induce the differentiation of Tera2.sp12 cells towards derivatives which posses neural cell surface markers.

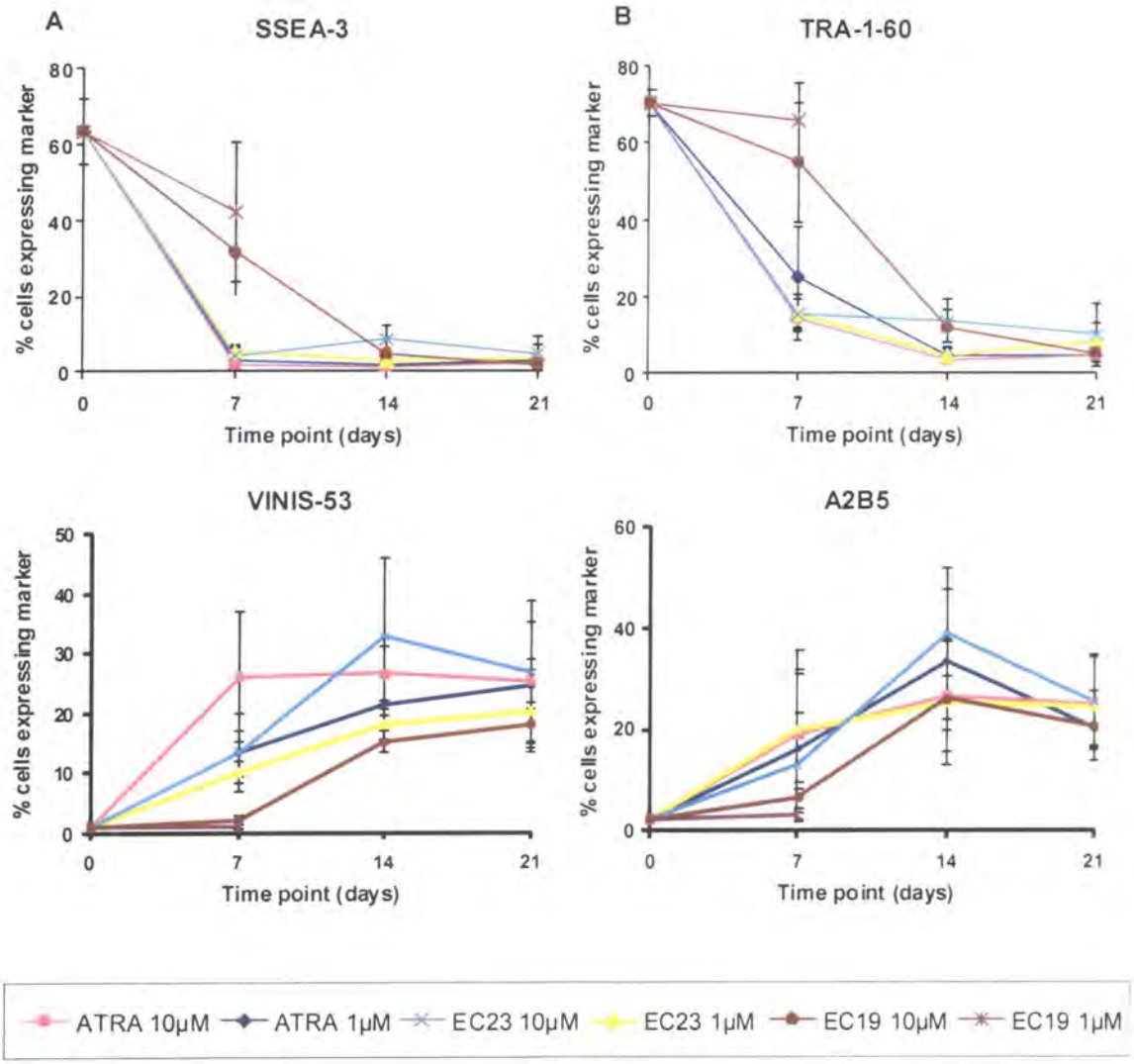


Figure 3.3.5: Flow cytometric analysis of markers for stem cells and neural differentiation. The percentage of Tera2.sp12 cells expressing the stem cell markers SSEA-3 **(A)** and TRA-1-60 **(B)** and neural differentiation markers VINIS-53 **(C)** and A2B5 **(D)** was measured at 7 day time-points following treatment with 10μM or 1μM ATRA, EC23 or EC19, alongside undifferentiated control cultures. Both concentrations of ATRA and EC23 resulted in a rapid decrease in expression of the stem cell markers and an increase in neural marker expression. Similarly 10μM EC19 induced differentiation as evidenced by complete absence of the stem cell markers after 14dys, although this down regulation occurred at a slower rate than that induced by EC23 and EC19. On the other hand treatment with 1μM EC19 appeared not to induce neural differentiation with cells continuing to proliferate and express stem cell markers whilst remaining negative for neural markers. Cultures treated with 1μM EC19 were maintained until 7 days whereupon cells became over confluent. Values represent ±SDM, n=3.

3.3.2.2 Immunocytochemical staining

Immunocytochemical staining was performed on 21-day-old retinoid-treated cultures, in order to further evaluate differentiation patterns. The neural progenitor marker nestin was first examined (**Fig. 3.3.6**). Nestin expression was evident in undifferentiated control cells (**Fig. 3.3.6F**), and became up-regulated in cells treated with both concentrations of ATRA and EC23 (**Fig. 3.3.6 A-D**). The neural-like rosettes, seen throughout ATRA and EC23 cultures under light microscopy, were positively reactive for nestin expression. In 10 μM EC19 cultures, nestin expression was observed in areas containing the small, highly proliferative cells. On the other hand, the large plaques consisting of large flat cells were all negative for nestin expression (**Fig. 3.3.6E**). In ATRA and EC23 cultures these plaques, which were fewer and smaller, also stained negative for nestin expression.

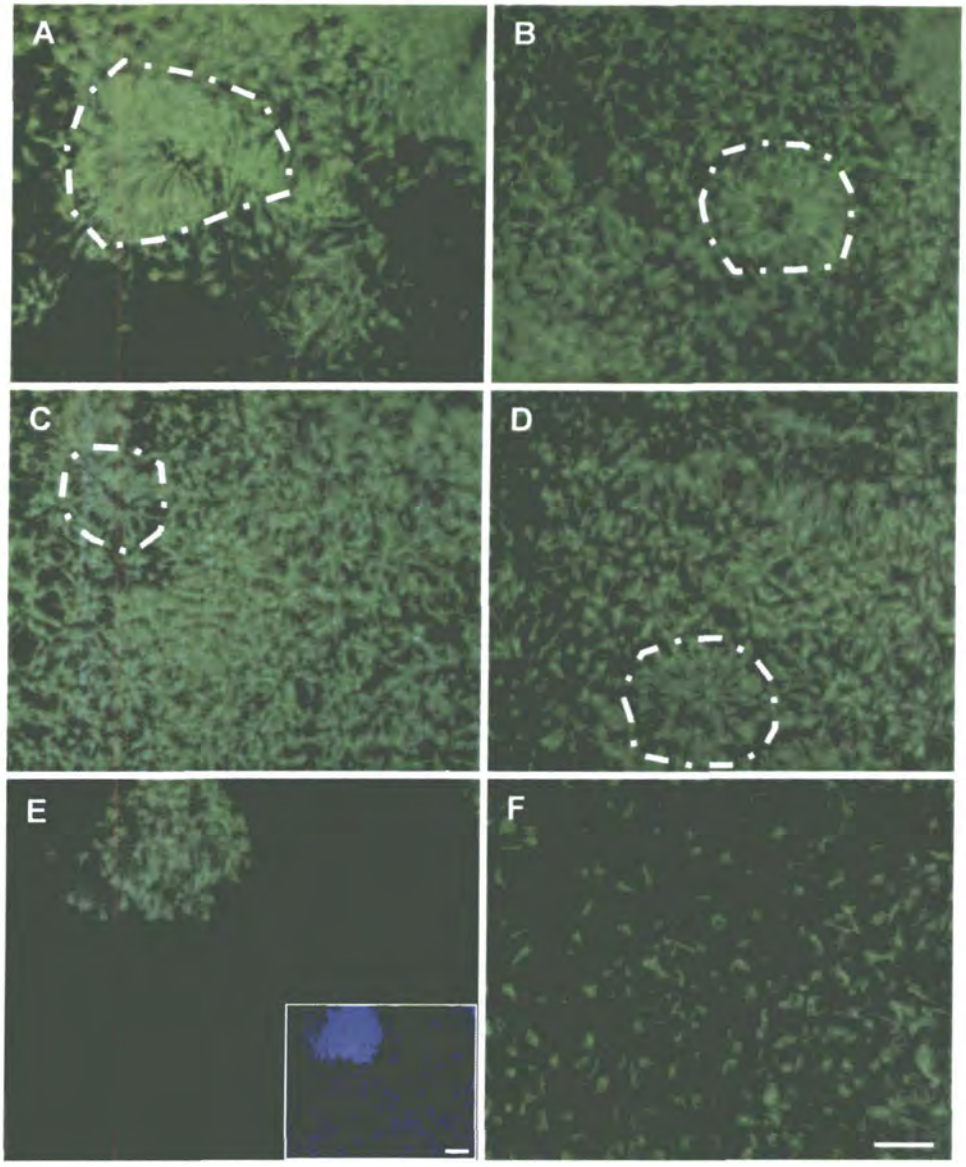


Figure 3.3.6: Induction of neural differentiation in cultures of Tera2.sp12 embryonal carcinoma stem cells incubated with **(A)** 1μM or **(B)** 10μM ATRA, **(C)** 1μM or **(D)** 10μM EC23, **(E)** 10μM EC19 or **(F)** undifferentiated controls. Undifferentiated and 21 day-differentiated cultures were immunocyto-stained for the neural progenitor marker nestin. Undifferentiated cells displayed relatively low expression levels of nestin **(F).** 1μM 21 day ATRA-treated cultures contained areas of high nestin expression **(A)** as did 10μM cultures **(B).** Both concentrations of 21 day EC23 **(C, D)** exhibited similar expression profiles to 10μM ATRA. In 21 day EC19 cultures **(E)** nestin expression was restricted to areas containing highly proliferative cells, and the large flat cells which dominate the EC19 cultures were negative. Marked areas in micrographs **A-D** indicate the presence of neural rosettes. Micrograph **E** includes the nuclear counter-stain Hoechst 33342 (Blue) image. Scale bar: 50μm.

Expression of neuronal specific proteins was examined using antibodies raised against the neuronal specific β-III-tubulin marker TUJ1 (**Fig. 3.3.7**) and the late neuronal marker neurofilament 200 (NF200, **Fig. 3.3.8**). Both markers were highly expressed in 1 μM and 10 μM ATRA and EC23 cultures (**Fig. 3.3.7A-D & 3.3.8A-D**), with many neuronal processes visible in both. However, very few TUJ1 or NF-200 positive cells were seen in 10 μM EC19 cultures, and very few neuronal processes were evident (**Fig. 3.3.7E & 3.3.8E**). Plaque regions were also negative in all cultures. As would be anticipated, undifferentiated cell cultures stained negative for both markers, indicating that induction of expression in ATRA and EC23 cultures occurred in response to the respective retinoid treatment. The number of NF200 positive cell bodies (perykarya) in each culture treatment was calculated as the mean from nine randomly selected immunostained images, with the aid of ImageJ software (**Fig. 3.3.9**). The highest number of NF200 positive perykarya, and by inference mature neurons, was seen in 10 μM EC23 cultures, decreasing as follows: 10μM EC23 >10μM ATRA > 1μM EC23 > 1μM ATRA >> 10μM EC19. Thus, at equal concentrations and under the conditions tested, EC23 appears to be a more potent inducer of neuronal differentiation than ATRA. The results also indicate a degree of neuronal differentiation in 10 μM EC19 cultures, however, this occurs at far lower frequency than observed following ATRA or EC23 treatment.

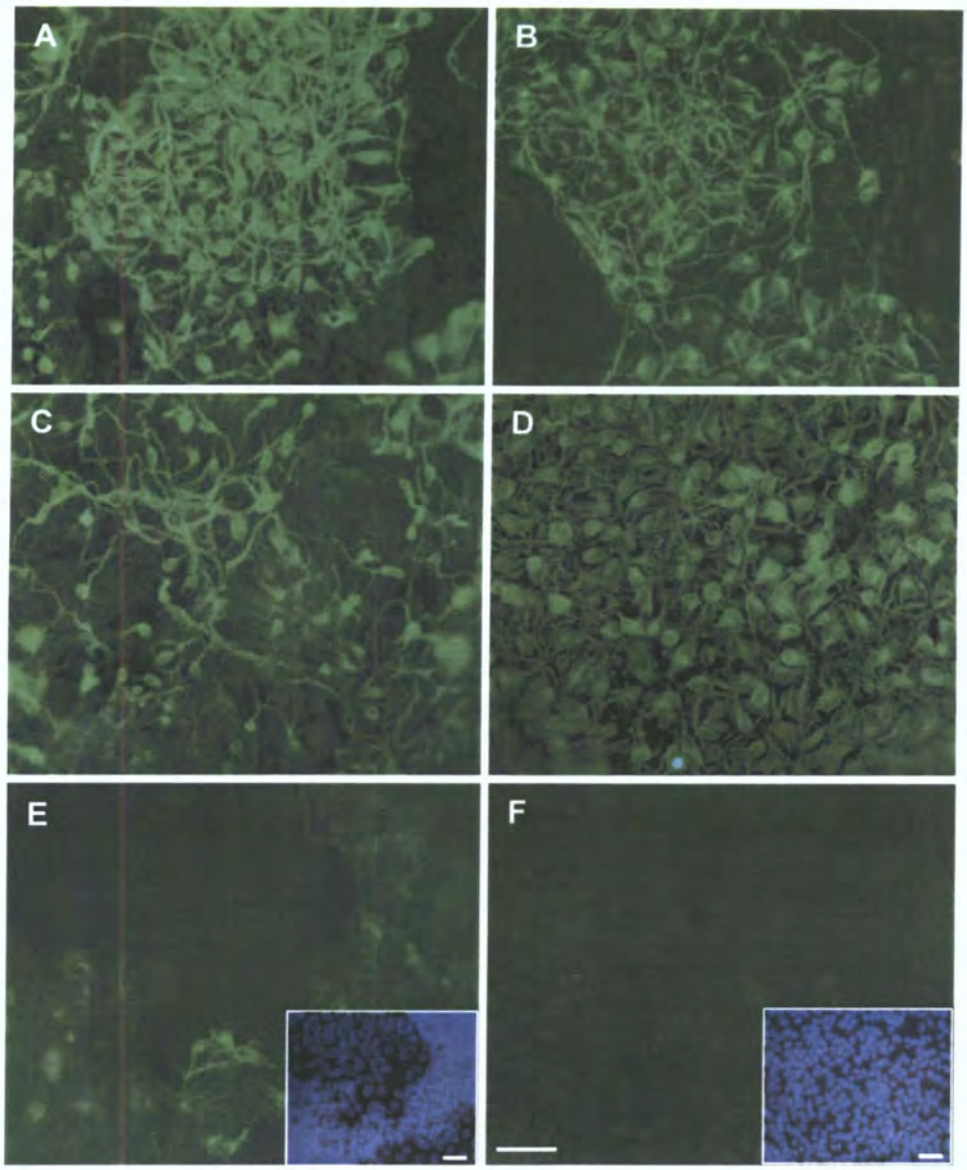


Figure 3.3.7: Induction of neural differentiation in cultures of Tera2.sp12 embryonal carcinoma stem cells incubated with **(A)** 1μM or **(B)** 10μM ATRA, **(C)** 1μM or **(D)** 10μM EC23, **(E)** 10μM EC19 or **(F)** undifferentiated controls. Undifferentiated and 21 day-differentiated cultures were immunocyto-stained for the neuronal marker TUJ1. Undifferentiated cells displayed no expression of TUJ1 **(F).** Both concentrations of 21 day ATRA-treated cultures contained areas of high TUJ1 expression **(A, B)**. Both concentrations of 21 day EC23 **(C, D)** exhibited similar expression profiles to ATRA. In 21 day EC19 cultures **(E)** TUJ1 expression was restricted to areas containing highly proliferative cells, and the large flat cells which dominate the EC19 cultures were negative. Micrograph E and F includes the nuclear counter-stain Hoechst 33342 (Blue) image. Scale bar: 50μm.

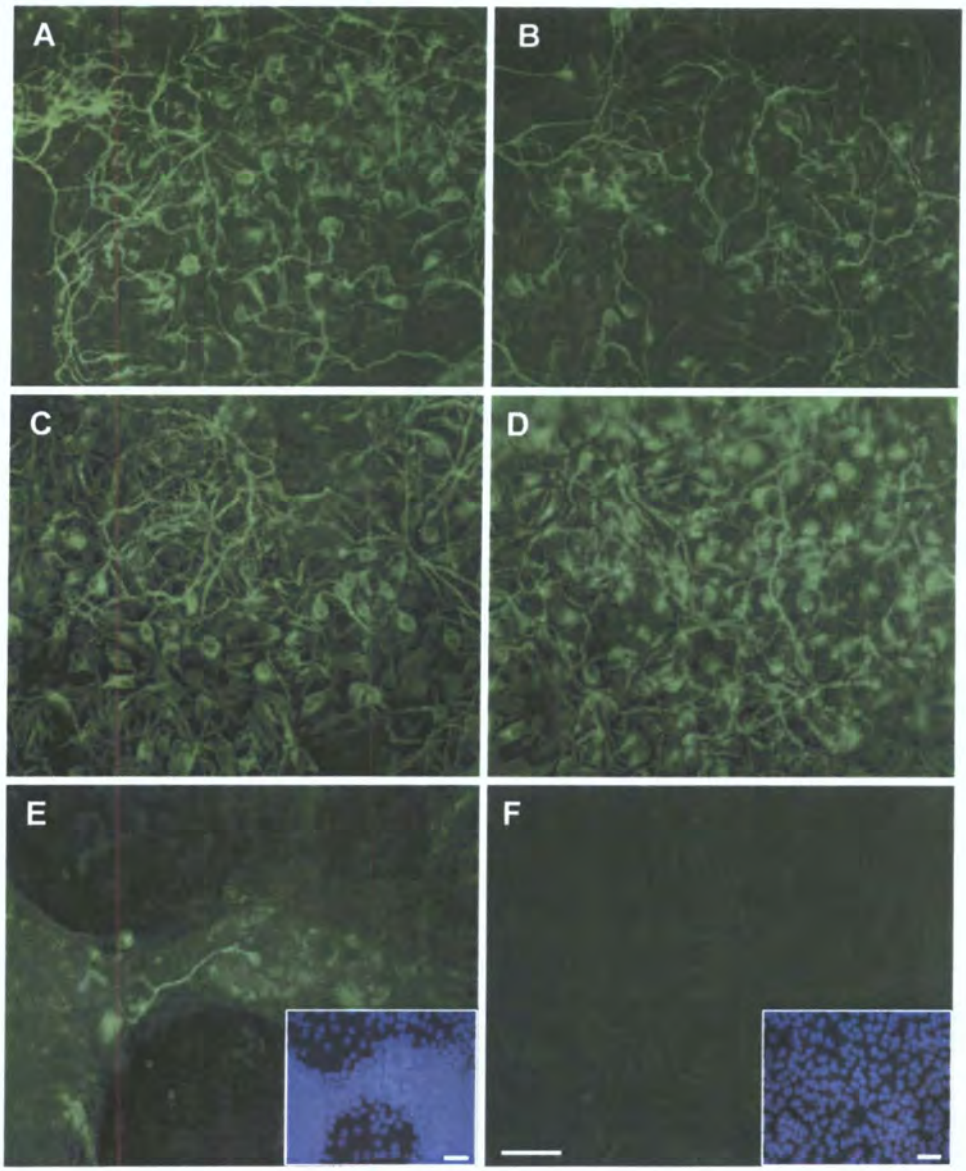


Figure 3.3.8: Induction of neural differentiation in cultures of Tera2.sp12 embryonal carcinoma stem cells incubated with **(A)** 1μM or **(B)** 10μM ATRA, **(C)** 1μM or **(D)** 10μM EC23, **(E)** 10μM EC19 or **(F)** undifferentiated controls. Undifferentiated and 21 day-differentiated cultures were immunocyto-stained for the late neuronal marker NF-200. Control undifferentiated cells displayed no NF-200 expression **(F).** However, Both concentrations of 21 day ATRA-treated cultures highly expressed NF-200 **(A, B)**, with long neuronal processes visible. Both concentrations of 21 day EC23 cultures **(C, D)** exhibited similar expression profiles to ATRA, also with many neuronal processes. In 21 day EC19 cultures **(E)** very few NF-200 positive neurons were visible and only appeared in the areas of highly proliferative cells. The large flat cells which dominate the EC19 cultures were negative for NF-200. Micrographs E & F include the nuclear counter-stain Hoechst 33342 (Blue) image. Scale bar: 50μm.

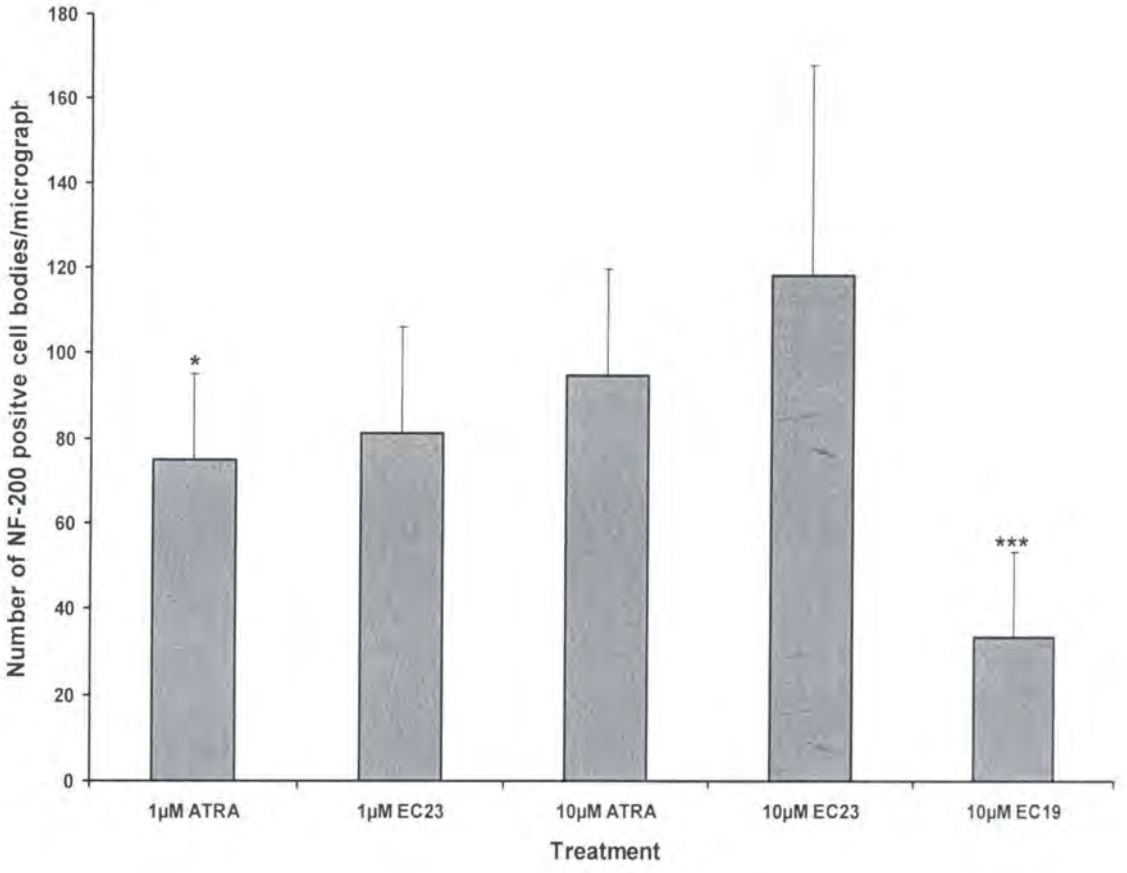


Figure 3.3.9: Induction of neural differentiation in cultures of Tera2.sp12 embryonal carcinoma stem cells incubated with 1μM or 10μM ATRA, 1μM or 10μM EC23 or 10μM EC19. 21 day-differentiated cultures were immunocyto-stained for the late neuronal marker NF-200. Using ImageJ software the number of NF-200 positive cell bodies was counted on 9 random fields of view per condition. Results were averaged and represented graphically. Increasing the concentration of ATRA and EC23 increased the number of neurons seen, with the most neurons being visualised in the 10μM EC23 condition. Compared to the natural retinoid ATRA control EC19 induced the differentiation of far fewer neurons. Error bars represent standard deviation of the mean n=9. * $p \leq 0.05$, *** $p \leq 0.0005$, student's t-test compared to 10μM ATRA.

3.3.3 Proteomic Analysis of Tera2.sp12 EC Cells Exposed to Natural and Synthetic Retinoids.

3.3.3.1 MALDI-TOF analysis

All data collated up to this point has indicated a similarity in the differentiation products induced by culture of Tera2.sp12 EC cells with either the natural retinoid ATRA or the synthetic retinoid EC23. Differentiation products induced by the synthetic retinoid EC19 were distinctly different. As a complimentary approach to further investigating the

similarity of natural/synthetic retinoid responses, we applied clinical biomarker analysis technology. Protein profiling has emerged as a potentially powerful tool for the measurement of diagnostic cohorts of biomarker proteins in the clinical arena. Here, we have investigated the applicability of biomarker profiling technology to characterise and compare the response of the EC stem cell model to natural and synthetic retinoids, an approach which this technology hasn't been used for before. It was hypothesised that this technology would allow the rapid and simultaneous detection of multiple protein/peptides present in EC cells before and after retinoid treatment and statistically analyse the similarities and differences between the sample groups. In principle this method should show the degree of similarity between the different cell treatments based on these protein signatures.

Total protein lysates were obtained from undifferentiated Tera2.sp12 EC cells and their 7 day retinoid differentiated counterparts. Only the 10μM retinoid concentration was used for these experiments. Total protein lysates underwent matrix assisted laser desorption ionisation time of flight (MALDI-TOF) analysis and resultant protein peak spectra were obtained and shown in triplicate (**Fig. 3.3.10**). Analysis of the raw spectra data shows obvious differences in protein profiles were visualised between the different sample groups. Undifferentiated control samples were easily distinguished from the retinoid differentiated samples, and also differences were visualised between the EC19 protein profiles and those obtained from the other two retinoid conditions. Confirming above data, ATRA and EC23 protein profiles appeared very similar. Using the associated Clinprot biomarker analysis software a four-way analysis of the grouped triplicate sample profiles was performed. A list of discriminatory biomarkers which could significantly distinguish between at least two of the sample groups was compiled, and the top ten are shown in **Table 3.3.1.** Initially, the top 2 discriminatory biomarkers were analysed, and visualised on a 2D distribution plot (**Fig. 3.3.11**). All of the retinoid sample cultures were distinct from the undifferentiated control, again indicating a departure of these treated cells from the undifferentiated stem cell state. EC19 was also distinct from ATRA and EC23, backing up previous data that EC19 did induce differentiation, but the induced cell products were distinct from treatment with ATRA and EC23.

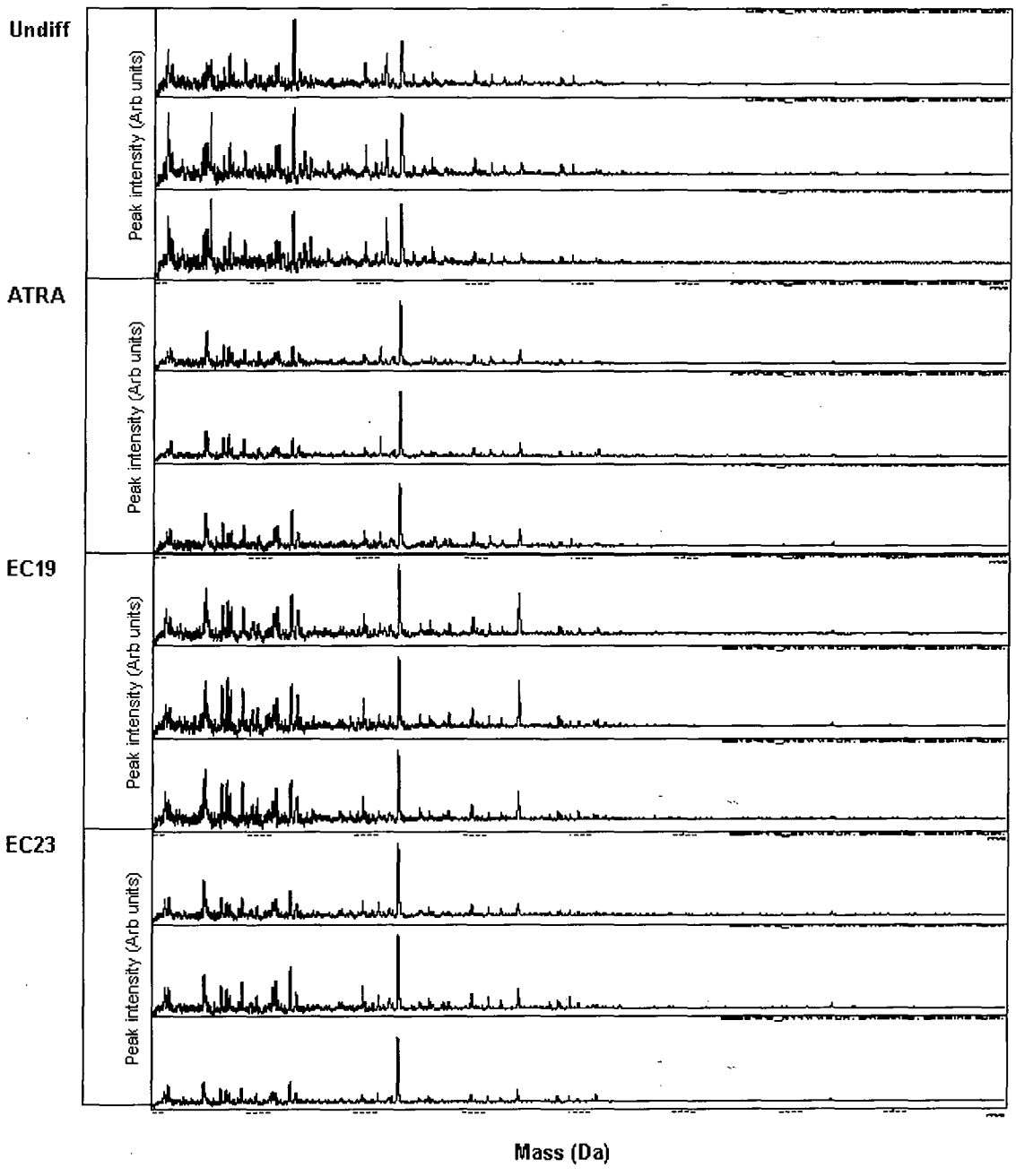


Figure 3.3.10: Triplicate MALDI protein spectra showing the similarities and differences between profiles obtained from control undifferentiated and retinoid differentiated EC cell lysates. Total protein lysates were analysed from undifferentiated EC cells and EC cells cultured with 10μM ATRA, EC19 or EC23 for 7 days. Obvious differences in protein profiles are visualised in the undifferentiated control compared to the differentiated samples, also differences are seen between the EC19 condition and the other two retinoids. ATRA and EC23 protein profiles appear very similar. n=3

Index No.	Observed m/z	Average peak area				*p*-value
		Undiff	ATRA	EC19	EC23	
3	4944.19	92.72	190.99	108.14	177.68	0.0137
22	12000.43	13.2	30.97	40.57	38.49	0.0281
5	5273.8	101.26	211.96	214.88	220.26	0.0438
26	16802.57	10.88	38.99	21.81	46.92	0.0438
4	4970.04	20.5	194.3	205.61	183.14	0.0536
18	10858.03	56.27	143.61	214.2	125.46	0.0586
14	8199.17	44.57	150.28	36.96	91.55	0.0586
21	11839.44	50.39	47.3	26.48	66.22	0.0586
25	12779.6	12.76	8.27	10.46	16.62	0.0586
2	4286.22	90.04	128.25	75.63	134.1	0.0671

Table 3.3.1: Table of the top ten discriminatory protein biomarkers as analysed by Clinprot biomarker analysis software. Marker proteins/peptides are statistically ranked (p-value of ANOVA test). Total protein lysates were analysed from undifferentiated EC cells and EC cells cultured with 10μM ATRA, EC19 or EC23 for 7 days. Markers in bold type displayed average peak area changes of > 1.5-fold compared with control undiff samples . n=3

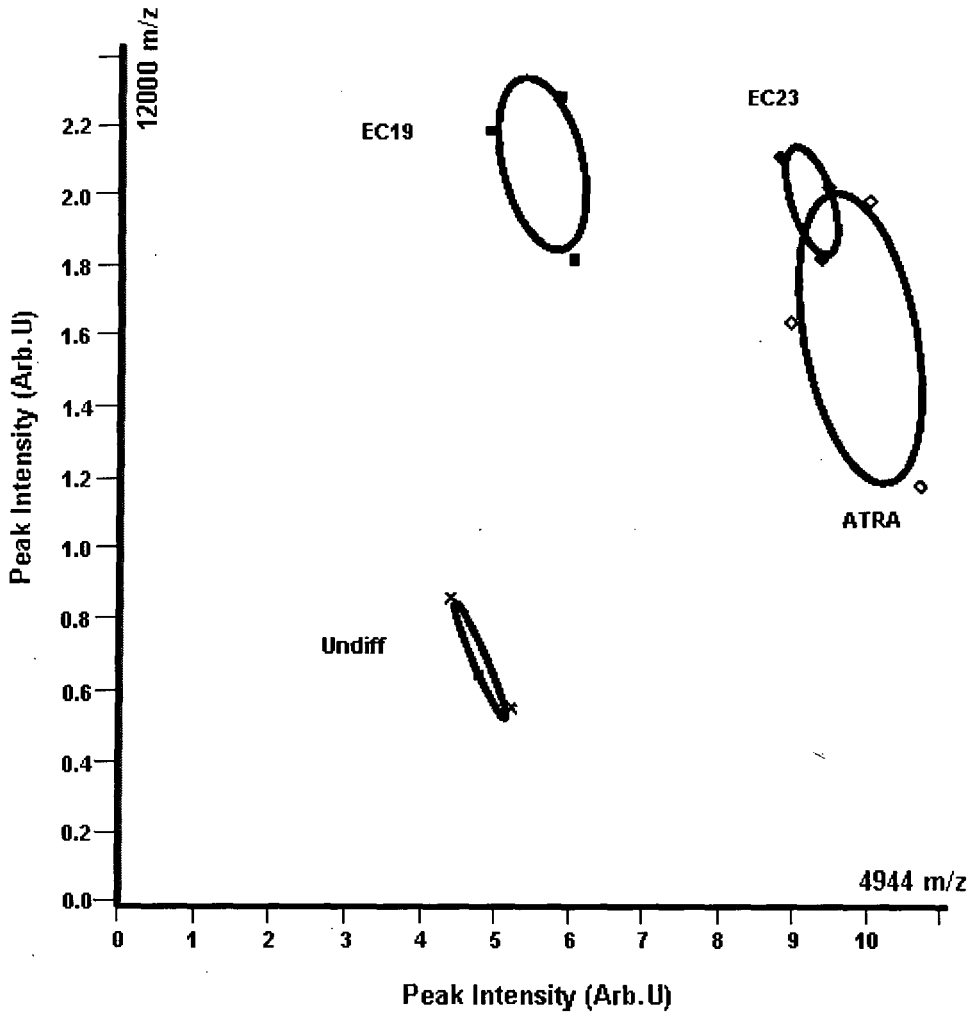


Figure 3.3.11: 2D distribution plot showing the top two biomarkers that were able to distinguish between the sample groups. Analysis was carried out using Clinprot biomarker analysis software. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. All of the retinoid conditions distinct from the control undifferentiated control. EC19 was also distinct from ATRA and EC23. The Clinprot biomarker software was unable to separate effectively ATRA and EC23 treated conditions, indicating the similarity in their protein profiles. n=3

Even when considering the top two discriminatory biomarkers, the Clinprot biomarker software was unable to separate effectively ATRA and EC23 treated samples, thus further verifying the similarity in the differentiation response between these two treatments. It was noted that the 'spread' of the three biological repeats was a lot tighter in both EC19 and EC23 samples compared with ATRA. This may be an indication that the induced-differentiation products visualised with the synthetic compounds are more tightly regulated and reproducible than in the ATRA counterparts. When considering the top ten discriminatory biomarkers in graphical form (**Fig. 3.3.12**) the similarity between

ATRA and EC23 treated cultures becomes even more apparent. In nine out of the ten biomarker profiles ATRA and EC23 elicit similar trends in protein expression levels (only Idx 25 is different). These top ten biomarkers also back up the differences seen with EC19 culture. Seven out of the ten hits show a difference in expression levels between EC19 profiles and the other retinoid samples (only Idx 5, 4, 25 are similar). Showing these data in a Venn diagram indicates a possible novel way to interpret the findings (**Fig. 3.3.13**). The Venn diagram was constructed by focussing on markers of differentiation which are up- or down-regulated by one or more of the retinoid conditions greater than 1.5 fold, with a statistical value of $p<0.06$. A total of nine peaks meet these criteria (indicated in bold in **Table 3.3.1**). Seven out of the nine peak profiles are shared between ATRA and EC23, indicating their effects are similar albeit not identical. Two of these markers are exclusive to ATRA and EC23. It is possible to hypothesise that these are neuronal differentiation markers, however this would have to be verified by further analysis. The five markers shared between each condition could be viewed as general differentiation markers as they are up-regulated in all conditions. The biomarker exclusive to EC19 could potentially be a non-neural differentiation marker. This biomarker platform technology has proved to be a very efficient way for screening novel retinoid compounds against control natural samples as the resultant protein profiles can be compared for similarities and differences. This is also a significantly quicker method for detection as samples can be analysed after only 7 days of culture compared to 21 days for immuncytochemical staining methods outlined above.

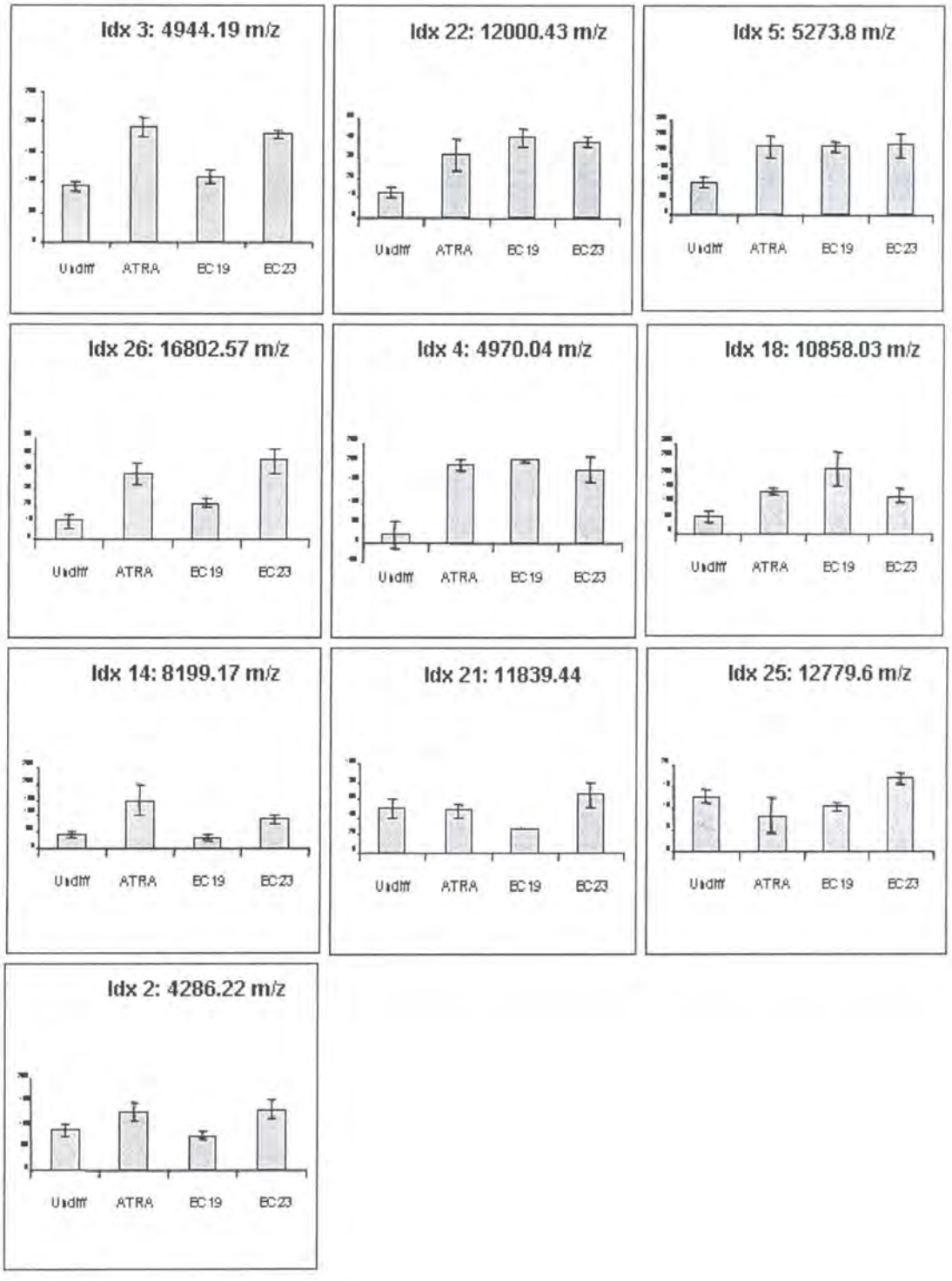


Figure 3.3.12: Graphs showing the average peak distribution areas of the top ten discriminatory protein biomarker hits of undifferentiated EC cells and their differentiated derivatives. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. In nine out of the ten profiles ATRA and EC23 elicit similar trends in protein expression levels (Idx 25 is different). Seven out of the ten hits show a difference in expression levels between EC19 and the other retinoid samples (Idx 5, 4, 25 are similar). n=3

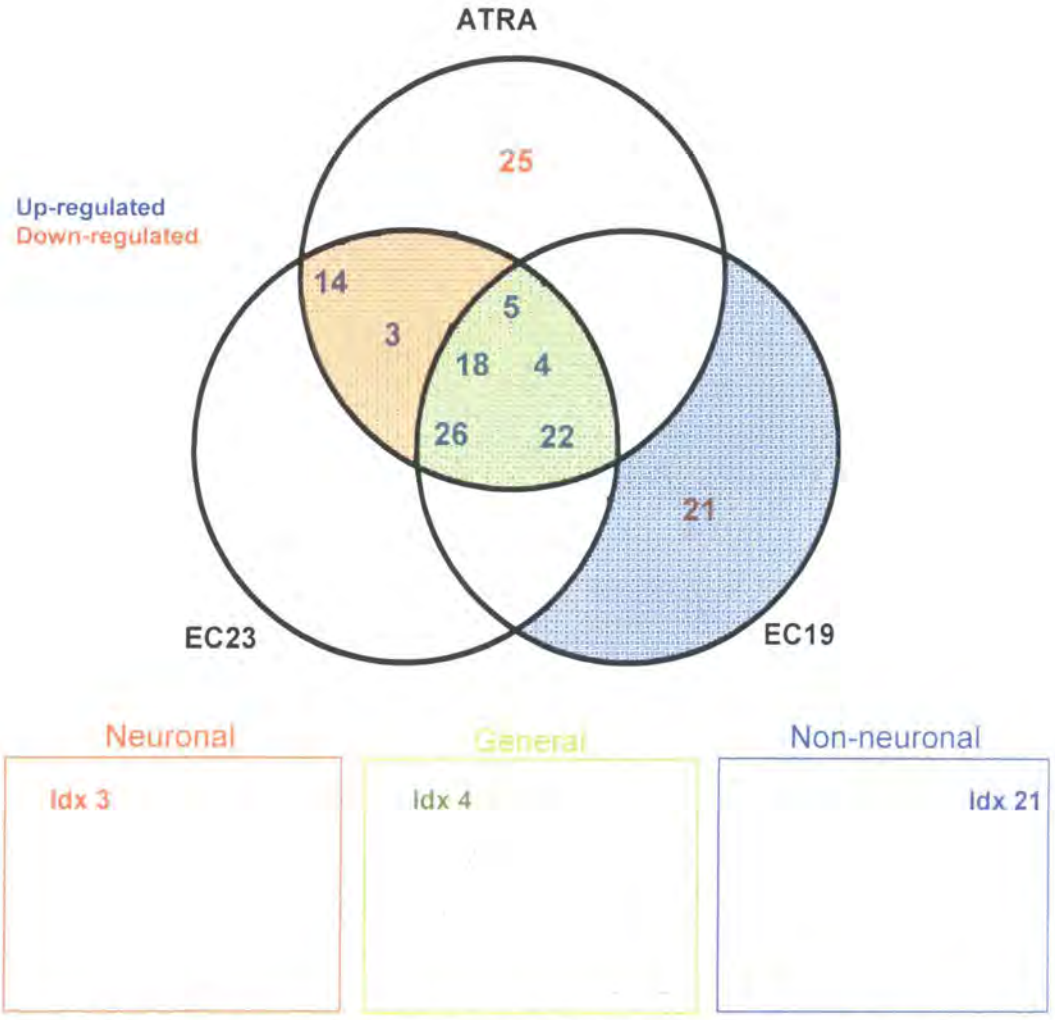


Figure 3.3.13: Venn diagram focussing on markers of differentiation which are up- or down-regulated by one or more of the retinoid conditions >1.5 fold, $p<0.06$. A total of nine peaks meet these criteria. Seven out of the nine peak profiles are shared between ATRA and EC23, indicating their effects are similar but not identical. Two of these are exclusive to ATRA and EC23. A hypothesis could be that these are neuronal differentiation markers. The five markers shared between each condition could be general differentiation markers. The biomarker exclusive to EC19 could potentially be a non-neural differentiation marker. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

3.3.3.2 Analysis and identification of proteins up-regulated after retinoid treatment.

To try and simplify the protein profiles obtained from each sample group and to help aid the identification of some of these up-regulated proteins, fractionation of the total protein lysates was performed. Samples were fractionated using strong anion exchange columns and underwent a NaCl step elution process. The resultant protein fractions were run on SDS-Page gels together with the total protein lysates (**Fig. 3.3.14**). Protein bands were visualised using Coomassie blue protein stain. A protein of interest of mass 15kD (marked with an arrow in the total lysate lane) was seen to be up-regulated in both ATRA and EC23 samples in the total lysate lane, however, it had not shown up on the protein

spectra obtained during the previous total lysate MALDI-TOF analysis. This protein band was tracked to a fraction (marked with a star in 0.1M NaCl lane), and this fraction was re-submitted for MALDI-TOF analysis. Another protein band at 17 kD which elutes in all retinoid conditions but not the undifferentiated control was noted, (marked with star in 0.25M NaCl lane) and this fraction was also re-submitted for further MALDI-TOF analysis. The up-regulation of protein in the ATRA and EC23 conditions with a mass of 15kDa was of particular interest, as it is known that the molecular weights of the cellular retinoic acid binding proteins are around the 15kDa mark.

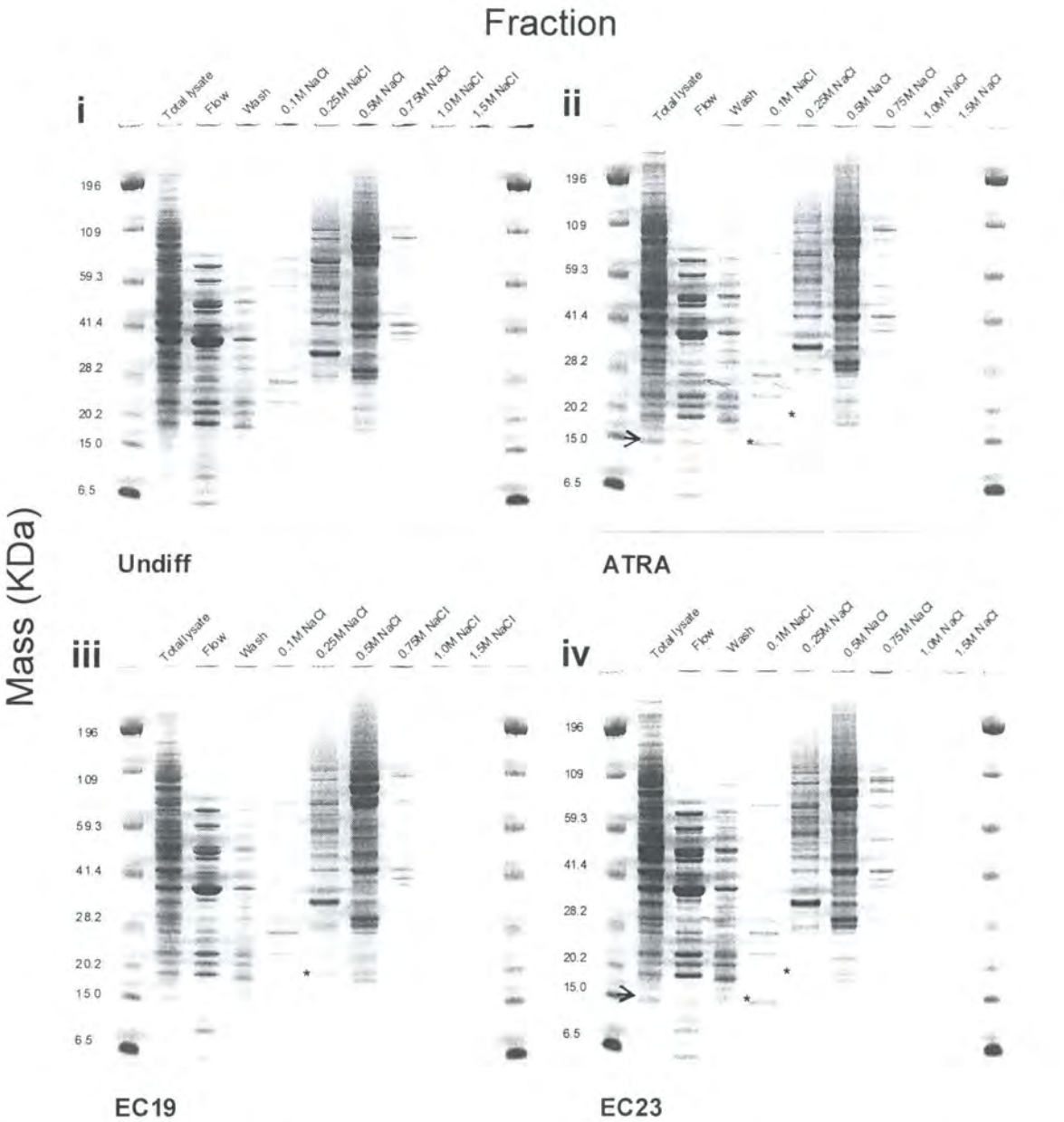


Figure 3.3.14: SDS-Page gels run with total protein lysates and protein fractions obtained after strong anion exchange chromatography and NaCl step elution. A protein of interest of mass 15kD (marked with an arrow in total lysate lane) did not show up on total lysate MALDI analysis. This was tracked to a fraction (marked with a star in 0.1M NaCl lane), and the fraction was re-submitted for MALDI analysis. Another protein band at 17 kD which elutes in all retinoid conditions but not the undifferentiated control was noted, (marked with star in 0.25M NaCl lane) and this fraction was also re-submitted for further MALDI analysis. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

MALDI-TOF re-analysis of total protein lysates alongside fractionated samples revealed that some proteins which *were* visualised readily on SDS-Page gels, were not being visualised in the total protein spectra obtained after MALDI-TOF analysis. However, these specific protein profiles were being visualised after fractionation (As shown in **Fig. 3.3.15A & C**). This phenomena is explained by ion suppression within the total lysate samples, due to the abundance of proteins being analysed at one time. This was overcome when the protein content of the samples were 'thinned out' after fractionation. Pseudo gel images and those actually visualised on the SDS-Page gels from the fractionated samples showed identical patterns of expression (**Fig. 3.3.15B & D**). Further analysis of the proteins within these bands of interest was carried out using fractionated protein lysates.

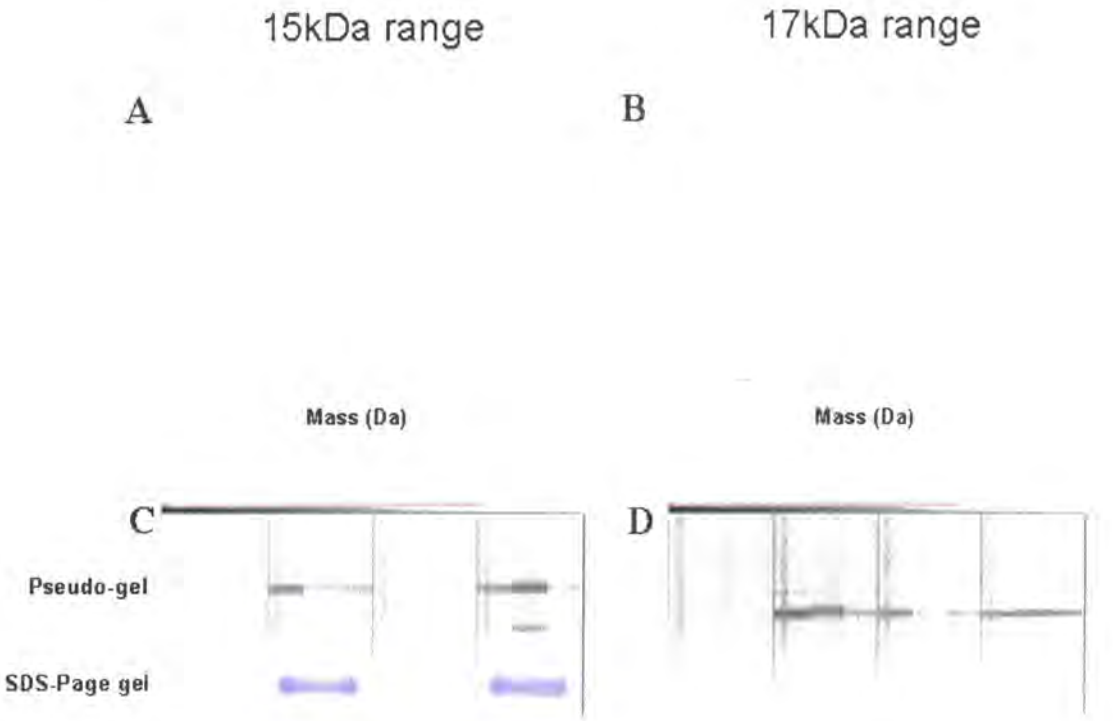


Figure 3.3.15: A & B: Comparison of MALDI spectra analysis from total cell lysate (red line) and specific fractionated cell lysate (green line) as chosen off gel analysis (A: 0.1M NaCl fraction, B: 0.25M NaCl fraction) (see figure 14). It appears that in the total cell lysate the protein peaks are not visualised due to ion suppression. C & D: Corresponding pseudo gel bands of the triplicate fractionated MALDI data are identical to actual fractionated gel images from SDS-page analysis. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

3.3.3.3 Stathmin identification

MALDI-TOF analysis of the 0.25M NaCl fraction showed two proteins were present within the 17kDa protein band visualised on the SDS-Page gels (**Fig. 3.3.16**). The fact that the two proteins were separated by only 80 Da meant they were not resolved separately on a standard SDS-Page gel. This is where the sensitivity of MALDI-TOF analysis becomes apparent. Overlaying all the corresponding fractionated sample spectra at this particular molecular weight indicated that both proteins were up-regulated in ATRA and EC23 cultures, just the 17,223 m/z peak was present in the EC19 condition albeit at a lower intensity than in ATRA and EC23 samples, and none were present/expressed in the undifferentiated control. Corresponding gel bands were digested with trypsin and resultant peptides were analysed by MALDI-TOF MS and submitted for peptide mass fingerprint searches (Mascot). Six matching identifications were obtained from monoisotopic peptide masses (singly charged, [M+H+]+) and were listed in **Table 3.3.3** alongside the corresponding molecular weight search (MOWSE) scores and sequence coverage against the intact matching database sequence. Predicted molecular masses provided from the database searches for each matched database protein sequence, were compared alongside observed intact protein m/z values from our MALDI-TOF protein profiles. From all the searches and analysis it was deduced that the identity of the proteins we were studying in the 17 kDa band and MALDI-TOF traces were stathmin. The exact 80 Da shift in molecular size of the two proteins, and the fact that only one protein ID was obtained, strongly suggested that this was the same protein but with a single phosphorylation modification [141]. Quantitative analysis of the expression profiles of these proteins in the different sample groups was shown in graphical form; the bars represent the mean peak area of the triplicate protein samples analysed via MALDI-TOF (**Fig. 3.3.17 & Table 3.3.2**). The patterns of protein expression were very similar between the ATRA and EC23 cultured cells, again indicating the similarity of the activity induced by these retinoids. Both conditions showed an up-regulation in stathmin expression compared to the undifferentiated control sample, and also a smaller but identical increase in phosphorylated stathmin was recorded. Protein collected from EC19 cultures only exhibited a minor increase in non-phosphorylated stathmin, which was not as pronounced as in ATRA or EC23 treated cultures.

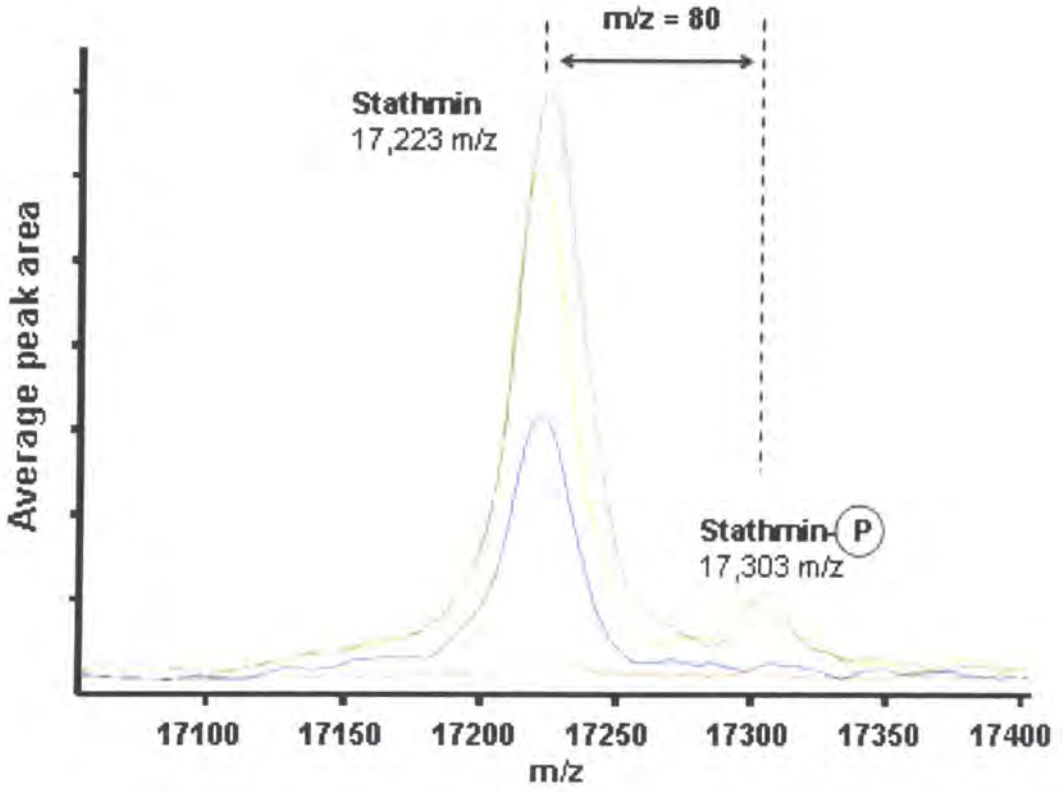


Figure 3.3.16: MALDI protein peak spectra obtained from the 0.25M NaCl SAX fraction from undifferentiated (red), 10μM ATRA treated (green), 10μM EC19 treated (blue), and 10μM EC23 treated cultures (yellow). The 17,223 kDa peak is identified as stathmin (Mass spec analysis- see table 3) and is up-regulated in all retinoid samples, but mainly ATRA and EC23. A smaller protein peak was identified in both the ATRA and EC23 conditions. As it has an exact 80kDa shift it can be tentatively assigned to phosphorylated stathmin, a protein known to be involved in tubulin remodelling during neuronal differentiation. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

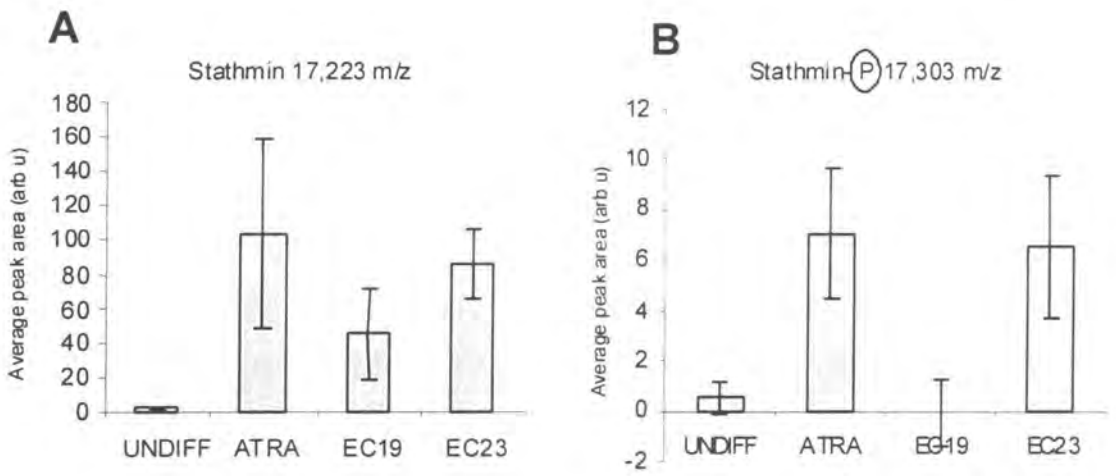


Figure 3.3.17: Quantification of the average peak area plot of stathmin (A) and phosphorylated stathmin (B) peak profiles obtained from MALDI-TOF spectra of fractionated protein lysates. Stathmin is up-regulated in all retinoid conditions, to similar levels in both ATRA and EC23, whereas phosporylated stathmin is only up-regulated in ATRA and EC23, also to a similar level. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

Assignment	Observed m/z	Average peak area				*p*-value
		Undiff	ATRA	EC19	EC23	
~15 kDa (0.1 M NaCl)						
CRABPI	15446.27	-0.02	6.26	0.78	33.57	0.0179
CRABPII	15574.83	0.46	81.01	0.5	79.13	0.131
CRBPI	15730.06	0.34	8.31	1.21	6.63	0.556
Profilin-1	15860.54	0.11	13.79	3.2	22.9	0.365
~17 kDa (0.25 M NaCl)						
Stathmin	17222.8	2.43	103.54	45.5	86.0	0.246
Stathmin-P	17303.26	0.55	7.06	-0.3	6.51	0.298

Table 3.3.2. Peak statistic data for enriched retinoid-induced proteins. Observed mass and average (n=3) MS peak area data for six retinoid-regulated proteins. Four proteins were enriched in 0.1M NaCl eluates from SAX columns and two were enriched in 0.25M NaCl fractions. Statistical p-values (ANOVA) reflect the discriminatory power of each protein to separate the four sample groups (Undiff, ATRA, EC19 and EC23).

Protein Accession number (NCBI)	Gel band	PMF peptides Observed [M+H⁺]⁺	[1]Mowse score [2]Seq. cover.	MS/MS [M+H⁺]⁺	Ions score	[1]Calc. MW [2]Calc. MW - N-term M [3]Calc. pI	Obs. MW
CRABPII (Cellular retinoic acid-binding protein 2) P29373	A	949.3633; 1490.7919; 1108.5153; 1000.5060; 1016.5325; 2449.3147; 1100.4382; 1219.6325	[1]81 [2]44%	-	-	[1]15693 kDa [2]15561 kDa [3]5.42	15574 m/z
	B	1490.7616; 1108.5171; 1016.5327; 2449.2228; 1100.4887; 1822.7433; 2324.9523	[1]82 [2]64%	949.3985 1108.5314	64 53		
CRABPI (Cellular retinoic acid-binding protein 1) P29762	A	852.3503; 1181.5658; 1423.6121	*[1]64 [2]21%	-	-	[1]15566 kDa [2]15434 kDa [3]5.30	15446 m/z
	B	920.3968; 1181.5774; 960.4376; 1447.8814; 1113.4973; 852.3873; 1423.6608; 1551.7852	[1]107 [2]51%	1423.6608 1551.7852	73 71		
CRBPI (Cellular retinol-binding protein 1) P09455	A	-	-	1556.7180 1572.7112	84 58	[1]15850 kDa [2]15719 kDa [3]4.99	15730 m/z
	B	970.4851; 1112.5020; 1556.6618; 1572.6701; 2434.1636; 2650.0905; 1438.6100; 2015.8420	[1]59 [2]65%	-	-		
Profilin-1 P07737	B	1643.8575; 1914.9667; 1470.7026; 1625.6672; 1641.6741; 1870.7272; 1379.6589; 1234.5866	[1]78 [2]52%	-	-	[1]15054 kDa [3]8.44	15860 m/z
Stathmin P16949	C	1388.7062; 1541.7772; 1326.6163; 1074.4746; 1202.5873; 1165.4705	[1]88 [2]31%	1388.7062	97	[1]17302 kDa [3]5.76	17223 m/z

Table 3.3.3. Peptide data and database search results for proteins identified from SDS-PAGE gels. Proteins bands at approximately 15 kDa (0.1M NaCl SAX fraction) and 17 kDa (0.25M NaCl SAX fraction) were excised from gel profiles for ATRA and/or EC23 treated samples. Gel bands were digested with trypsin and resultant peptides were analysed by MALDI-TOF MS and submitted for peptide mass fingerprint searches (Mascot). Matching monoisotopic peptide masses (singly charged, [M+H+]+) are listed alongside corresponding MOWSE scores and sequence coverage against the intact matching database sequence. Peptides analysed using MS/MS fragmentations are detailed alongside corresponding ion scores. Calculated masses are provided in kilo Daltons (kDa) for each matched database protein sequence, alongside observed intact protein m/z values from MALDI protein profiles. In the case of CRABPII, CRABPI, and CRBPI, additional calculated masses are shown, which account for the post-translational removal of N-terminal methionine. Peptide mass fingerprint searches were performed under a mass tolerance of 100 ppm unless stated otherwise in the text (*). A ATRA, 0.1 M NaCl SAX fraction (15 kDa band); B EC23, 0.1 M NaCl SAX fraction (15 kDa band); C ATRA, 0.25 M NaCl SAX fraction (17 kDa band).

3.3.3.4 Cellular retinoid binding proteins identification

MALDI-TOF analysis of the 0.1M NaCl fraction indicated that there were four separate proteins within the 15kDa protein band visualised on the SDS-Page gels (**Fig. 3.3.18**). Again, the fact that the four proteins were separated by only 414 Da meant they were not sufficiently resolved on a standard SDS-Page gel. This is where the sensitivity of MALDI-TOF analysis became apparent again. However, the 15 kDa band visualised in the SDS-Page gel was bigger than the previously analysed 17 kDa band, so was divided into two sections, the top half and the lower half, and underwent separate mass spec analysis. This was done in an attempt to correctly assign protein identification to the corresponding MALDI-TOF spectra.

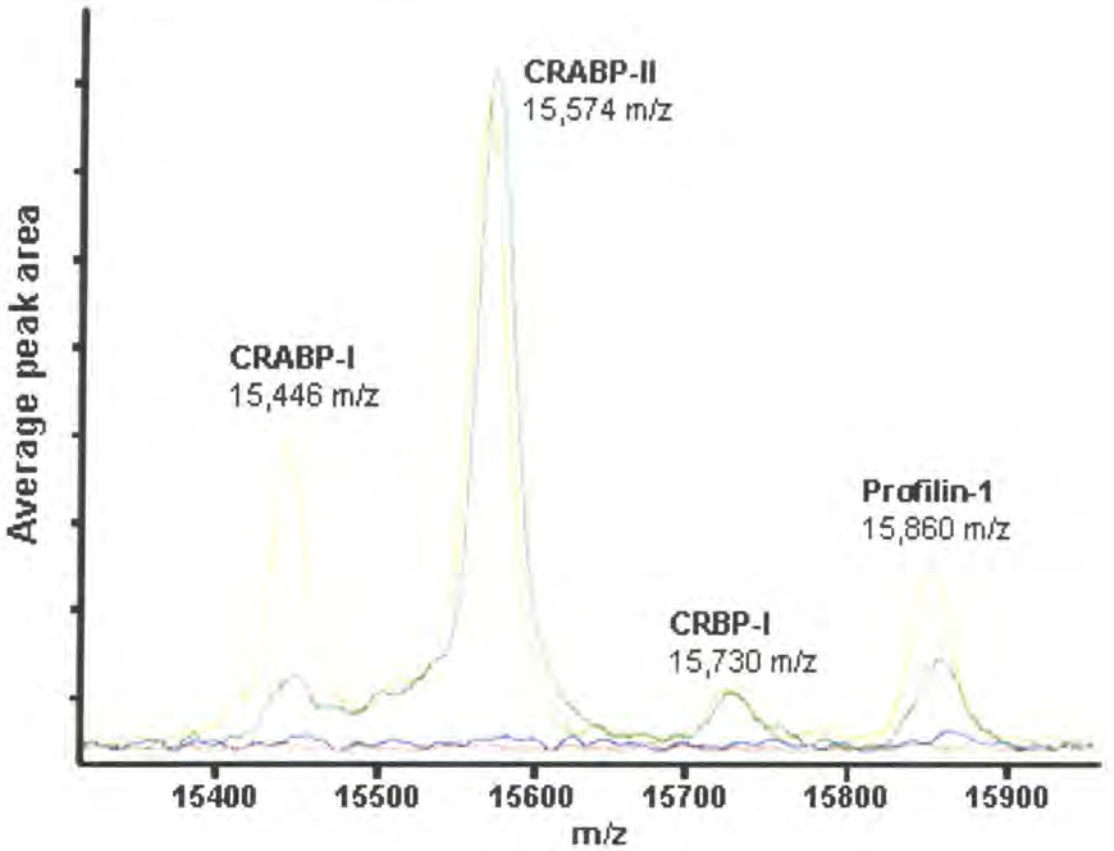


Figure 3.3.18: MALDI-TOF protein peak spectra obtained from the 0.1M NaCl SAX fraction from undifferentiated (red), 10μM ATRA treated (green), 10μM EC19 treated (blue), and 10μM EC23 treated cultures (yellow). The 15,446 kDa peak is identified as cellular retinoic acid binding protein-1 (CRABP I) and is up-regulated in both ATRA and EC23 conditions. The 15,574 kDa peak is identified as cellular retinoic acid binding protein-2 (CRABP II), and is strongly up-regulated in both ATRA and EC23 conditions. This identity assignment is backed up by the 128kDa difference between the first 2 peaks, which is identical to the difference in their predicted masses in database searches. The third peak at 15,730 kDa is identified as cellular retinol binding protein-1 (CRBP I), which again has an identical increase in mass size (156kDa) from the previous peak compared to the predictive mass change. The final peak in the group at 15,860 kDa is identified as profilin-1, a protein involved in actin remodelling. (All protein identities are assigned after mass spec analysis and the associated predictive masses, see table 3). Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

Analysis of the lower portion of the excised gel band identified three proteins. The gel band was digested with trypsin and resultant peptides were analysed by MALDI-TOF MS and submitted for peptide mass fingerprint searches (Mascot). Matching identifications were obtained from monoisotopic peptide masses (singly charged, [M+H+]+) and were listed in **Table 3.3.3** alongside the corresponding MOWSE scores and sequence coverage against the intact matching database sequence. Predicted molecular masses provided from the database searches for each matched database protein sequence, were compared alongside observed intact protein m/z values from our MALDI-TOF protein profiles. The first protein to be identified was cellular retinoic acid binding protein II (CRABP-II). This ID had eight matching peptides from the ATRA gel and 7 matching peptides from the EC23 gel with corresponding sequence coverage's scores of 44% and 64% respectively. The next protein identified was cellular retinoic acid binding protein I (CRABP-I). This ID had 3 matching peptides from the ATRA gel and 8 matching peptides from the EC23 gel with corresponding sequence coverage's of 21% and 51% respectively. The calculated masses of CRABP-I and CRABP-II are 15,566 and 15,693 Da respectively, the difference between them being 127 Da. This mass difference corresponded very closely with the observed m/z difference between the first two MALDI-TOF spectra peaks (128m/z), thus enabling a tentative assignment to these two peaks as CRABP-I and II (**Fig. 3.3.18**). The third protein to be identified elicited eight matching peptides from the EC23 gel with sequence coverage of 65%. Initially no matching peptides were found from the ATRA gel, however, further MS/MS mass fingerprinting did elicit 2 peptides of similar mass. The protein ID for the third hit was identified as cellular retinol binding protein I (CRBP-I). The calculated mass for this protein was 15,730 Da, 156 Da more than the calculated mass of CRABP-II. This mass difference again, corresponded very closely with the observed m/z difference between the second and third peaks visualised on the MALDI-TOF protein spectra (156m/z), therefore the third peak was again tentatively assigned with this protein ID (**Fig. 3.3.18**).

Quantitative analysis of the expression profiles of these proteins in the different sample groups was shown in graphical form; the bars represent the mean peak area of the triplicate protein samples analysed via MALDI-TOF (**Fig. 3.3.19 & Table 3.3.2**). All four proteins are up-regulated in both ATRA and EC23 samples compared to the undifferentiated controls, with EC23 showing a higher expression level of both CRABP-I and the as yet unidentified protein, compared to ATRA. EC19-induced cultures displayed

very little protein expression of both CRABP-I or –II, and only a slight increase in expression levels of CRBP-1 and profilin-1 above the base control level. However, neither protein amount reached that of either ATRA or EC23 samples. The patterns of protein expression were again relatively similar between the ATRA and EC23 cultured cells, indicating the similarity of the activity induced by these retinoids.

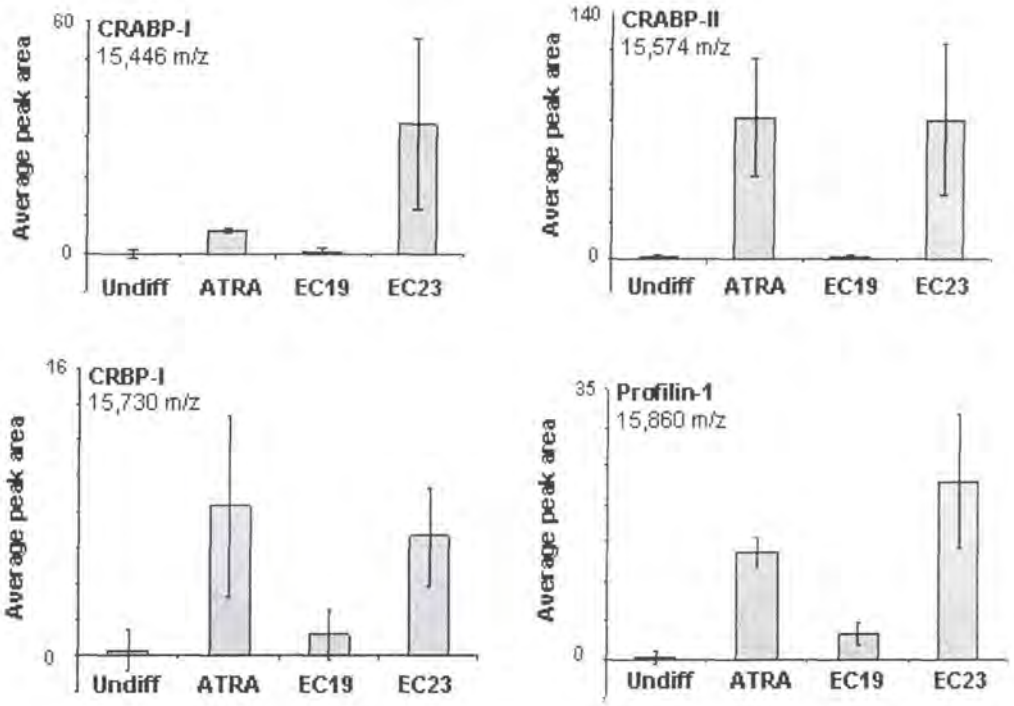


Figure 3.3.19: Average peak area plots of CRABP I, CRABP II, CRBP I and profilin-1 peak profiles obtained from MALDI-TOF spectra of fractionated protein lysates. CRABP I is up-regulated in both ATRA and EC23 conditions, but most strongly in EC23. CRABP II is equally up-regulated in both ATRA and EC23 conditions, as is CRBP I, which is also slightly up-regulated in the EC19 sample. Profilin-1 is up-regulated in all retinoid samples compared to the undifferentiated control, with the strongest up-regulation seen in EC23 followed by ATRA and EC19 respectively. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3

There was a consistent discrepancy between the observed and calculated masses of CRABPI, CRABPII and CRBPI (119, 118, and 120 Da, respectively). This was attributed to the post-translational removal of N-terminal methionine residues (131 Da). The N-terminal peptide for all three proteins was detected in their respective peptide mass fingerprints, and was found in each case to lack the starting methionine. Taking this into account the observed and calculated values differ by approximately 12 mass units only. Accordingly, we are confident that we have assigned these proteins to the correct peaks in the MALDI spectra.

3.3.3.5 Profilin-1 identification

To try and identify the final protein in the group of four visualised on the MALDI-TOF spectra the top half of the 15kDa band from the 0.1 M NaCl fraction underwent peptide mass fingerprinting. After initial database interrogation, 2 protein hits were returned; nine matching peptides were found for profilin-1 with sequence coverage of 52% and eight further peptides were found for CRBP-1 (**Table 3.3.3**). Unmatched peptides were resubmitted and returned a match with CRABP-II (eight peptides). Therefore the final protein in our MALDI-TOF spectra profile was assigned the identity of profilin-1. However, the calculated mass for profilin-1 is only 15,054kDa, significantly different from the assigned peak m/z of 15,860. We believe this assignment is correct as profilin-1 was only identified in the top half of the gel band, which corresponds to the higher m/z proteins, therefore we attribute this extra mass to some kind of post-translational modification. It is well known that profilin-1 can undergo phosphorylation, and also that this modification is likely to play a regulatory role [142]. Phosphorylation of profilin would help account for its co-elution with proteins of different calculated isoelectric points (see **Table 3.3.3**). Thus, although non-phosphorylated profilin (calculated p*I* 8.44) would be anticipated to be only weakly anionic under the SAX fractionation conditions (pH 9.0), multiple post-translational phosphorylation could potentially impart a significantly lower p*I* value. Profilin-1 has been shown to influence the dynamics of the actin cytoskeleton during neuronal differentiation [143]. Therefore we can tentatively suggest that the up-regulation of profilin-1 we see in the ATRA and EC23 samples could be attributed to actin re-modelling during neuronal differentiation (**Fig. 3.3.19**).

3.3.3.6 Immunological verification of CRABP identifications

To verify the identities of the CRABPs obtained via MALDI-MS, antibody detection was performed on western blot membranes. Blotted membranes were obtained from both the total cell lysate protein and the 0.1M NaCl fractionated protein samples from all three retinoid conditions and compared against the undifferentiated control (**Fig. 3.3.20A**). The antibody used was a monoclonal human specific antibody for CRABP-I, however, when the company were queried over the specificity of the antibody it could not be verified, and therefore is considered to be recognised by at least both the CRABP-I and –II isoforms and possibly the CRBPs as well. Immuno-reactive signals were quantified for the fractionated sample data and shown in graphical form (**Fig. 3.3.20B**). The highest

level of expression for this antibody was seen in the EC23 sample followed by ATRA, EC19 and undifferentiated cells respectively. This expression pattern is consistent with that seen in the MALDI data, however, in the MALDI data a greater difference was seen between the expression levels visualised in the ATRA and EC23 samples compared with EC19. Also no detection was visualised in the undifferentiated samples. This could be due to the non-specificity of the antibody used, or more likely, due to the greater sensitivity of detection in the western blot and the higher detection threshold in the MALDI-TOF spectra.

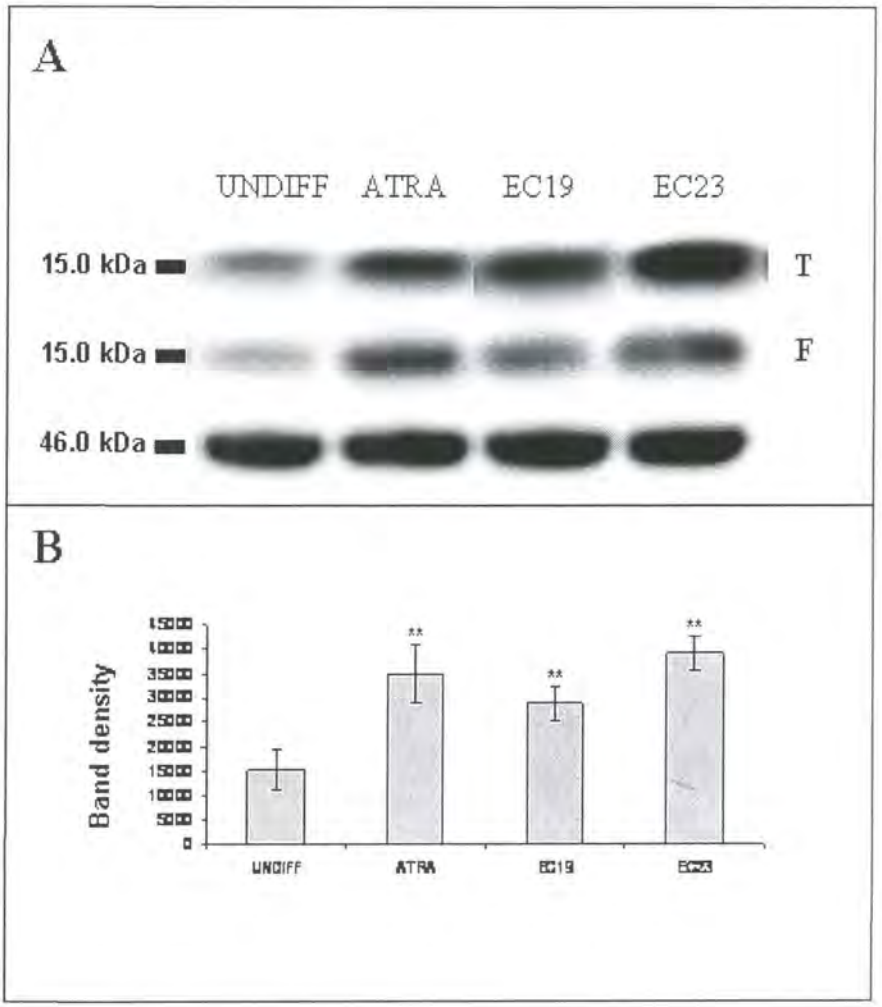


Figure 3.3.20: Verification of protein identifications obtained after mass spec analysis was carried out via western blot analysis. A: 10 % acrylamide SDS-page gels were probed for an antibody raised against CRABP-I. Protein profiles were obtained from total protein lysates (T) and the fractionated protein samples (F). The visualised bands from the fractionated sample underwent densitometric analysis as shown in graph B. Retinoid protein samples were obtained from EC cells cultured with 10μM retinoid for 7 days. n=3. . ** $p \leq 0.005$, student's t-test compared to the undifferentiated control.

3.3.4 The Biological Analysis of Natural and Synthetic Retinoid Activity on H9 Human Embryonic Stem Cells.

The culture of both synthetic and natural retinoids with the robust stem cell model system Tera2.sp12 provided insight into both the natural and synthetic retinoids mode of actions. Results obtained so far indicated that the synthetic retinoid EC23 elicited similar biological responses to the natural retinoid ATRA, potentially via similar retinoic acid induced pathways. Both retinoids induced a significant amount of neuronal differentiation within the culture conditions tested. However, the important difference between the two molecules is that EC23 is more stable under standard laboratory conditions, making it a more favourable alternative for *in-vitro* work. To further observe the biological similarity of EC23 to ATRA, experiments were conducted where the retinoids were cultured with pluripotent human embryonic stem cells, to further assess the potential of the compounds to induce cell development. Markers of neural differentiation were analysed to see if the retinoids were able to direct neuronal differentiation in this model system.

Two methods of inducing cell differentiation were carried out: a specific method for the induction of neuronal cell types; and a generic differentiation protocol involving the formation of embryoid bodies. These were chosen to see if the retinoids (both ATRA and EC23) had any effect on the differentiation products visualised in either circumstance. Control differentiation cultures were set up in each case and acted as the base level of differentiation induced with each protocol. One μM concentrations of retinoids were used in all experiments.

3.3.4.1 Culture techniques required for optimal ES cell proliferation

The culture of human H9 embryonic stem cells (ES cells) requires a defined culture condition, and deviation away from this may result in spontaneous differentiation. ES cells are cultured with the aid of a mouse embryonic fibroblast feeder layer (MEFs) which have been mitotically inhibited with exposure to mitomycin-C 24 hrs before use. Small colonies of ES cells expand in the proliferative media, 'pushing' the MEFs out of the way (**Fig. 3.3.21**). It is thought that the MEFs release important factors which are required for ES cell proliferation; however the exact properties of these factors are as yet

unknown. As the colonies expand areas of spontaneous differentiation occur and need to be scraped off to maintain a pure ES cell culture, this procedure is done every three days or as necessary. Cultures also needed to be plated onto freshly inactivated MEFs every 7 days to limit this spontaneous differentiation. Cultures underwent total removal of differentiated material before use in the differentiation experiments.

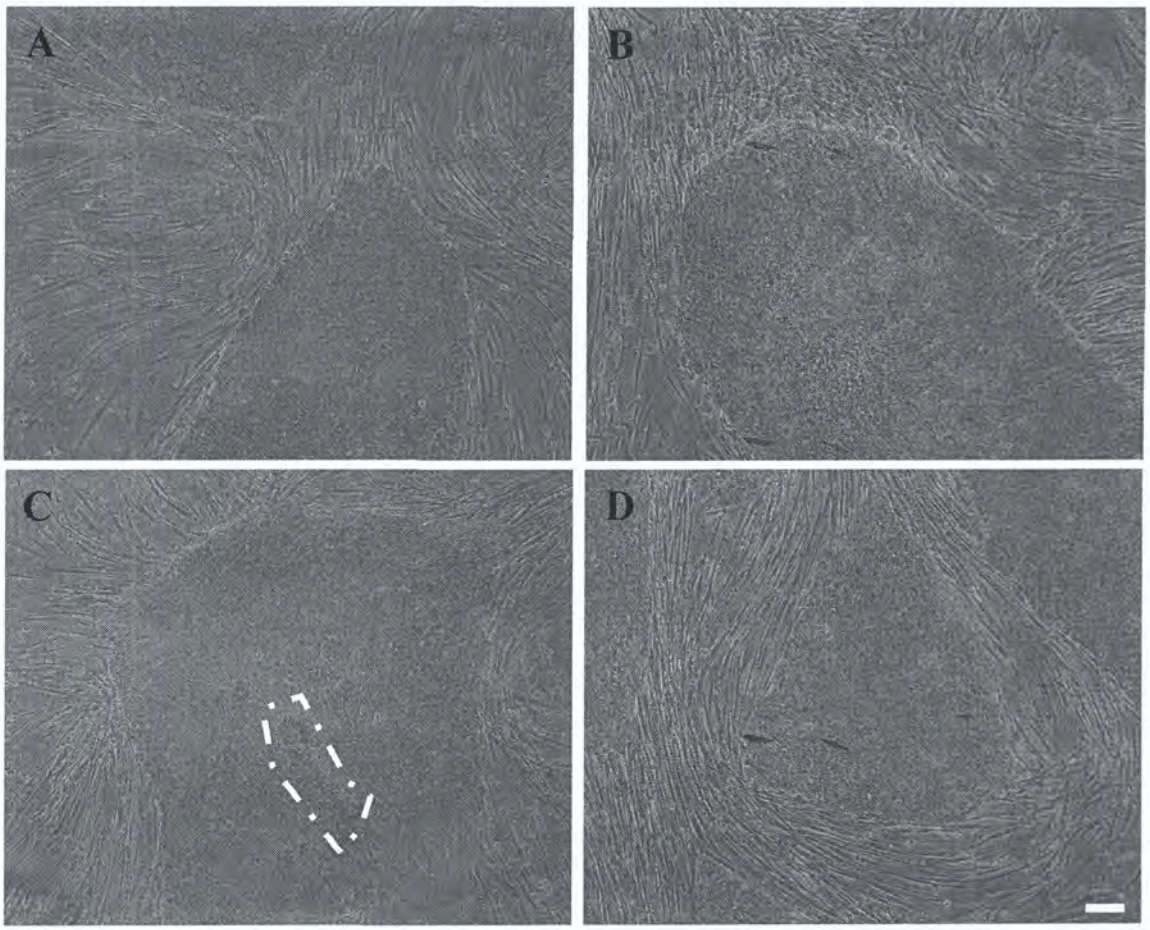


Figure 3.3.21: Undifferentiated human ES cells, cell line H9. The proliferation of the human ES cell line H9 was propagated with growth on an inactivated mouse fibroblast feeder layer. Colonies of ES cells are visualised surrounded by the feeder layer. Areas of spontaneous differentiation (outlined by the dotted line in C) were removed with a 100μL pipette tip using a dissection microscope. Scale bar represents 50μm. n=5

3.3.4.2 Specific neuronal-inducing differentiation protocol

Initially, cultures were induced to differentiate using a defined neuronal differentiation protocol, whereby cells were grown in monolayer on 1% gelatine coated plates in defined neuro-basal media. After 7 days, the cultures were switched to neuro-basal media plus bFGF, to induce outgrowth of neuronal cells, with or without retinoids, and left for a further two weeks. At this time point many neurons were visualised in all conditions

except the control cultures, which had been plated but left in proliferative media (**Fig. 3.3.22**). These cells had spontaneously differentiated but did not appear to be neural, they appeared to contain a high content of fat lipids; however this observation was not verified and analysis of these cultures was not taken any further.

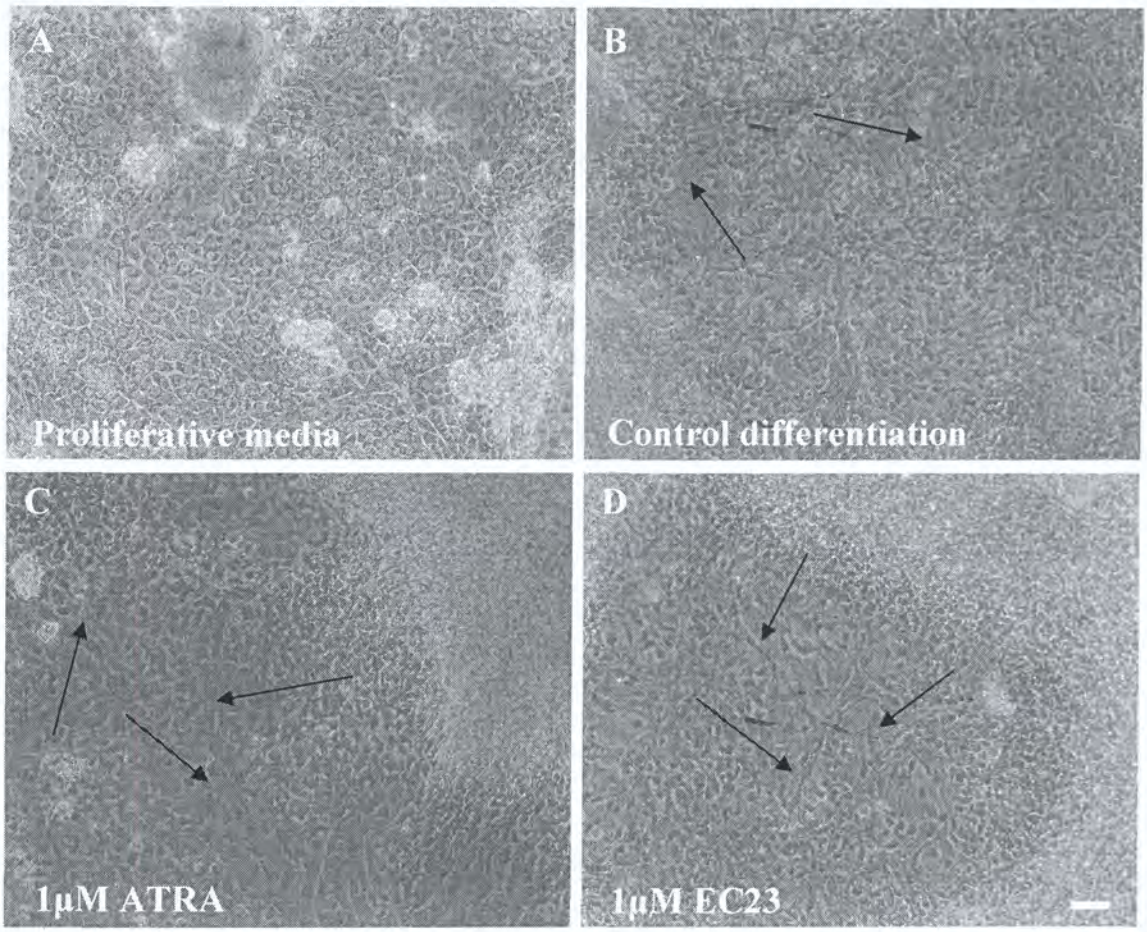


Figure 3.3.22: Differentiated human ES cells, cell line H9. The differentiation of the human ES cell line H9 was induced by plating on 1% gelatine coated tissue culture plastic (A). Neuronal differentiation was enhanced by incubation in defined neuronal medium (B), plus the addition of retinoids (B, C). Neuronal processes (arrows) are readily visualised in all three neuronal culture conditions. Scale bar represents 50μm. n=3

To try and quantify the extent of neuronal differentiation a panel of neural markers was used to immunocyto-stain the obtained cultures. Nestin, an early neural marker, clearly showed differential expression levels between the three different culture conditions. Many of the long neural processes were stained with nestin in the control differentiation condition compared with both the ATRA- and EC23-supplemented cultures, suggesting the presence of immature neuronal phenotypes (**Fig. 3.3.23**) In the retinoid conditions, the levels of nestin expression was decreased throughout the whole culture compared to control differentiation conditions, and very few of the long neural processes visualised

under light microscopy were nestin positive. This again, could indicate that the cultures obtained with retinoid supplementation are more mature than those seen in the control differentiation, without retinoid supplementation, alone. All cultures contained neuronal cells which were positive for the β-III-tubulin marker, TUJ1 (**Fig. 3.3.23**). There did not appear to be any differential expression of this marker between the different culture conditions tested. Next, two more mature markers of neuronal differentiation were analysed, namely the late neuronal marker neurofilament-200 (NF-200), and the motor neuron specific marker HB9 (**Fig. 3.3.24**). Low power (X4 objective) images showed the length and extent of NF-200 positive neuronal in all conditions tested, again showing no real distinction between the different conditions. However, the expression of the motor neuron marker HB9 did appear to be differentially expressed. HB9-positive cell nuclei were visualised in the control differentiation condition, but upon supplementation with either ATRA or EC23 the number of positive cells increased. This result was quantified using ImageJ software to count the number of positive cell nuclei/field of view in randomly selected images (**Fig. 3.3.25**). An increase in the number of HB9-positive cell nuclei upon ATRA or EC23 supplementation was confirmed. Both retinoids induced a similar increase in expression.

Following a defined neuronal-inducing culture protocol, it was shown that H9 human stem cells are able to differentiate down a neuronal lineage. Supplementation with either ATRA or EC23 into the culture media appeared to induce an increase in number of cell phenotypes indicative of a more caudal CNS location, as would be expected after ATRA introduction. The synthetic retinoid EC23 has again been shown to mimic ATRA action.

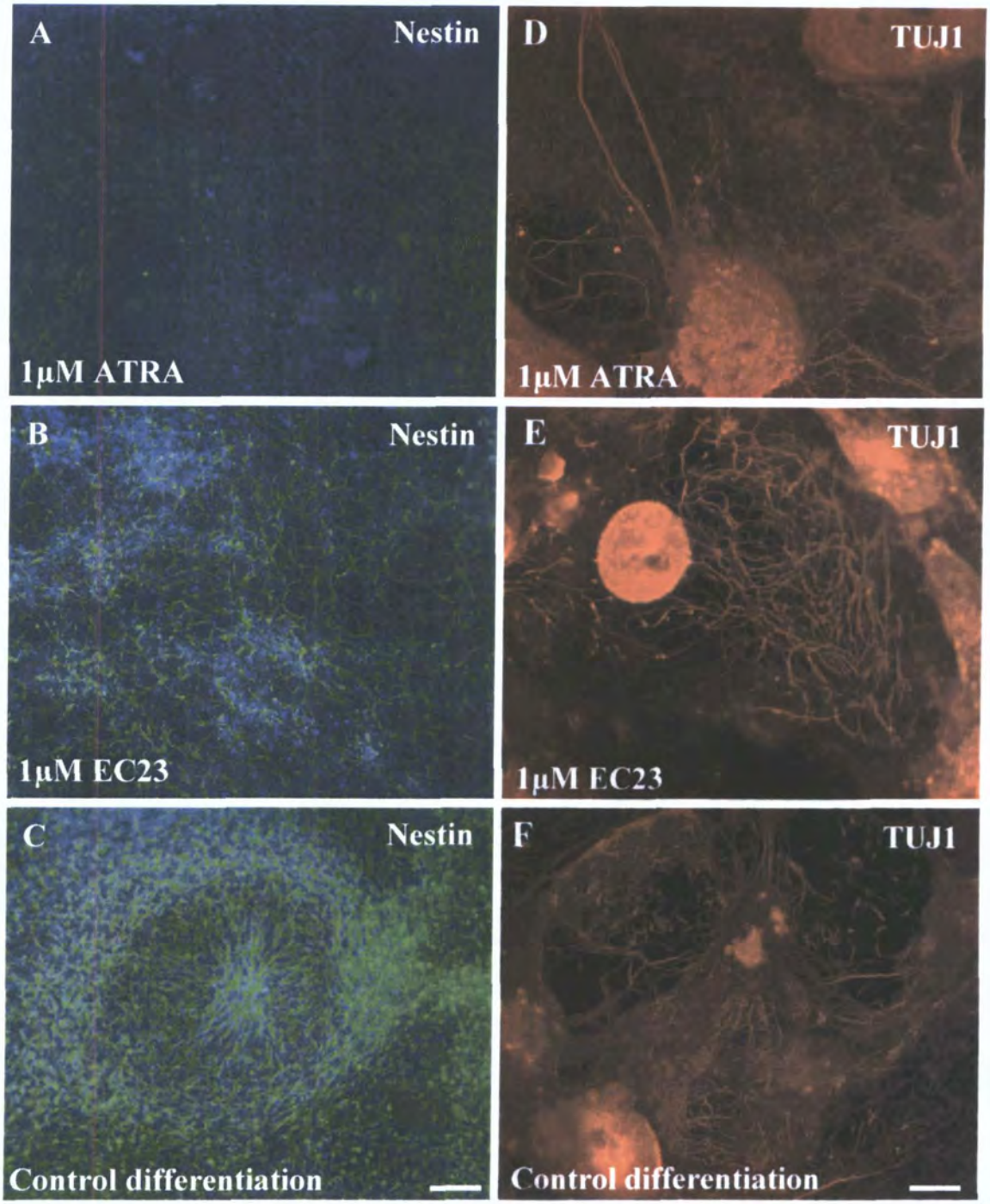


Figure 3.3.23: Nestin and TUJ1 immunostaining of differentiated H9 stem cells after 21 days of culture. ES cells were differentiated on 1% gelatine coated 12-well plates. Cultures were maintained in either differentiation media alone (C, F), or differentiation media plus 1μM ATRA (A, D) or 1μM EC23 (B, E). Nestin positive cells were found throughout the culture of the control differentiation condition. Nestin expression was visualised in a few distinct patches in both ATRA and EC23 conditions, with most of the cultures being negative for this antibody. TUJ1 positive neurons were found throughout the culture of all differentiation conditions. Images were captured after the 21 day culture period. Nestin: Scale bar represents 100μm. TUJ1: Scale bar represents 250μm. n=3

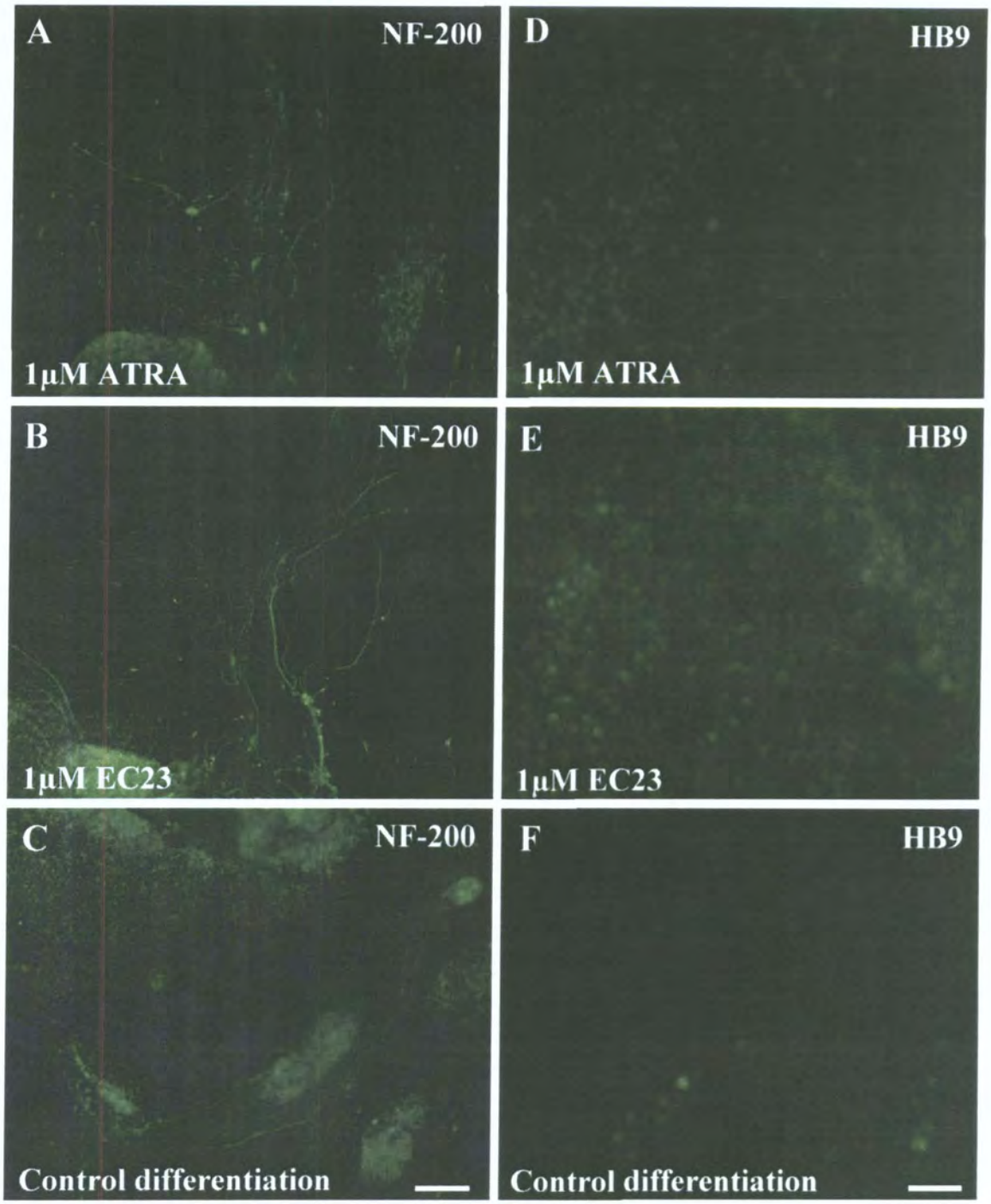


Figure 3.3.24: NF-200 and HB9 immunostaining of differentiated H9 stem cells after 21 days of culture. ES cells were differentiated on 1% gelatine coated 12-well plates. Cultures were maintained in either differentiation media alone (C, F), or differentiation media plus 1μM ATRA (A, D) or 1μM EC23 (B, E). NF-200 positive neurons were found throughout the culture of all differentiation conditions. HB9 positive nuclei were visualised in all conditions, with increased amounts in ATRA and EC23 supplemented cultures. Images were captured after the 21 day culture period. Scale bar represents NF-200: 250μm, HB9: 50μm. n=3

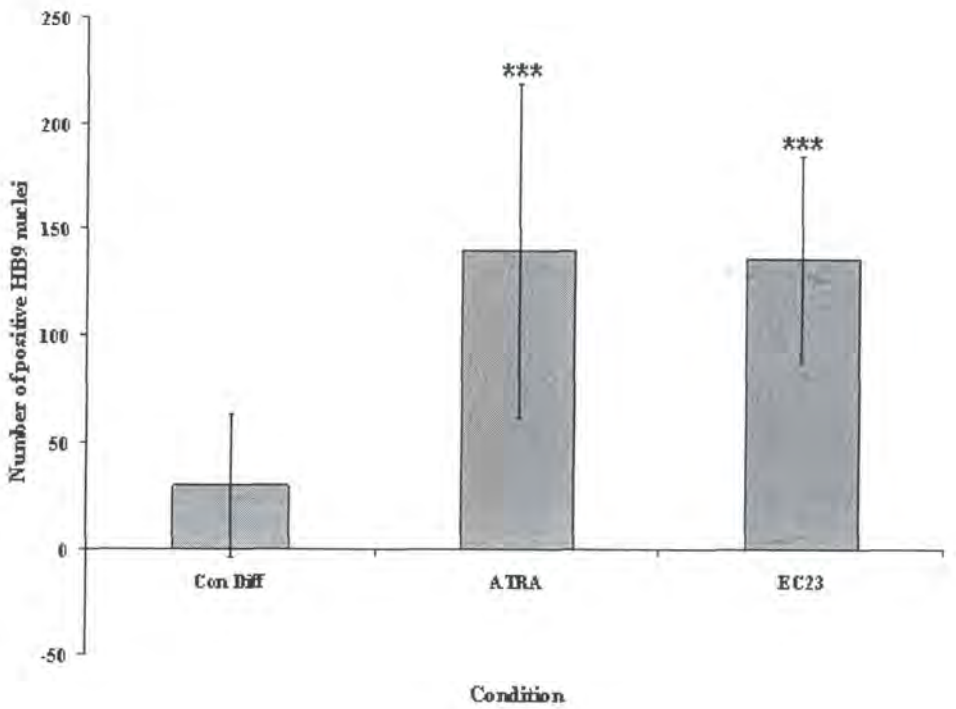


Figure 3.3.25: Quantitative analysis of HB9 positive cell nuclei in differentiated H9 stem cells. Cultures were maintained in either differentiation media alone (control differentiation), or differentiation media plus 1μM ATRA or 1μM EC23. Using ImageJ software the number of HB9 positive cell nuclei were counted on 13 random fields of view per condition. Results were averaged and represented graphically. An increase in the motor-neuron marker HB9 was visualised after cultures were supplemented with either ATRA or EC23. Error bars represent standard deviation of the mean n=13. *** $p \leq 0.0005$, student's t-test compared to control differentiation.

3.3.4.3 Generic differentiation protocol

This method was followed to observe the responses induced by the retinoids during a non-specific differentiation cue. Unlike in the Tera2.sp12 system, to obtain neurons from ES cells, very defined protocols are followed, i.e. the addition of just retinoids is not thought to be sufficient. As these cells retain the capacity to differentiate into all the cells of the body, this was another good test to ascertain how closely EC23 would mimic ATRA.

To initiate differentiation ES cell colonies were grown in generic differentiation media, with and without retinoids, in bacteriological dishes. As these dishes are low attachment, floating embryoid bodies (EB) were formed and maintained for two weeks. After the culture period EBs were transferred to poly-L-ornotine/laminin coated 24-well plates and

maintained for a further week, after which cells were fixed and immuncyto-stained. It was noted that measurements of diameter of the EBs formed after the two week culture period in the presence of both ATRA or EC23 were significantly smaller than those formed in just the differentiation media alone (**Fig. 3.3.26**). After plating out, cells had migrated away from the central EB in all conditions, but no obvious neural cells were visible under light microscopy, unlike in the neuronal-defined experiment (**Fig 3.3.27**).

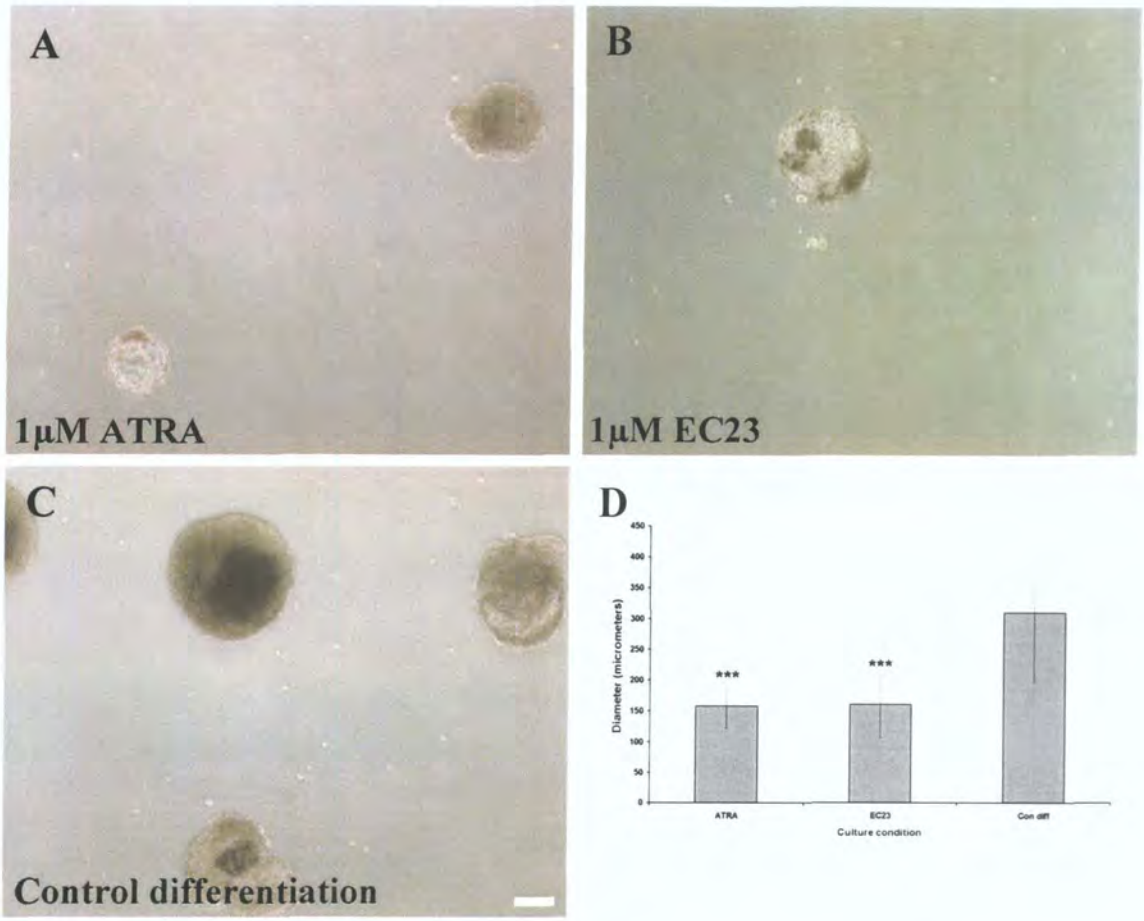


Figure 3.3.26: Human stem cells, H9, were induced to differentiate and form embryoid bodies. Cells were cultured in defined differentiation media in uncoated bacteriological dishes for 14 days. Cells were either cultured in differentiation media alone (C), or differentiation media plus 1μM ATRA (A) or 1μM EC23 (B). Images were captured of embryoid bodies after the 14 day culture period. The embryoid bodies in both retinoid treated cultures were smaller than those observed in control differentiation cultures. Scale bar represents 50μm. n=2 Using ImageJ software the diameter of the embryoid bodies was calculated and visualised in graphical form (D). On average the embryoid bodies were larger in the control differentiation condition. n= at least 8. Error bars represents ± standard deviation of the mean. Images were taken at X5 objective. *** $p \leq 0.0005$, student's t-test compared to control differentiation.

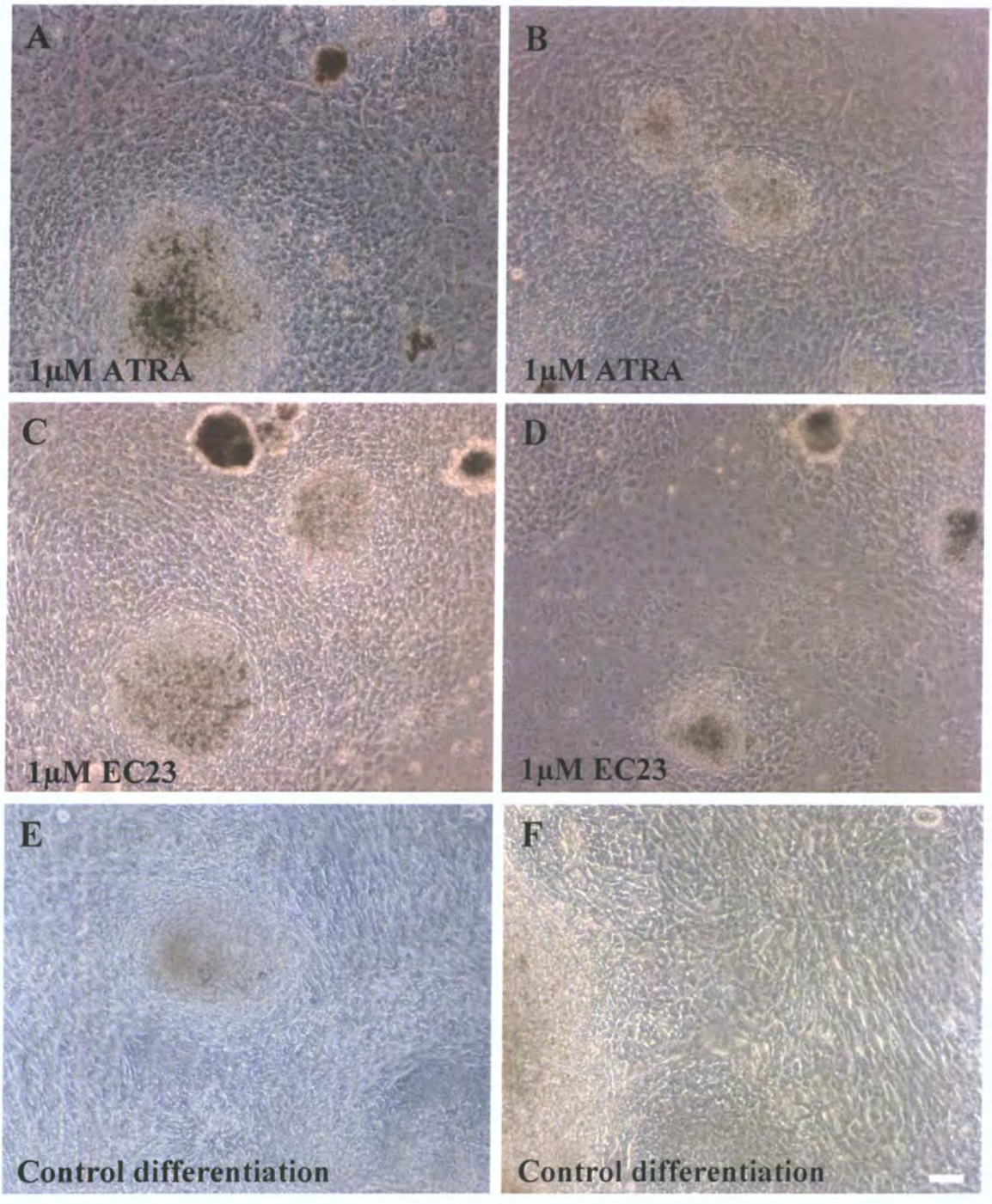


Figure 3.3.27: Differentiated H9 stem cells after 21 days of culture. After 14 days culture as embryoid bodies, the differentiated stem cells were then cultured on poly-L-ornothine/laminin coated 24-well plates for a further 7 days. Cultures were continued in either differentiation media alone (E, F), or differentiation media plus 1μM ATRA (A, B) or 1μM EC23 (C, D). Differentiated cells appeared to grow out from the aggregates in all conditions tested. Images were captured after the 7 day culture period. Scale bar represents 50μm. n=2

Nestin staining revealed differing patterns of expression between cultures incubated with the retinoids and those incubated without. In both ATRA and EC23 cultures small areas of nestin positive cells were visualised, normally associated around an EB, however nestin expression in the control differentiation condition was much more generic and ubiquitously expressed throughout the culture (**Fig. 3.3.28**). The neuronal specific marker TUJ1 was next examined. In both ATRA and EC23 cultures no visible neuronal differentiation had occurred, however the antibody was expressed in some large flat cells of unknown identity (**Fig. 3.3.29**).The expression of the glial marker GFAP was subsequently analysed to see if these cells were glia, possibly reactive astrocytes, however, no GFAP expression was seen in any of the conditions analysed. It is therefore possible that this antibody is not specific for neuronal cells and that ATRA and EC23 have induced some other form of differentiation within these ES cell cultures. Small areas of typical TUJ1 neuronal expression was visualised in the control differentiation culture. The neurons appeared to be emanating from the EBs, suggesting that the neural differentiation had been initiated during the EB stage of the culture protocol. It is interesting to note that the large flat positive TUJ1 cells were also visualised within this culture condition, but at a much lower frequency. It appears as if the addition of both retinoids in this differentiation protocol inhibits neuronal differentiation within the EBs. It is important to note that yet again EC23 is mimicking the effects of ATRA. As mentioned above ATRA is an important morphogen during many different embryonic developmental processes so it is unsurprising that when ATRA or EC23 is given to ES cells in an undefined culture media, a variety of cell types form.

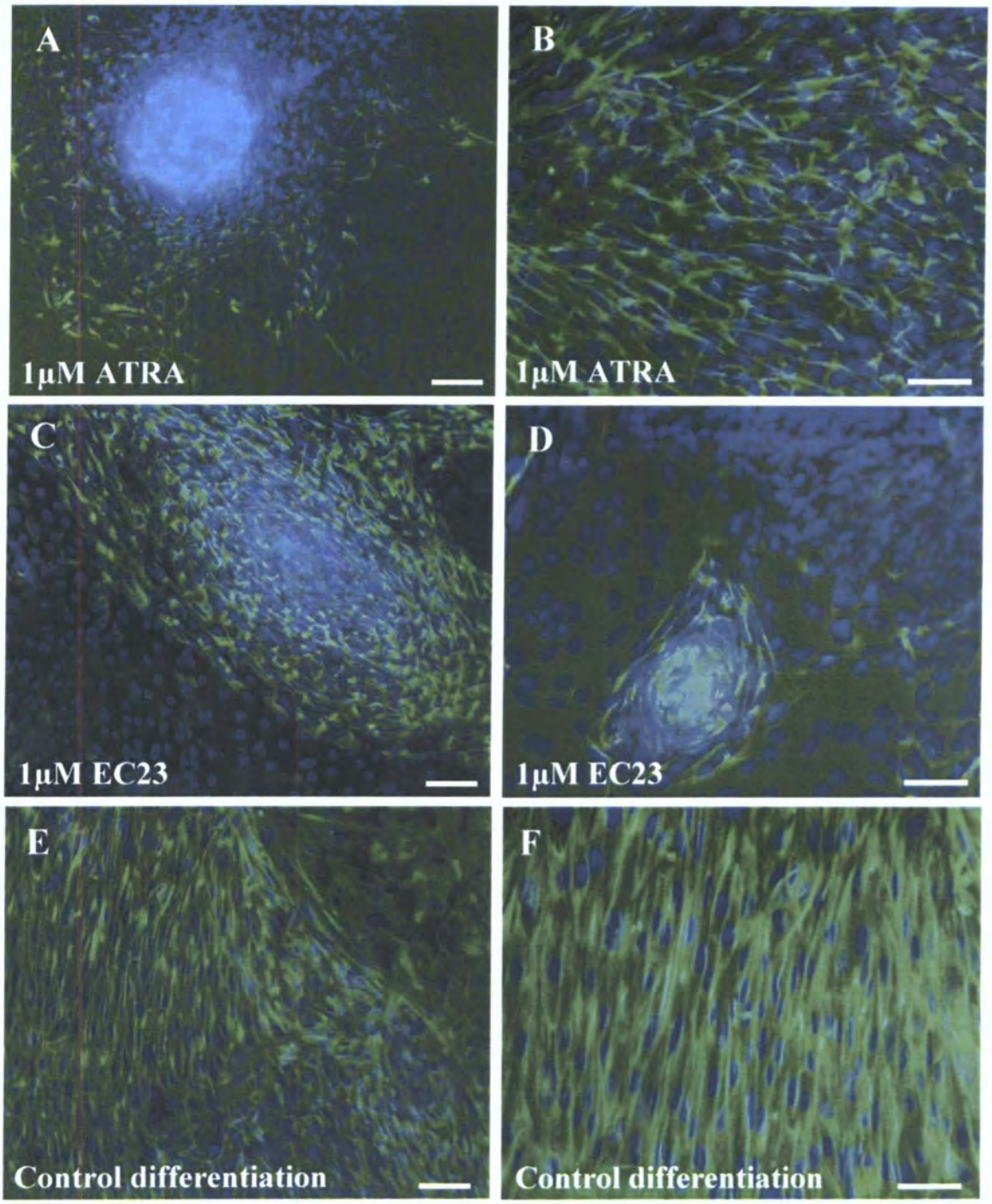


Figure 3.3.28: Nestin immunostaining of differentiated H9 stem cells after 21 days of culture. ES cells were differentiated for 14 days as embryoid bodies, then cultured for a further 7 days as monolayers on poly-L-ornothine/laminin coated 24-well plates. Cultures were maintained in either differentiation media alone (E, F), or differentiation media plus 1μM ATRA (A, B) or 1μM EC23 (C, D). Patches of nestin positive cells were visualised in both cultures exposed to retinoids, however the number of nestin positive cells were not equal to that found in the control samples. Images were captured after the 21 day culture period. Scale bar represents A, C, E: 100μm, B, D, F: 50μm. n=2

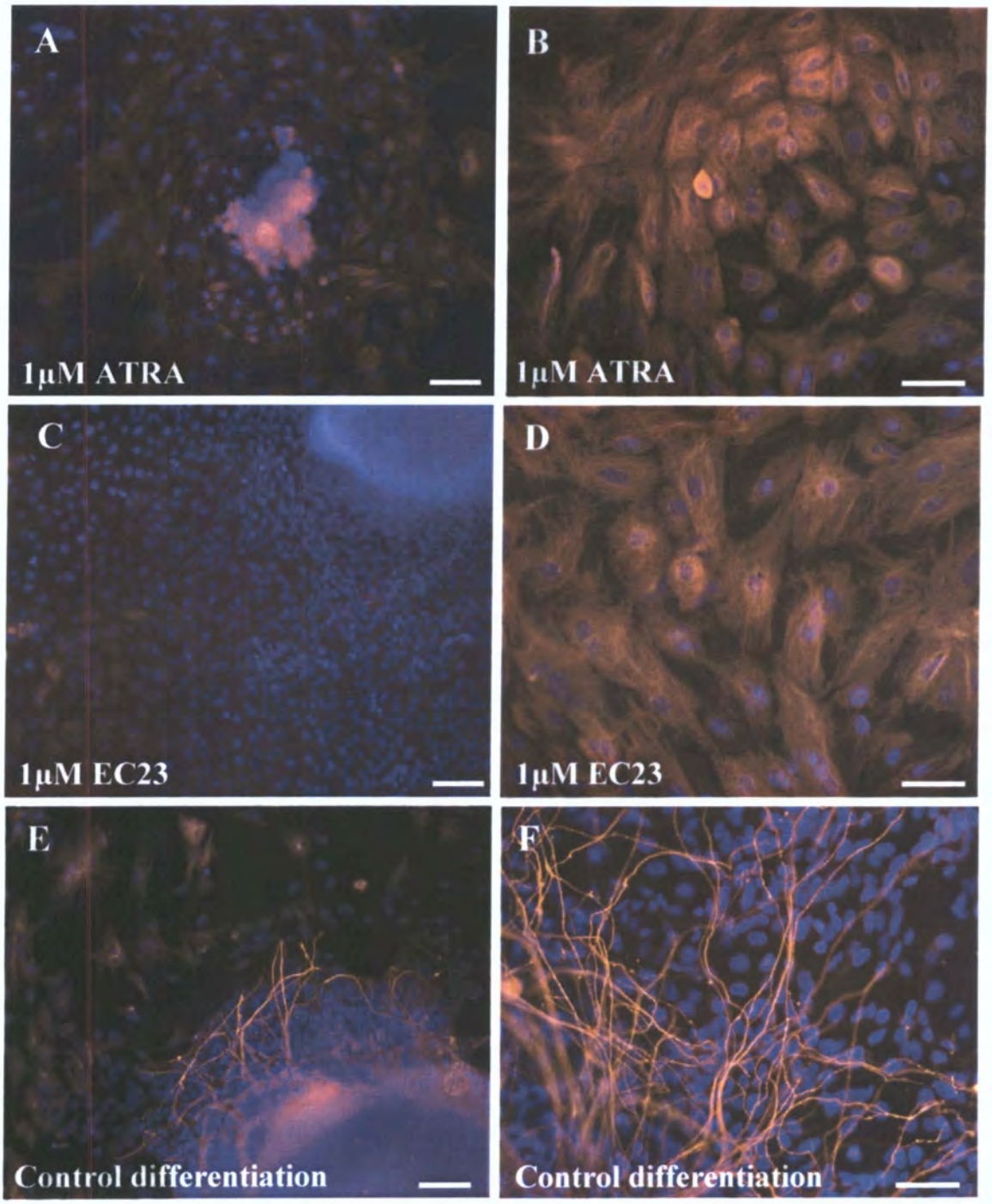


Figure 3.3.29: Neuronal specific β-III-tubulin (TUJ1) immunostaining of differentiated H9 stem cells after 21 days of culture. ES cells were differentiated for 14 days as embryoid bodies, then cultured for a further 7 days as monolayers on poly-L-ornothine/laminin coated 24-well plates. Cultures were maintained in either differentiation media alone (E, F), or differentiation media plus 1μM ATRA (A, B) or 1μM EC23 (C, D). TUJ1 positive cells were visualised in all conditions, however the morphology of positive cells was vastly different in ATRA and EC23 cultures compared to the control. Control cultures contained many neuronal cells. Images were captured after the 21 day culture period. Scale bar represents A, C, E: 100μm, B, D, F: 50μm. n=2

It was also noted that when cultures were stained with the late neuronal marker NF-200, very few positive cells were visualised in any of the conditions tested (**Fig. 3.3.30**). So although some neural differentiation was recorded in the control condition, this appeared to be representative of more immature cells than those visualised after defined neuronal medium was used.

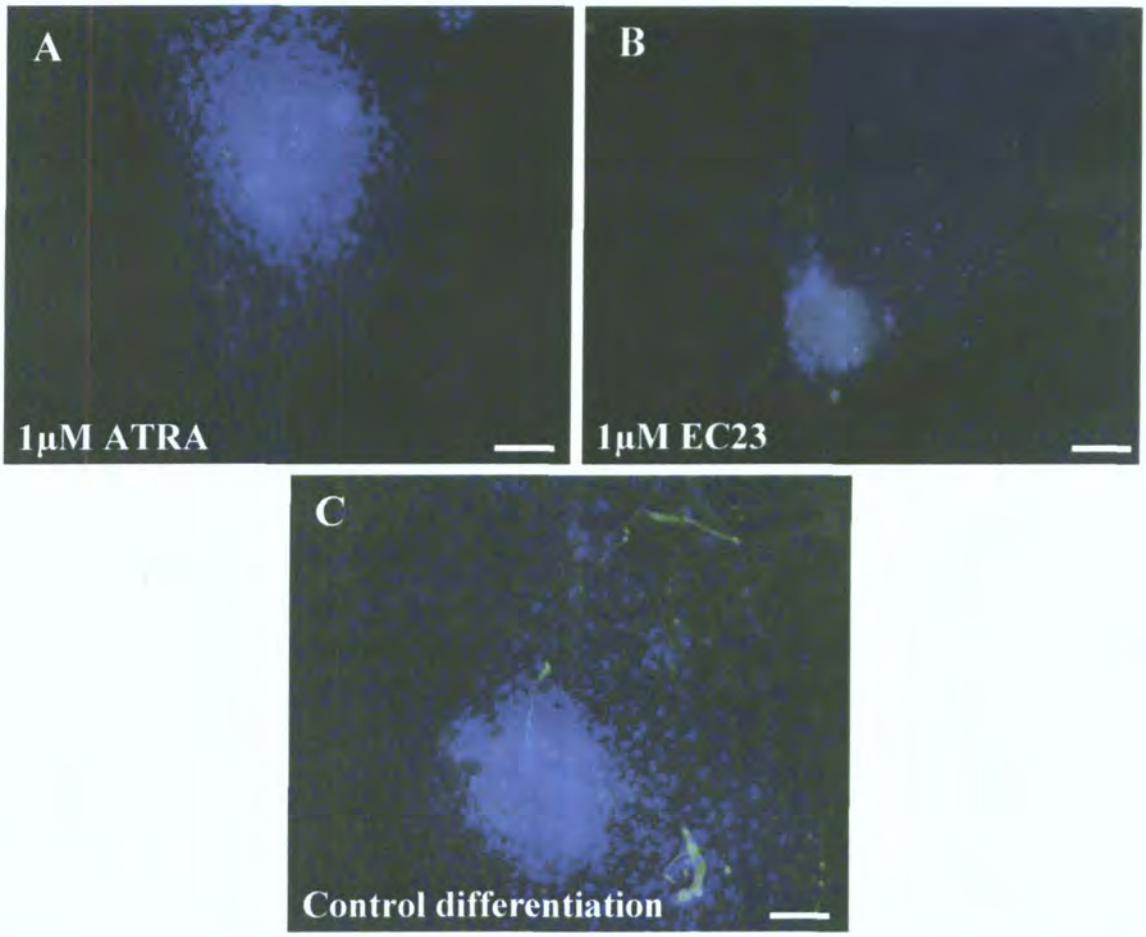


Figure 3.3.30: Neurofilament 200 (NF-200) immunostaining of differentiated H9 stem cells after 21 days of culture. ES cells were differentiated for 14 days as embryoid bodies, then cultured for a further 7 days as monolayers on poly-L-ornothine/laminin coated 24-well plates. Cultures were maintained in either differentiation media alone (C), or differentiation media plus 1μM ATRA (A) or 1μM EC23 (B). Very few NF-200 positive cells were visualised in the retinoid conditions. Slightly increased staining was visualised in the control differentiation condition. Images were captured after the 21 day culture period. Scale bar represents 100μm. n=2

3.4 Discussion and Conclusions:

The aim of this chapter was to test the biological activity of stable synthetic retinoids on model human pluripotent stem cells, and to compare the results with the known effects of the natural counterpart ATRA. We have demonstrated the design, synthesis and purification of two novel arotinoids, EC19 and EC23. Furthermore we have shown that these compounds are resistant to light conditions which cause isomerisation/degradation of ATRA. We therefore present them as potential alternatives to ATRA for *in vitro* use (Literature review, Chapter introduction and [123]). Arotinoids are not new compounds, they have been developed and synthesised since 1983 [144], when their ability to induce differentiation of EC cells was reported. However, the emphasis has always been on their use as chemotherapeutic agents (for example, see [145]), and not as a replacement for the *in vitro* use of ATRA. Consequently publications outlining their biological effects on stem cell models are limited. This quality was therefore investigated here, specifically their ability to induce neural differentiation in stem cell model systems. The ability of ATRA to induce neural differentiation in models of stem cells has been well documented (see literature review), and the specific neuronal-inducing qualities of ATRA has also been documented in the EC cell model system used here, Tera2.sp12 [131, 132]. This background formed the basis for experiments carried out into the synthetic retinoid modes of action.

Two effective concentrations of the synthetic retinoids EC19 and EC23 were chosen for initial treatment of Tera2.sp12 EC cell cultures. At the lower concentration (1μM), EC19 did not induce significant alteration in cell phenotype. On the other hand, 10μM EC19 did induce differentiation, albeit at slower rates and in a different manner to that observed for ATRA and EC23. In terms of cell proliferation, morphology, and marker phenotype, EC19 is quite distinct from ATRA and EC23 in its mode of action. For example EC19 treatment of this cell line gives rise to a dominance of 'plaque-forming' epithelial-like cells at the apparent expense of neural derivatives. The second synthetic retinoid, EC23, was an effective inducer of differentiation at both concentrations as shown by decreased cell proliferation and the concomitant increased expression of neural markers. EC23 expression patterns revealed similar responses to those of ATRA. Nonetheless differences were apparent between EC23 and the natural counterpart, for example, delayed shut-down of stem cell marker expression in the 10μM treatment, as well as

lower incidence of non-neural cell plaques. Perhaps the most significant difference however, was the increased number of mature neuronal cells compared with ATRA treatments at equivalent concentrations. Data presented here indicating the ability of the synthetic arotinoids EC19 and EC23 to induce differentiation in EC cells backs up previous studies on arotinoid-induced differentiation of EC cells and teratogenicity in whole embryo culture [144, 146]. Bechter *et. al.* published data indicating the ability of synthetic arotinoids to effect the differentiation of limb buds in the developing rat embryo, and also showed that some of the dysmorphogenesis visualised by ATRA teratogenicity was recapitulated *in vitro* by the synthetic arotinoids. As these abnormalities arise from incorrect CNS development it can be concluded that the arotinoids tested in those experiments were affecting neural cell development, as was observed in this investigation. Neuronal differentiation has also been induced by the synthetic retinoid TTNBP in P19 EC cells [147]. They presented that the neuronal differentiation induced occurred via activation of the RARs, and not the RXR receptors, i.e. those which are activated by ATRA. It is already known that EC23 is able to activate all of the RAR subtypes, similar to ATRA [122]. Therefore it is possible to hypothesise that the increased levels of neuronal differentiation visualised with EC23 compared to ATRA could be a consequence of the compounds' enhanced stability (i.e. following media supplementation, EC23 has a longer active half-life than ATRA, resulting in an increased level of RAR activation).

MALDI protein profiling and subsequent biomarker analyses described herein support the above findings into the response of EC cells to ATRA and the synthetic retinoids EC19 and EC23. Immunological detection of a limited number of marker proteins showed similar differentiation patterns in response to ATRA and EC23 treatment, while EC19 was found to elicit distinctly different phenotypes. The MALDI data revealed common markers of differentiation which were elevated following treatment with all three compounds when compared with the untreated stem cell control. These experiments also showed a close functional relationship between ATRA and EC23. However, the simultaneous detection of multiple protein constituents also revealed differences between ATRA and EC23 which might otherwise be overlooked using conventional methods. The discriminatory power of biomarker profiling has the potential to provide novel subtle insights into different modes of retinoid action. Surface enhanced laser desorption ionisation time of flight (SELDI-TOF) mass profiling has previously been carried out on

this particular cell line, enabling successful discrimination between stem cell samples and their differentiated derivatives [148], SELDI-TOF data also discriminated between different pathway sets of differentiation [149]. Therefore the MALDI data presented here not only verifies the use of these technologies for this type of investigative experimentation, but also presents the method used as a novel tool for discerning similarities and differences between stem cell samples and their retinoid-induced differentiated counterparts which would otherwise be overlooked. For example, 2D distribution plots of collated protein expression data has indicated enhanced experimental reproducibility obtained with the synthetic compounds compared to ATRA. On these plots, tighter protein expression levels were witnessed between biological repeat samples of the synthetic retinoid repeats than with the ATRA repeats. This could again be the result of greater stability of these compounds, leading to more consistent concentration levels throughout the culture period. This observation could easily have been over-looked if analysis had been restricted to traditional methods. MALDI profiling is more commonly associated with investigations into biomarker discovery for disease states, or for the hunt for biomarkers of specific cell types (for example, see recent review of MS proteomic technology in the search for biomarkers of neurodegenerative diseases [150]). However, here it has been shown to be applicable for the screening of retinoid-mediated protein profile changes in cell lysates. This technology will provide an effective tool for the quick and efficient screening and analysis of future synthetic retinoid compounds. It is hypothesised that once a comprehensive cohort of samples are analysed and the results collated and grouped, it may be possible to classify new retinoids on this basis alone.

Subsequently, work in this investigation, focused our analysis on two protein bands which displayed strong up-regulation in response to retinoids as viewed on SDS-PAGE profiles of subfractionated cell lysates. Using MALDI-TOF MS methods we have identified from these bands two groups of proteins. The first group comprised of four proteins – CRABPI, CRABPII, CRBPI and profilin-1, and the second group consisted of phosphorylation isoforms of stathmin.

These findings provided the first strong evidence that EC23 exerted its biological effects in a similar manner to ATRA, i.e. signalling via cellular retinoid response proteins downstream of the RAR receptor. Previously published data provides evidence for the up-regulation of these binding proteins upon ATRA stimulation and activation of the

retinoic acid receptor pathway in nerve regeneration [151]. ATRA-mediated up-regulation of CRABP-II has also been previously demonstrated in the P19 EC cell system [152], where proteomic techniques were used to evaluate up-regulated proteins after the induction of neural differentiation. As reported herein, CRABPI and II, and CRBPI play central roles in the metabolism and gene regulatory effects of ATRA and retinol (**Fig. 3.4.1**). Although the exact functions of the CRABPs are not fully understood, it is widely held that they facilitate the metabolism and gene regulatory effects of ATRA in the cell, while also playing an important role in maintaining the cellular concentration of free ATRA within a suitable physiological range (as outlined in the literature review, Chapter 1). Briefly, it is thought that the predominant role for CRABP-I is to facilitate the metabolism of ATRA to more polar metabolites which can be excreted by the cell [23], while CRABP-II primarily functions to facilitate the transfer of ATRA to the nuclear envelope where it can interact with its specific receptors [25]. From the results obtained so far it can be tentatively concluded that EC23 appears to act on cells via a similar mode of action to ATRA as it up-regulates the associated binding proteins which facilitate this process. EC19 does not appear to function in the same way as ATRA and may be inducing cellular responses by an alternative mode of action, for example via the retinol binding proteins or by other pathways altogether. These proposed roles for the CRABPs may explain the different inductive effects of ATRA and EC23 on CRABP-I expression. By definition we know that EC23 is more stable than ATRA therefore it is feasible that it could be non-metabolisable by the cell, and therefore the cell increases the level of CRABP-I to try and compensate. Alternatively, EC23 may simply be more potent than ATRA, in which case the cell may similarly synthesise more CRABP-I to lower the effective free [RA].

That EC19 did not appear to effect the expression levels of the CRABPs compared to the undifferentiated controls supports the conclusion that the position of the carboxyl group is critical to the activity of these retinoids. The *para-* configuration seen in EC19 does not appear to be effective at activating this particular retinoid signalling pathway. However, since we do see morphological changes to cells cultured with this retinoid it can be concluded that EC19 must elicit its effect via an alternative route to either ATRA or EC23. It is known that other arotinoids are able to elicit biological effects through pathways which do not involve the retinoid nuclear receptors [153], and this would have to be evaluated in the case of EC19. The fact that all three retinoids induce an increase in expression of CRBP-1 implies a feedback mechanism may be initiated to inhibit further

formation of ATRA from retinol, and EC19 must still, in part, activate certain retinoid mediated responses. CRBP-1 is thought to facilitate the storage of retinol into retinal esters rather than its oxidation to ATRA (**Fig. 3.4.1**).

Stathmin and profilin are involved in the dynamics of actin filament and microtubule assembly, and as such they play important roles in cytoskeletal reorganisation during neurogenesis. Our results show ATRA and EC23-mediated regulation of profilin-1 and stathmin expression *in vitro,* and this may reflect direct regulation of these proteins by ATRA *in vivo*. Indeed there is close correlation between areas of natural high expression of these proteins and of ATRA localisation in the brain. Further investigations would need to be performed in *in-vivo* experiments to establish this hypothesis.

Stathmin and its phosphorylated isoforms are involved in a number of different pathways related to cell proliferation and differentiation. It is a small regulatory protein which acts as a relay, integrating diverse intracellular signalling pathways [154, 155]. Stathmin's specific role is understood to be involved in the regulation of tubulin polymerisation. Unphosphorylated stathmin binds to two free tubulin αβ-heterodimers in a T2S formation thus sequestering the tubulin monomers and inhibiting polymerisation. Once stathmin becomes phosphorylated a conformational change means it is unable to bind to free tubulin monomers thus allowing the elongation of tubulin polymers. Stathmin is ubiquitously expressed throughout the body but there are specific areas where high levels of expression are visualised. For example in the embryonic and juvenile brain where there is a creation and turnover of neural networks for learning and memory, i.e. areas that are associated with a high level of tubulin assembly and disassembly [156]. Stathmin has also been identified via proteomic screening in 11.5 day cultured rat embryos, where high levels of cytoskeletal rearrangements occur during development [157]. In agreement therefore, an up-regulation of phosphorylated stathmin in ATRA and EC23 treated samples is predictable in these cultures due to the high level of neuronal differentiation and neurite outgrowth, which in turn would require a high level of tubulin assembly for neurogenesis. As far as we are aware, this is the first time that stathmin and phosphorylated stathmin have been directly linked to retinoic acid mediated induction of differentiation. The novel link between the two pathways is visualised in **Figure 3.4.1**.

Profilin-1 is involved in the regulation of actin polymerisation. It appears to work in a number of different ways to modulate actin modelling which depends upon a complex combination of tissue specific signalling pathways found in all cells [142]. Profilin-1 can bind to g-actin monomers in a 1:1 ratio, inhibiting/slowing down actin polymerisation, or it can promote filament elongation at the free end by dissociating the inhibiting capping proteins capG and capZ, or finally, it can play an important role in accelerating the ADP-ATP exchange on g-actin there-by also increasing the elongation of filaments [142]. Profilin-1 has been shown to influence the dynamics of the actin cytoskeleton during neuronal differentiation [143]. Therefore we can tentatively suggest that the up-regulation of profilin-1 recorded in the ATRA and EC23 treated samples could be attributed to actin re-modelling during neuronal differentiation. Proteomic techniques used to evaluate protein expression in P19 EC cell samples induced to differentiate down a neural lineage in response to ATRA, also indicated up-regulation of proteins related to actin re-modelling [152]. The actin-regulating protein cofilin 1 was up-regulated after ATRA-induced differentiation, which has a similar role as profilin-1 during the cytoskeletal re-modelling during neurite outgrowth. Indeed, in another earlier proteomic study, the up-regulation of profilin-1 was recorded in cancer cells, treated with ATRA, suggesting a major role in the inhibition of cell proliferation and migration and induction of differentiation [158]. Taken together, these data suggest that profilin-1 is up-regulated in response to either ATRA or EC23-induced differentiation of EC cells and appears to be involved in the actin re-assembly of the cytoskeleton during neurite outgrowth.

Of particular interest was the observed differential effect between ATRA and EC23 on both CRABPI and profilin-1. Both proteins were expressed at a higher level in cultures exposed to EC23 compared to ATRA-treated cultures. As noted above, this suggests that resistance to cellular metabolism may be a contributing factor to the observed enhanced potency of EC23, which has a knock-on effect in stimulating enhanced neural differentiation within these cultures. While Western blot analysis was able to verify immunologically the presence of the CRABPs, this technique alone was insufficient to draw the above conclusions since cross reactivity of the antibody with the different isoforms is likely, and this would mask the differential effect of these two compounds specifically on CRABPI.

Protein profiling analysis is a useful tool to discriminate the different responses of stem cells to related retinoid compounds. Nonetheless, analysis of total cell lysates is liable to reveal a relatively minor proportion of the total available information, and this work has demonstrated that additional strong markers become accessible following only rudimentary sample prefractionation. It is anticipated that more sophisticated means of fractionation and characterisation, such as LC-MALDI and top-down MS techniques, may provide even more in-depth analysis of these sets of samples. In the meantime, protein profiling can serve as a useful approach enabling simple quality control and classification of new retinoid compounds.

Further verification of retinoid EC23 to mimic the biological activity of ATRA was carried out using the human ES cell line H9. ATRA is known to enhance the yield of neural cell types when incorporated into differentiation protocols of stem cells [159, 160], increasing the percentage of neurons recovered from just a few percent without ATRA conditioning to nearly 40% with ATRA conditioning. In this investigation, similar biological results were seen in the human ES cell cultures when incubated with either ATRA or EC23. Under defined neuronal-inducing differentiation cues, both retinoids were able to stimulate neuronal differentiation. Moreover, the number of cells expressing the motor-neuron marker, HB9, increased when samples were cultured with either ATRA or EC23, compared to control differentiation samples. The ability of stem cells to differentiate into motor-neurons has also recently been demonstrated by two independent laboratories [140, 161]. Both studies showed that unless the caudalising agent ATRA was present in the culture media, cells of a posterior location within the neural tube, i.e. motor-neurons, were not induced to differentiate. In another study, motor-neurons generated from human stem cells were transplanted into rat spinal cord and were shown to integrate with host tissue, extending choline acetyltransferase positive fibres [162]. This incorporation of transplanted, functional neurons is paramount for the development of stem cell-based therapies of neurodegenerative diseases. Therefore our results corroborate with these findings, and also indicate the close correlation of biological activities of the natural retinoid ATRA and the synthetic arotinoid EC23. These data further exemplify the more stable synthetic retinoid as an attractive alternative to ATRA. We propose that the more stable EC23 analogue could be incorporated into a commercially viable neural supplement, replacing ATRA, which may to added to culture

media to induce motor-neuron formation. This use of EC23 would be advantageous over ATRA, as EC23 is more stable.

Under generic differentiation cues both ATRA and EC23 appeared to block neuronal differentiation in favour of another as yet unidentified cell phenotype. These initial experiments again indicate the ability of ATRA and EC23 to direct differentiation in a human ES cell line which is capable of generating all cells of the three embryonic germ layers, and to do so in similar ways.

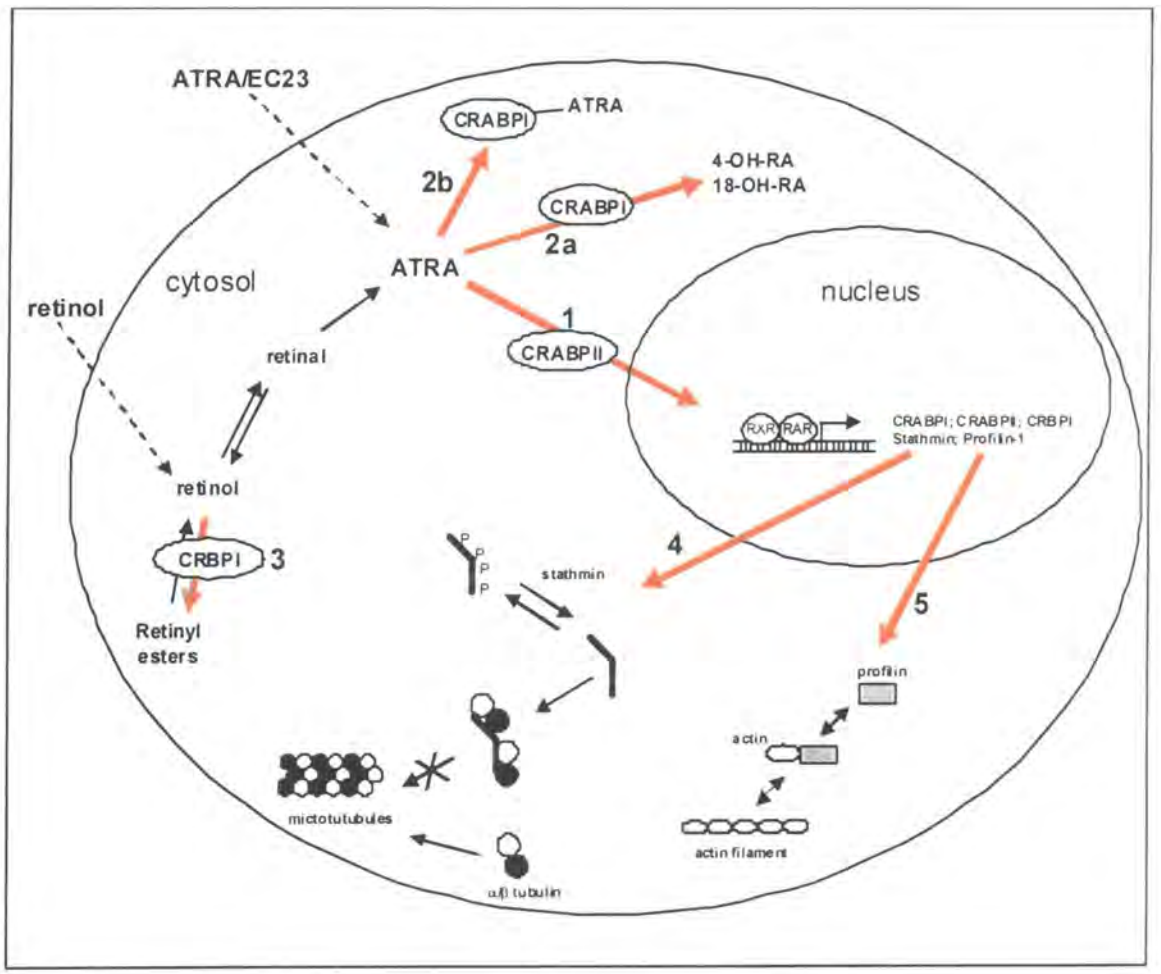


Figure 3.4.1: Proposed modified retinoic acid receptor pathway including new link between retinoid activation of the actin re-modelling protein profilin-1 and the tubulin re-modelling protein stathmin.

3.4.1 Conclusions

We have shown that a subtle structural difference between EC19 and EC23 (namely, the isomeric placement of the carboxylate function) has a major effect on the biological function of two closely related compounds. It is possible that this structural difference may change the chemical alignment/structure of the molecule sufficiently that it either does not bind to the same panel of RARs as ATRA or EC23, or does so with altered affinity/potency. It may be that EC19 activates different RARs or even RXRs and further research is required to investigate this.

The observed similarity of biological-induced activity of EC23 to ATRA highlights this molecule as an exciting new retinoid, the potential of which must now receive further attention in alternative cell systems to further verify these interesting findings.

The synthetic ATRA analogues reported herein may form the basis for the development of a range of novel compounds for use as alternatives to ATRA in stem cell research. Key to the benefits of these photostable compounds is the opportunity to exactly define their concentration in solution, a problem which poses a major hindrance when using ATRA. For instance, physiological concentrations of ATRA across the neural tube are critical for correct embryonic neural development (as outlined in Chapter 1), and work attempting to replicate physiological gradients *in vitro* would require that the exact working concentration of ATRA is known. It is anticipated that the availability of more stable alternatives to ATRA will contribute significantly to our ability to control retinoid-induced neurogenesis *in vitro,* and may play an important role in identifying further downstream molecules involved in retinoid-mediated biological activities.

4 Chapter 4: The Role of Natural and Synthetic Retinoids in Neural Progenitor Cell Differentiation:

4.1 Introduction:

The ability of the synthetic retinoids EC19 and EC23 to induce neural differentiation in pluripotent embryonic stem cell models was considered in the previous Chapter. This Chapter will address their ability to direct differentiation in cells which are already committed to specific neural cell lineages. Two models of neural progenitor cells will be analysed: an adult neural progenitor line and a neural progenitor line derived from embryonic neural tissue.

4.1.1 Adult neural progenitor cells

The importance of retinoid activity in the adult CNS is slowly being realised (refer to Chapter 1 and [163]). As mentioned, the hippocampus is one of a few areas in the adult brain where the active remodelling of neuronal pathways occurs throughout life into adulthood. As in embryonic systems, the retinoic acid signalling pathway has been shown to be involved, with ATRA playing a vital role in this very tightly regulated process [80]. Once this adult neurogenesis was discovered and verified, there was interest into how to exploit this novel area for the benefit of therapeutic applications. As a result many animal models of adult neural progenitor cells were established to try and increase the knowledge base in this relatively new area of neuroscience. Most model systems are derived from animal models and not human tissues due to the obvious inability of ethically obtaining living adult 'normal' brain material. There has recently been a paper indicating the ability to derive neural progenitor cells from human adult brains after death [164], and this could potentially establish an attractive cell culture based system for investigating adult neurogenesis in humans. However, it can be assumed that evidence obtained in animal models will relate to human systems, as has been seen in embryonic stem cell studies. However, it must also be noted that differences may also occur and will have to be taken into consideration. For example, differences are seen between mouse and rat progenitor cell lines derived from whole brain samples [165]. It was found that ATRA incubation induced both neurons and glia in rat cultures but mainly induced astrocytes in mouse cultures.

One such animal model cell line was created in 1995 from the rat hippocampus by Fred Gage's laboratory at the Salk Institute [166], and further characterised a few years later [167]. This cell line was transfected with the fluorescent protein GFP and termed HCN-nitGFP. This adult cell line was shown to proliferate *in-vitro* in response to bFGF media supplementation and was also shown to respond to ATRA by differentiating down a neural lineage [168]. This cell line is used throughout this investigation and termed adult hippocampal progenitor cells (AHPC). Most *in-vitro* studies have relied on synthesised ATRA to investigate retinoid action in adult CNS differentiation and neural cell homeostasis. However, as stated throughout this investigation, ATRA is extremely heat and photo-sensitive, degrading into multiple isomers which could possibly elicit different results from intact ATRA, due to ATRA's morphogenic nature.

4.1.2 Embryonic neural progenitor cells

Human embryonic neural progenitor cells derived from aborted foetuses have been transplanted into animal models of neurodegenerative disorders with great success [169, 170]. Therefore these cell lines hold great therapeutic potential for the treatment of numerous brain disorders. However, as many such cell lines are derived from whole brain samples, and as it is already known that cells from specific locations respond to stimuli differently, a need for more brain-region specific cell lines is required. Cell lines derived from specific brain areas have been transplanted into brain models and have been shown to respond differently. For example in models of stroke, transplanted cells derived from striatal and cortex both integrated with the host tissue but differentiated into differing proportions of neural subtypes [171]. Therefore to realise the full therapeutic potential of cell lines derived from specific brain areas, more basic investigation is required to understand their differentiation capabilities under specific differentiation induction cues. As outlined in Chapter 3, ATRA is known to have a caudalising effect on neuronal differentiation of embryonic stem cells; therefore the ability of cells to respond to this signal could be important. It was analysed here whether the addition of retinoids would have any effect on embryonic neural progenitor cells which are associated with a specific area of brain formation. The cell line used for this part of the investigation was a commercially available cell line derived from the ventral mesencephalon of a human foetus. The cell line was created by ReNeuron Group plc and was termed ReNcell VM

[172]. ReNcell VM is an immortalized human neural progenitor cell line with the ability to readily differentiate into neurons and glial cells with removal of growth factors from the culture medium. The cell line was immortalised by retroviral transduction with the v-myc oncogene, and retains normal karyotype even after continued proliferation. Cells have been maintained in an undifferentiated state in culture when grown on laminin for >45 passages. In experiments carried out by ReNeuron Group plc, ReNcell VM were shown to differentiate *in vitro* into dopaminergic neurons. These neurons have furthermore been shown to be electophysiologically active. It was not known what effect, if any, the addition of retinoids would have on this particular cell type.

4.1.3 Aim and Objectives

Aim

The aim of this chapter was to study the effect of stable analogues of ATRA on an adult hippocampal neural progenitor cell line and an embryonic neural progenitor cell line derived from the ventral mesencephalon. Synthetic retinoids were designed and synthesised in the Department of Chemistry, Durham University with emphasis on stability and a RAR mediated mode of action [122]. The rationale was to create an ATRA alternative which elicited the same biological effects but which was much more stable making it a promising alternative to ATRA in *in vitro* investigations.

Objectives

- The initial objective was to establish a valid adult neural progenitor cell model system. This would involve the use of natural retinoids (ATRA and 13-cisRA) to induce differentiation along a neural lineage and to demonstrate a concommital change in morphology and expression profiles. 13-cisRA was incorporated into the experimental plan as it is known to have a deferential effect on adult cells compared to ATRA *in vivo*.
- Subsequently this model would be used to test the action of synthetic retinoids EC19 and EC23. It was proposed that these molecules may enhance the knowledge of adult neurogenesis by inducing differentiation in a more controlled manner than the natural counterpart ATRA.

- It was hypothesised that EC23 would induce differentiation in a similar manner to ATRA, but given EC19s differing activity in the EC cell system it was hypothesised that it may induce differing sub-types in adult progenitor cells too.

- The next objective was to analyse the effect of both the natural and synthetic retinoid compounds on embryonic neural progenitor cells derived from the ventral mesencephalon. While there is a large body of work demonstrating the importance of ATRA in embryonic CNS development, to the best of our knowledge retinoids have not been tested in this particular cell line.
- It was hypothesised that ATRA may induce a specific type of neural differentiation due to its caudalising nature, and it was tested whether EC23 would again be able to mimic ATRA.
- The activity of the arotinoid EC19 was again hypothesised to be different.

4.2 Methods

4.2.1 Tissue Culture

4.2.1.1 HCN-nitGFP cells

Cells were cultured under standard laboratory conditions as described in Chapter 2. Before use, all plasticware was coated with 10μg/ml poly-L-ornothine (Sigma, diluted in H_2O) for 24rs at room temperature (RT), followed by 5μg/ml laminin (Sigma, diluted in PBS (Sigma)) for another 24hrs at 37°C. Plates were then washed thoroughly with PBS before use.

Cells were cultured under serum-free conditions at all times as outlined in protocols from the Salk Institute. To expand the cell cultures proliferation media used, consisting of DMEM/F12 (high glucose) (Irvine scientific), supplemented with 1X N_2 supplement (Gibco), 2mM L-glutamine (Sigma), 20ng/ml bFGF (Sigma, added just before use) and 100 active units of penicillin/streptomycin (Gibco). To maintain optimum cultures, media was changed every 3 days, and cultures were passaged at ~90% confluence. To passage cells, the media was aspirated, 1ml trypsin-versene solution (Cambrex) added and rocked over cells and the excess was immediately removed. Cells were dislodged from the plasticware by gentle tapping, and resuspended in 10mls DMEM/F12. The suspension was pelleted by centrifugation at 12000rpm for 3 mins. The supernatant was removed and the pellet resuspended in 1ml proliferation media and triturated to gain a single cell suspension. Cells then underwent a 1:5 to 1:10 split depending on cell numbers and were transferred to new poly-L-ornothine/laminin coated plasticware.

Differentiation protocol

HCN-nitGFP cell cultures were induced to differentiate with the introduction of 1μM ATRA (Sigma, diluted in DMSO (Sigma)) or 1μM 13-cisRA (Sigma, diluted in DMSO) into the media, and the reduction of bFGF concentration down to 1ng/ml. In preparation for differentiation, cultures which had reached ~90% confluence were treated with trypsin-versene solution to obtain a single cell suspension as outlined above. Cell number was subsequently determined using a haemocytometer under a light microscope. Unless otherwise stated, cells were seeded at 100,000 cells/well in a 4-well plate. Cultures were maintained for up to 14 days with media being refreshed every 3 days.

EC19/23-induced differentiation

The synthetic retinoids EC19 and EC23, produced by the Chemistry Department was also assessed for their ability to induce differentiation. Alongside corresponding control experiments using the natural retinoids, a range of retinoid concentrations was added into the differentiation media, 0.01μM, 0.1μM, and 1μM. Cells were seeded at 100,000 cells/well of a 4-well plate and maintained for up to 14 days with media being refreshed every 3 days. In a separate experiment cells were seeded at varying densities and cultured with differentiation media containing 0.5μM EC23. Cell densities used were 20,000, 60,000, 120,000, and 200,000 and a confluent T25 (~2-5,000,000 cells). The cultures were subsequently maintained as outlined above.

4.2.1.2 ReNcell VM

Cells were cultured under standard laboratory conditions as described in Chapter 2. Before use plastic-wear was coated with laminin solution: 1ml laminin (Sigma) was diluted in 49ml unsupplemented DMEM:F12 media. 4ml was used to coat a T75. Laminin solution was incubated in the flask at 37°C for at least 6 hrs. Flasks were then washed twice with media prior to use.

ReNcell VM progenitor cells were cultured in serum free conditions using methods previously described. Briefly, DMEM:F12 (1:1, Gibco) was supplemented with 10mls B27 (Invitrogen), 2mM L-Glutamine (Sigma), 0.5ml Gentamycin (Gibco), and 1ml 50mg/ml Heparin solution (Sigma). For proliferation, growth factors were added from stock solution (for 10ml media; 10μL FGF and 20μL EGF). Cultures were passaged using 0.25% trypsin EDTA solution and trypsin action was inhibited by incubation with trypsin inhibitor (5mg trypsin inhibitor dissolved in 1ml supplemented media then made up to 20ml with non-supplemented media. Solutions were then f/s through a 0.2μm filter.). Cultures were passaged 1:3 up to 1:6 as appropriate.

Differentiation protocol

Cultures were incubated with trypsin to obtain a single cell suspension as outlined above, then 100,000 cells/well were seeded into 12 well plates. Plates were allowed to get 75% confluent then were switched to differentiation media with or without retinoid supplementation as indicated below.

- 2 lanes were resuspended in proliferative media (+ growth factors)
- 2 lanes were cultured in media minus growth factors (control diff)
- 2 lanes were cultured minus growth factors + 1μM ATRA

- 2 lanes were cultured minus growth factors + 1μM EC23
- 2 lanes were cultured minus growth factors + 1μM EC19

Cultures were incubated for 7 days then MTS analysis and immunocytochemical staining was performed as outlined in the Methods Chapter.

4.2.2 Flow cytometry

For complete methods refer to Intracellular staining, Chapter 2, Section 2.4

Antibodies used:

Antibody	Company	Dilution	Incubation time	Secondary
GFAP	Sigma	1:200	1hr	Alexafluor 488

4.2.3 Immunocytochemistry

For complete methods refer to Chapter 2, Section 2.6

Antibodies used:

Antibody	Company	Dilution	Incubation time	Secondary
Nestin	Chemicon	1:100	1hr	Alexafluor 488
TUJ1	Covance	1:500	1hr	Cy3
NF-200	Sigma	1:200	1hr	Alexafluor 488
GFAP	Sigma	1:200	1hr	Alexafluor 488
Phalloidin	Sigma	2 Units	1hr	-

4.2.4 Real-time PCR

For complete methods refer to Chapter 2, Section 2.15

Primers used: CYP26A1, RAR-β

4.3 Results:

4.3.1 Analysis of Natural and Synthetic Retinoid Induced Neural Differentiation of AHPCs:

4.3.1.1 Cytological analysis of natural retinoid-induced differentiation

Initial experiments were carried out to monitor the cellular response to the natural retinoids ATRA and 13-cis retinoic acid (13-cisRA). Alongside these experiments a preliminary run of the synthetic retinoid EC23 was carried out. Cells were exposed to either 1μM ATRA, 1μM 13-cisRA or 0.5μM EC23. It was decided to start with a lower dose of EC23 compared to that of the natural retinoids due to results obtained in previous experiments with EC cells, where EC23 appeared to be more potent/toxic (Chapter 3). Cultures were maintained for up to 14 days. At specified time points the cell viability assay MTS was carried out to determine what effect the retinoids were having on cell number over the time course. The MTS assay works via a colorimetric change that can be quantified on a spectrometer. MTS is a yellow tetrazolium dye that is reduced by live (and not dead) cells to a purple formazam compound. Surviving cultures also underwent immunocytochemical analysis with neural markers to monitor differentiation. After 14 days exposure to ATRA, cell numbers remained constant, an indication that the culture may have stopped proliferating, (**Fig. 4.3.1B**) and had been induced to differentiate. Expression of the neuronal specific β-III tubulin marker, TUJ1, was evident throughout the culture (**Fig 4.3.1A**) with the majority of cells expressing this protein. A few cells were positive for the glial marker, glial fibrillary acidic protein (GFAP), and were distributed sporadically throughout the culture. After 14 days exposure to 13-cisRA it was noted that reproducibly more TUJ1 positive cells were seen compared with ATRA cultures (n=3) (**Fig 4.3.1A**). However the number of GFAP positive cells appeared to remain roughly the same (**Fig 4.3.1A**). As noted above, cell numbers did not increase in ATRA treated cultures over the time course, possibly an indication that the cells had dropped out of the cell cycle and had been induced to differentiate. However, when cells were exposed to 13-cisRA, although immunocytochemical data suggested that the cells had differentiated down a neural lineage, the MTS assay indicated that the number of viable cells was still increasing with time. This could possibly indicate that 13-cisRA is not as potent as ATRA or could be eliciting its effect via a different mechanism.

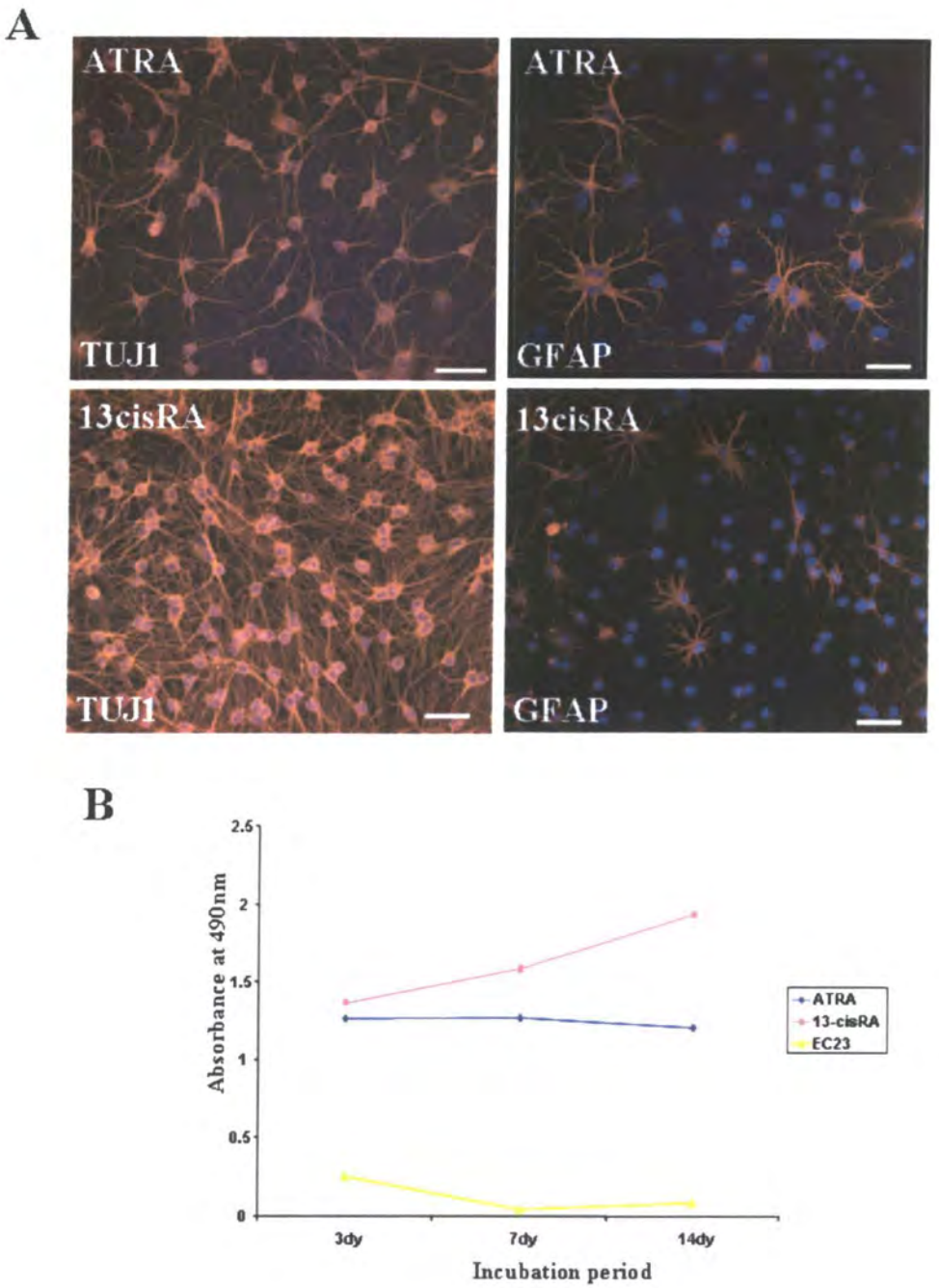


Figure 4.3.1: A: Adult hippocampal progenitor cells differentiate down a neural lineage in response to the natural retinoid compounds all-trans retinoic acid (ATRA) and 13-cis-retinoic acid (13cisRA). Cells were cultured with 1μM retinoid for 14 days, and subsequently underwent immuno-cytochemical analysis for the neuronal marker TUJ1 and the glial marker GFAP. Extensive neural differentiation is observed in both cultures. Reproducibly, more cells in total and more neuronal cells are present in cultures exposed to 13cisRA. All micrographs are counterstained with Hoechst. n=3

B: Cell viability of AHPCs after exposure to natural and synthetic retinoid compounds. MTS cell viability test was carried out on AHPCs after exposure to either 1μM ATRA, 1μM 13-cisRA or 0.5μM EC23. Cells were seeded at 20,000/well in a 4-well plate. At this cell density EC23 was toxic to cell cultures. n=3

4.3.1.2 Seeding cell density effects EC23 biological activity in AHPCs

The synthetic retinoid EC23 was initially supplemented into AHPC cultures at half the concentration of the natural retinoids to see if any morphological changes indicating differentiation were seen. Cultures were exposed to 0.5μM EC23, and run alongside the above experiments for up to 14 days. At this concentration very few viable cells were seen in the EC23 cultures compared to the natural retinoid cultures (**Fig. 4.3.1B**), and therefore no immunocytochemical analysis could be performed. However, in simultaneous experiments, 0.5μM EC23 or 1μM ATRA was simply added to confluent cultures of the AHPCs in flasks, for a period of 7 days, followed by morphological analysis (**Fig. 4.3.2**) Phase photomicrographs indicated that in the confluent culture, significantly fewer cells were visible in EC23 cultures compared to the undifferentiated control. Cell morphology had also changed significantly, resembling cultures exposed to ATRA. The neuronal specific marker TUJ1 was analysed and was found to be expressed by these cells, also similar to ATRA cultures.

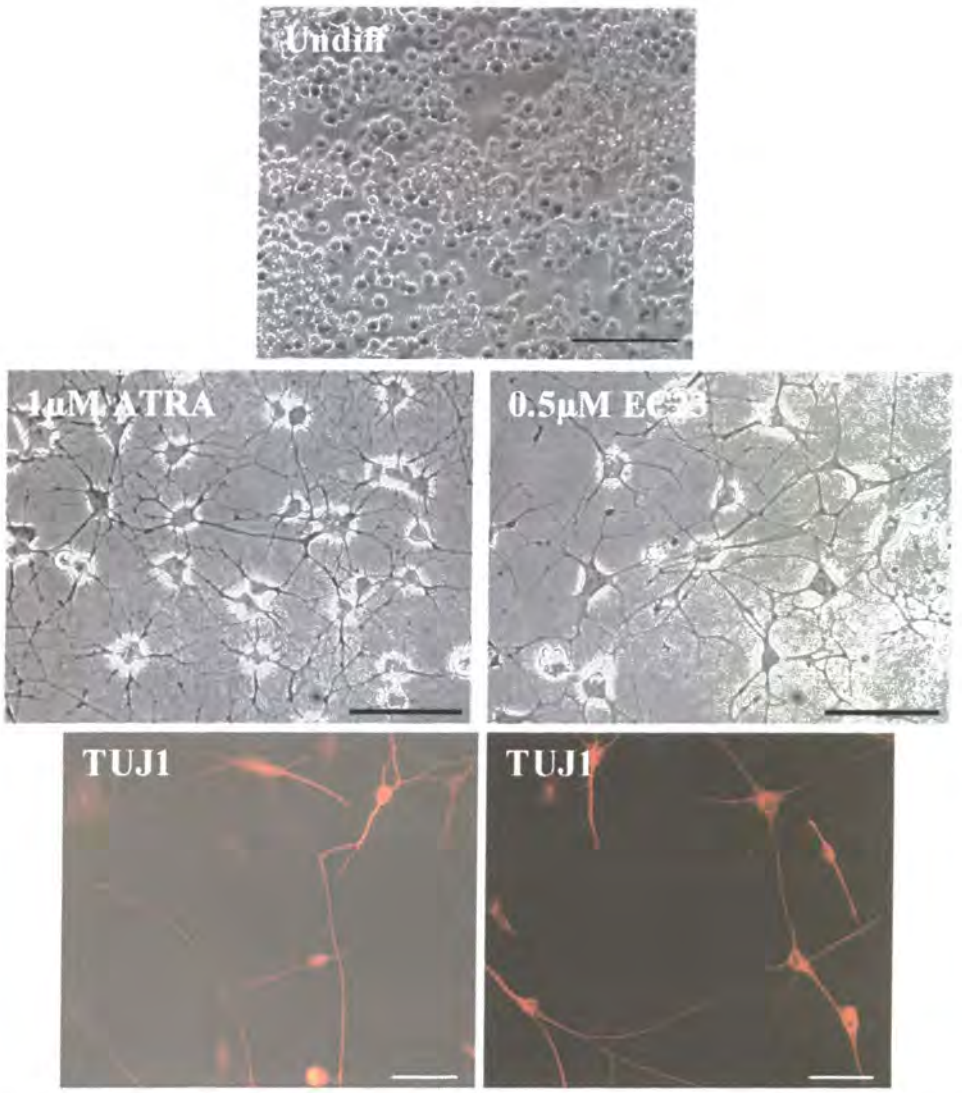


Figure 4.3.2: Neuronal differentiation was induced with the synthetic retinoid EC23. When AHPCs are seeded at a high enough density and cultured with 0.5μM synthetic retinoid EC23 for 7 days neuronal differentiation is visualised. Immunocytochemical staining with the neuronal-specific marker TUJ1 revealed positive neuronal expression within the cultures tested. n=3, scale bar represents 50μm.

On the basis of this morphological and immunological evidence it appeared that AHPC cells could be sufficiently induced to differentiate by EC23 exposure, but only if they were seeded at a high starting density. The issue of cell density was studied further in order to optimise culture conditions. 0.5μM EC23 was added to cultures of AHPCs seeded at varying densities in 4-well plates (20,000, 60,000, 120,000 and 200,000 cells/well) and samples were subsequently analysed after 7 days in culture. Both Figures 3 and 4 provide evidence suggesting that the biological effect of EC23 indeed appeared to be dependent on cell density. Cells seeded at 20,000 and 60,000 cells/well did not resemble normal healthy undifferentiated or differentiated culture morphologies. In phase photomicrographs (**Fig. 4.3.3**), and after immunostaining for phalloidin, a cytoskeletal

marker, cells appeared as round ball shaped cells with no visible cellular processes (**Fig. 4.3.4**). However, when the cells were seeded at 120,000 or 200,000 cells/well their morphological appearance resembled cells which had started to differentiate i.e. they possessed long, extended processes and appeared significantly different from undifferentiated cultures (**Fig. 4.3.3**). Phalloidin immunostaining, also indicated the presence of long processes (**Fig. 4.3.4**). Therefore it can be inferred that given the appropriate culture conditions, AHPCs can be induced to differentiate down an apparently neural lineage by EC23 supplementation, similar to the effects seen when the AHPCs were cultured with the natural retinoids ATRA and 13cisRA. To study this apparent similarity in differentiation responses, experiments were next carried out directly comparing cultures induced to differentiate with both the natural retinoid ATRA and the synthetic retinoid EC23.

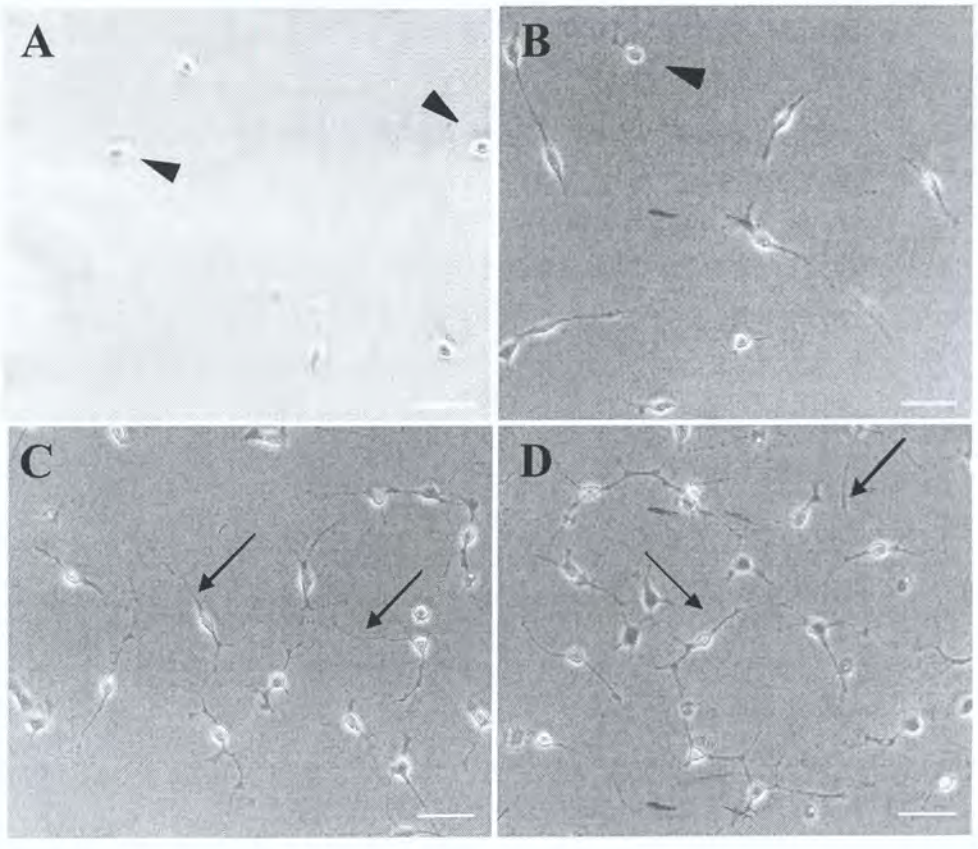


Figure 4.3.3: EC23 biological effects on AHPCs appears to be cell density dependent. AHPCs were seeded at varying densities and exposed to 0.5μM EC23 for 48hrs. At low densities few cells survived and those present appeared to stay rounded (arrow heads). At higher cell densities cells appeared to be more viable and put out processes which resemble neurites (arrows). Cells were seeded at A:20,000, B:60,000, C:120,000, D:200,000/well in a 4 well plate. Scale bars: 50μm. n=3

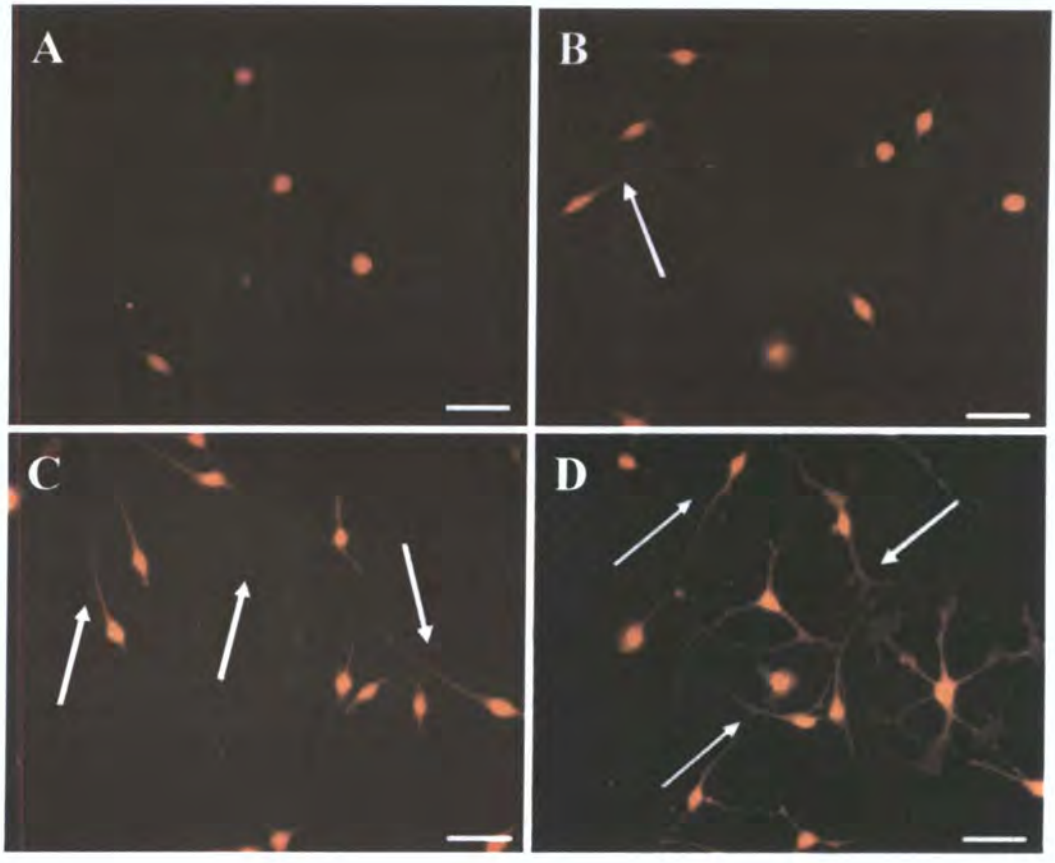


Figure 4.3.4: Cytoskeletal analysis of cultures exposed to EC23. Immnocytochemical staining of AHPC cultures which have been seeded at increasing cell densities and exposed to 0.5μM EC23 for 7 days. The cytoskeletal marker phalliodin has been used to indicate cell area. It can be seen that with increasing cell density more cells appear to have started to extend long processes (arrows). Cell densities are A: 20,000 B: 60,000 C: 120,000 D: 200,000. All cultures were seeded in 4-well plates. Scale bars: 50μm. n=3

4.3.1.3 Concentration study of EC23

Earlier data established that EC23 could induce differentiation in AHPCs. A concentration study was carried out on cultures seeded at constant cell densities to try and determine whether EC23 had an enhanced effect compared to the natural counterpart ATRA, as seen in embryonic stem cell cultures (Chapter 3). It was decided that 100,000 cells/well would be used, as this appeared to be the lowest level at which the cells could be seeded whilst still eliciting the desired effect.

Cultures were incubated with either 0.01μM, 0.1μM or 1μM EC23 or ATRA for 7 days. After this time period cells underwent MTS analysis (**Fig. 4.3.5**). As in previous investigations, the vehicle control showed no attenuation of number of viable cells

compared to the undifferentiated sample. The control natural retinoid, ATRA, induced a decrease in the number of viable cells compared to undifferentiated samples, and this effect was enhanced as the concentration of the retinoid increased. Cells in all ATRA conditions tested appeared healthy, looked like they had dropped out of the cell cycle, and appeared to have been induced to differentiate. EC23 samples showed similar attenuation of number of viable cells, with numbers decreasing with increasing retinoid concentration. The level of attenuation was greater in the 1μM EC23 samples compared with 1μM ATRA counterparts, and morphological analysis of these EC23 cultures revealed cells which appeared sub-optimal and were beginning to die. These data further indicate that the synthetic retinoid EC23 has an enhanced effect compared to the natural retinoid ATRA, on which it is modelled. It appears that the toxic threshold of this cell type for EC23 is lower than that of ATRA.

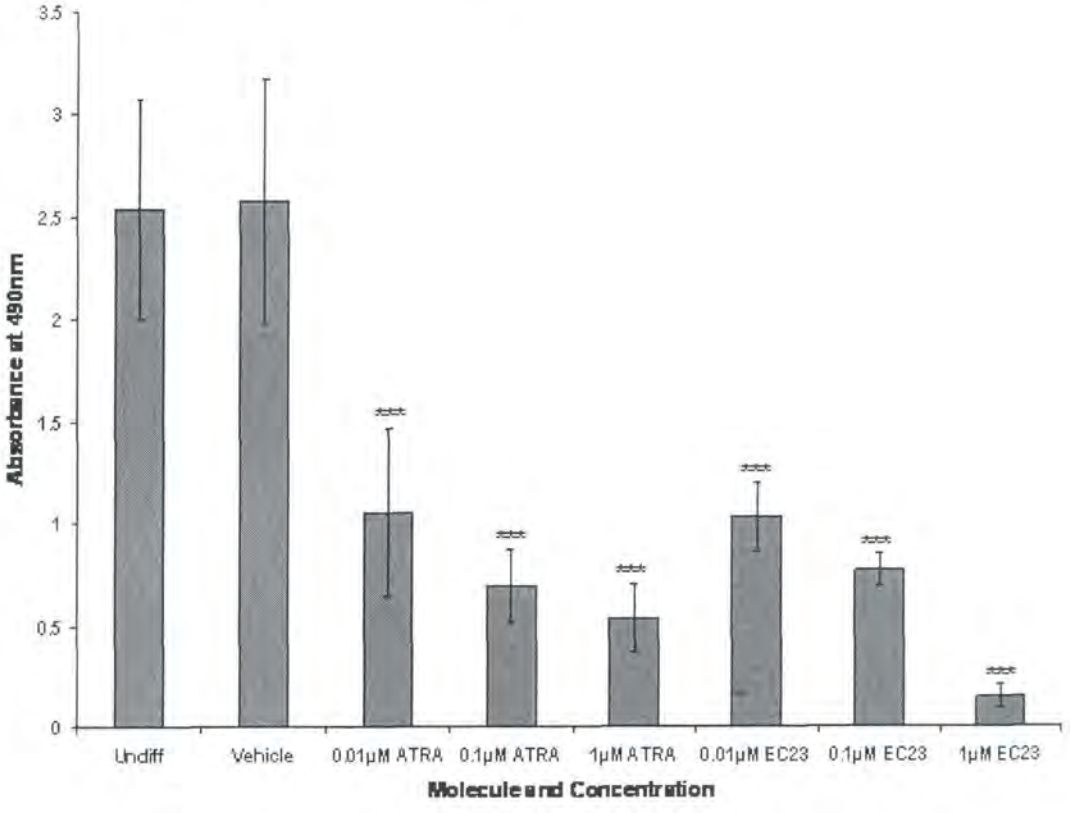


Figure 4.3.5: Number of viable cells determined on AHPCs cultured with a range of retinoid concentrations for 7 days. 100,000 cells/well were cultured with either 0.01, 0.1 or 1μM ATRA or EC23 and subjected to the MTS cell viability assay. EC23 appeared to be more potent than ATRA with fewer cells surviving at the highest concentration studied. n=3, error bars represent ± standard deviation from the mean. *** $p \leq 0.0005$, student's t-test compared to the undifferentiated control.

Immunocytochemical analysis of a panel of neural markers was performed to investigate levels of specific neural differentiation visualised. Since 1μM EC23 cultures were not viable or healthy, the 1μM EC23 and ATRA treatments were not analysed further. The antibodies investigated were the neural progenitor marker nestin, the neuron specific marker β-III tubulin (TUJ1) and the glial marker GFAP (**Fig. 4.3.6**). Undifferentiated cultures exhibited low level expression of both TUJ1 and GFAP and highly expressed the progenitor marker nestin. Morphologically, cells appeared rounded with very small projections protruding out from the main cell body. After ATRA and EC23 treatment this rounded appearance was lost and long neural processes appeared. At both concentrations tested the cells with long processes were TUJ1 positive, and therefore neuronal in phenotype. It is concluded that under suitable culture conditions EC23 is able to mimic ATRA-induced neuronal differentiation within this adult neural progenitor cell model system.

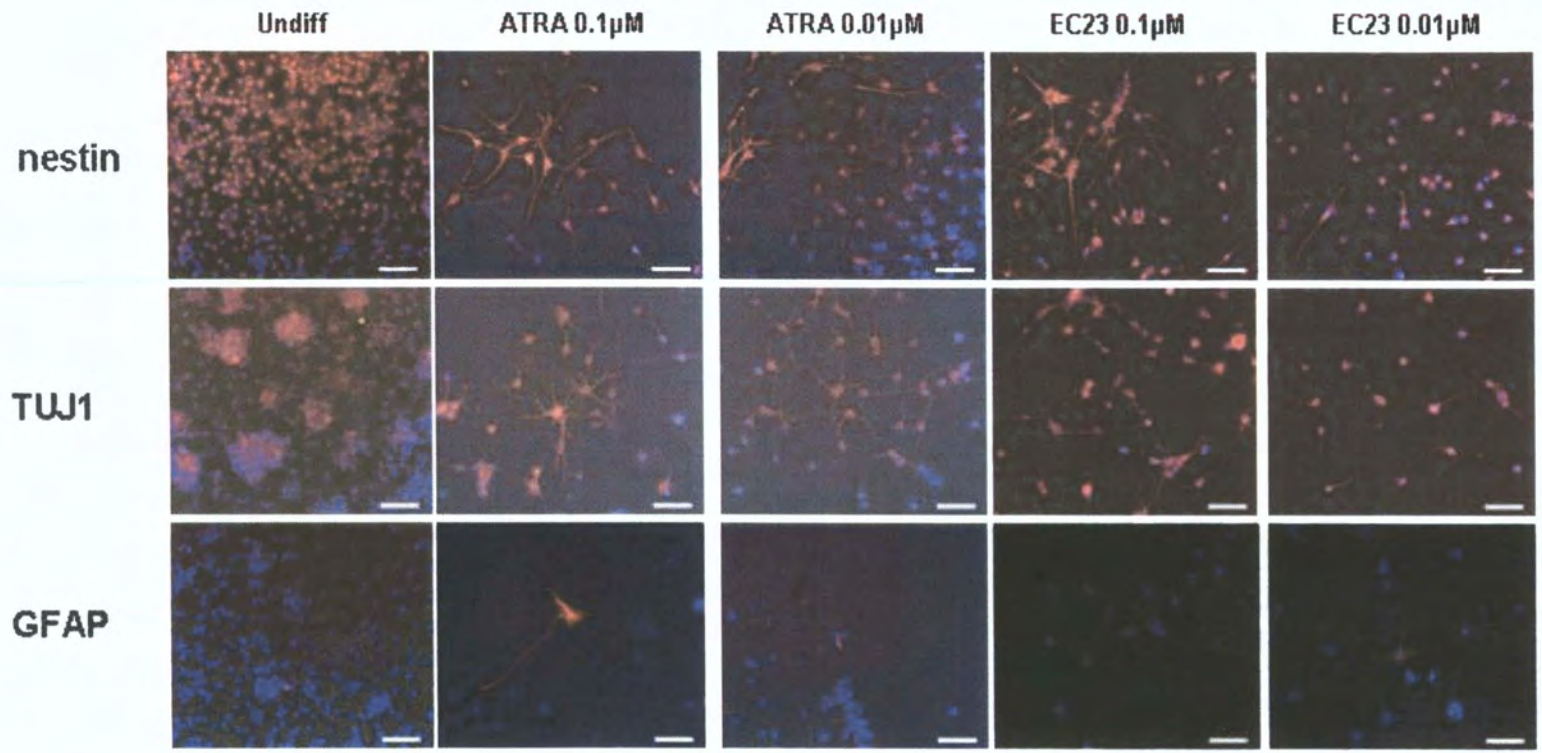


Figure 4.3.6: Immunocytochemical staining of AHPCs exposed to varying concentration of ATRA or EC23 for 7 days. Similar marker expression is seen with both natural and synthetic retinoid. Markers studied were nestin, a neural progenitor marker, TUJ1, a neuronal marker and GFAP, a glial marker. Scale bars 50μm. n=3

4.3.1.4 Concentration study of EC19

Analysis of the synthetic retinoid EC19s activity was also assessed at similar concentrations. In previous experiments (Chapter 3), EC19 was shown to induce differentiation in stem cell model systems, however, the cellular sub-types induced were distinct from the control natural retinoid ATRA and the synthetic analogue EC23. The effect of this retinoid on adult neural progenitor cells is described here. Initially a range of concentrations was administered to cultures, and after a 7 day time period, samples underwent both MTS and immunocytological analysis. Concentrations studied were 0.01μM, 0.1μM and 1μM, and all cell cultures were seeded at 100,000 cells/well, in a 4-well plate. MTS analysis revealed, as found above, that the vehicle control showed no attenuation of number of viable cells, compared to the undifferentiated sample (**Fig. 4.3.7**). As previously, the control natural retinoid ATRA, induced a decrease in cell number compared to undifferentiated samples, and this effect became enhanced as the concentration of the retinoid increased. Again, morphological analysis of cells in all ATRA conditions tested looked healthy and appeared to have dropped out of the cell cycle and begun to differentiate. EC19 cultures responded differently to the ATRA control. At the lowest concentration tested, 0.01μM, EC19 induced a relatively small decrease in cell number compared with the ATRA counterpart. However, at the highest concentration tested, 1μM, very few cells remained viable after the 7 day period suggesting that the retinoid was toxic at this level. Complementing previous findings, it appeared that EC19 exerted its biological effects in a narrow range of concentration, but was nonetheless able to induce differentiation in this adult neural progenitor model. Morphological analysis of the cultures indicated that at the 0.1μM concentration it did appear as though cells exposed to EC19 had been induced to differentiate, they had lost their typical small round appearance and had developed long processes.

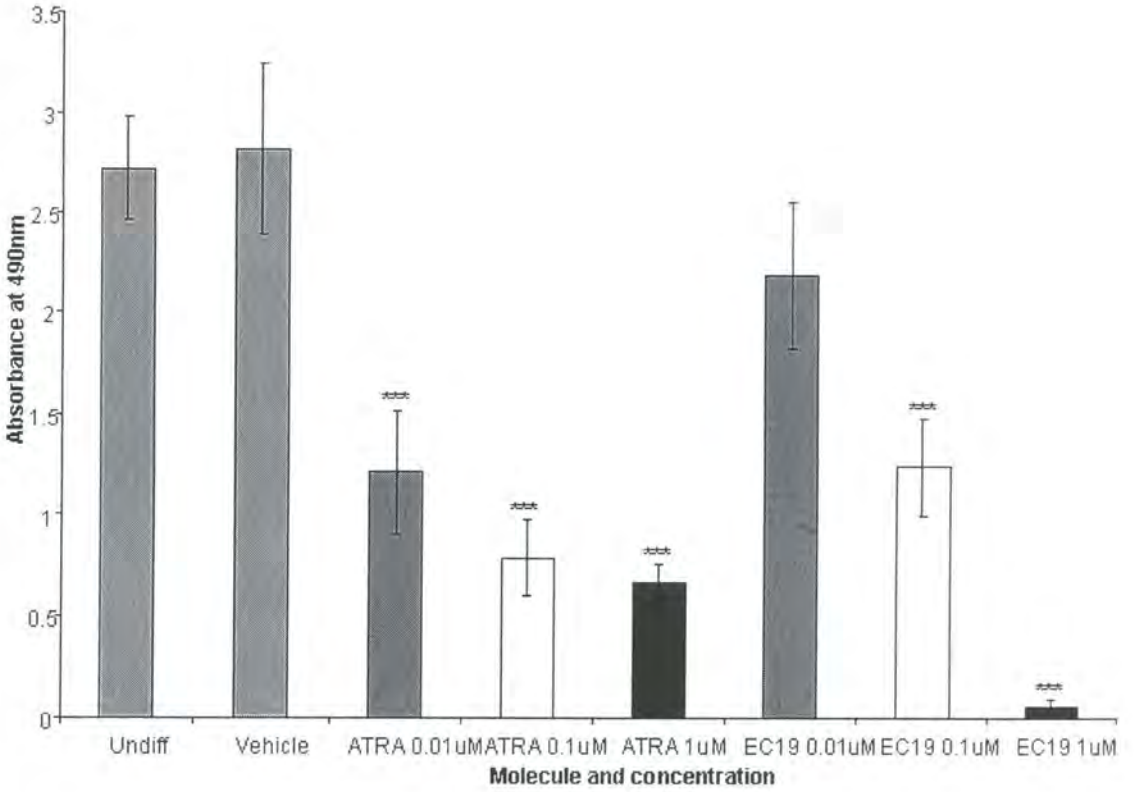


Figure 4.3.7: Number of viable cells determined on AHPCs cultured with a range of retinoid concentrations for 7 days. 100,000 cells/well were cultured with either 0.01, 0.1 or 1μM ATRA or synthetic retinoid EC19 and subjected to the MTS cell viability assay. EC19 appeared to be more potent than ATRA with fewer cells surviving at the highest concentration studied, however, at the lowest concentration studied cells continued to proliferate, subsequently increasing the final cell number in the EC19 condition. At this concentration it appeared that EC19 was less potent than ATRA. n=3, error bars represent ± standard deviation from the mean. *** $p \leq 0.0005$, student's t-test compared to the undifferentiated control.

Expression of a panel of neural markers was next analysed to deduce the levels of specific neural differentiation visualised. As above, the markers analysed were the nestin, β-III tubulin (TUJ1), and GFAP (**Fig. 4.3.8**). At the lowest concentration studied, 0.01μM, cell morphology in EC19 cultures remained small with no obvious neurite outgrowth, typical of cells in the undifferentiated state. The marker expression visualised at this level of concentration was also similar to that seen in undifferentiated cultures. Low level of expression of both TUJ1 and GFAP was observed alongside a slightly elevated expression of nestin. This indicated that at this concentration no differentiation was induced by EC19. At the same concentration, ATRA cultures showed limited neural differentiation with a small proportion of cells possessing extended processes, and these cultures exhibited up-regulated expression of TUJ1 and GFAP compared to controls.

These data infers that EC19 is either un-reactive at this concentration, or less potent than the natural retinoid ATRA. At the highest concentration tested, 1μM, there were no viable cells in EC19 cultures to stain, again an indication that at this concentration, EC19 is toxic to AHPC cells. The ATRA counterpart culture showed an elevated level of expression of TUJ1, with lots of cells expressing this neuronal marker, and extending long neuronal processes. Some glial differentiation was also witnessed. In the 0.1μM condition, where MTS analysis indicated that EC19 does appear to elicit a response, extensive neural differentiation was witnessed with this compound. Compared to the ATRA counterpart EC19-induced differentiation appeared to be more glial in nature, with less cells expressing the neuronal TUJ1 marker, and more expressing the glial marker GFAP in comparison to ATRA. This observation was quantified via flow cytometry (**Fig. 4.3.9**). Flow cytometric analysis of the glial marker GFAP showed a 2 fold increase in expression between 0.1μM ATRA and 0.1μM EC19 samples, with only ~20% cells expressing GFAP in ATRA-induced cultures and ~40% cells expressing GFAP in EC19-induced cultures. These results indicate that the synthetic retinoid EC19 is able to induce differentiation within this adult neural progenitor cell system, however, the proportion of neural sub-types induced is different from the control natural retinoid ATRA and the synthetic retinoid EC23. It also appears as if the 'window' where this compound elicits an effect is smaller than ATRA, too low and nothing happens, too high and the cells die.

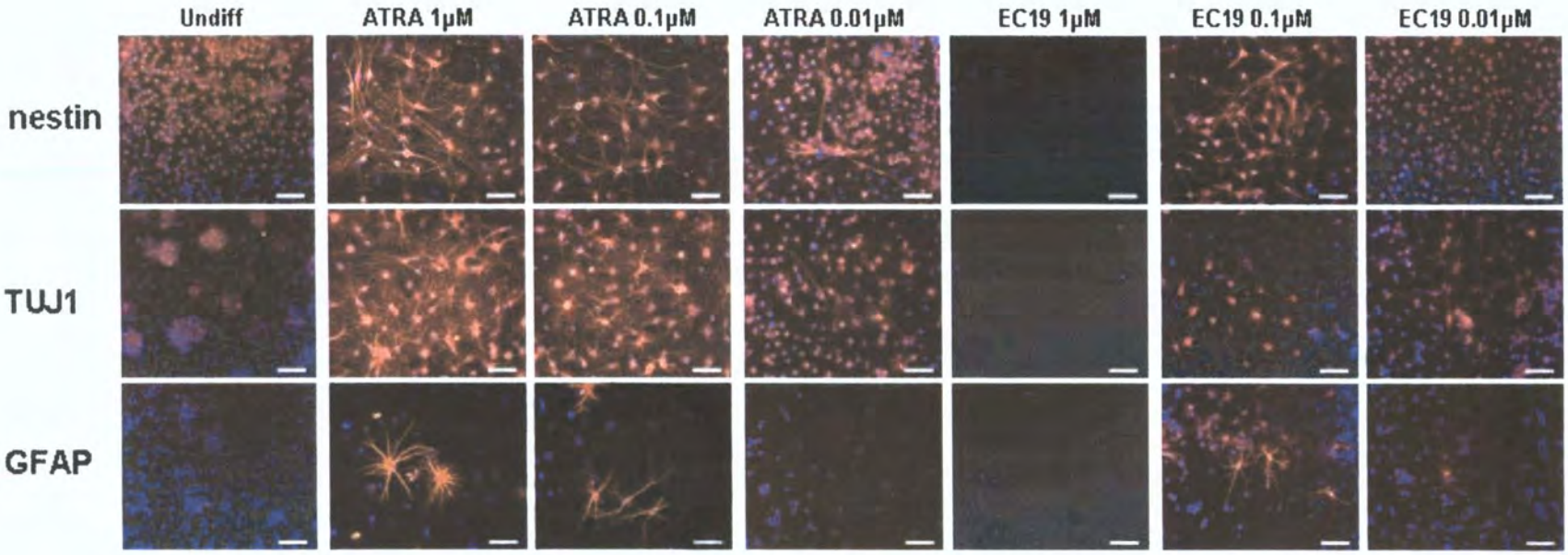


Figure 4.3.8: Immunocytochemical staining of AHPCs exposed to varying concentration of ATRA or EC19 for 7 days. Markers studied were nestin, a neural progenitor marker, TUJ1, a neuronal marker and GFAP, a glial marker. The highest concentration of EC19 studied, 1μM, was toxic to the cells and elicited no differentiation. The lowest concentration studied, 0.01μM, did not induce significant differentiation in this cell type, and cells continued to proliferate. When cells were cultured with 0.1μM, differentiation was induced down the neural lineage, with more glial differentiation being noted compared to its ATRA counterpart control. Scale bars 50μm. n=3

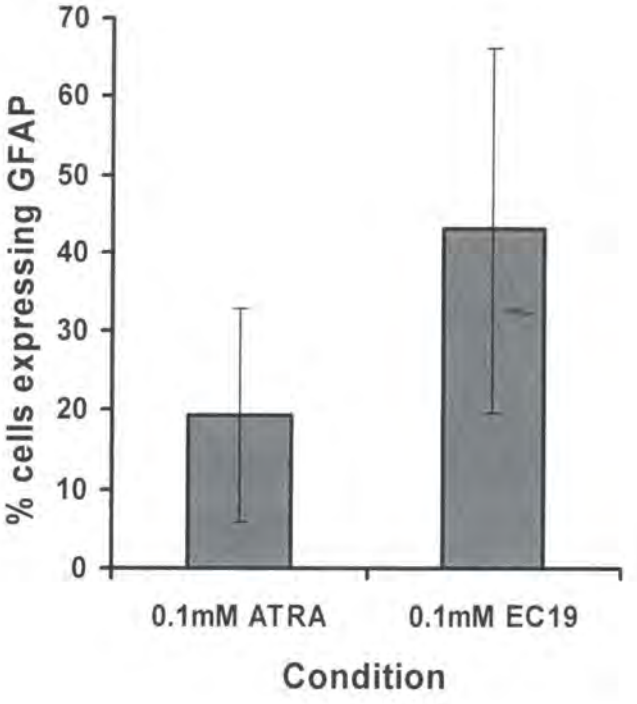


Figure 4.3.9: Flow cytometric analysis of the glial marker GFAP on cultures of AHPCs incubated with 0.1μM ATRA or EC19. In support of the morphological data, there is an increase in cell % expressing GFAP in 0.1μM EC19 culture compared to its ATRA counterpart. n=3, error bars represent ± standard deviation of the mean.

4.3.1.5 Real time PCR analysis of components of the retinoic acid receptor pathway

Experiments so far have indicated the ability of both synthetic retinoids EC19 and EC23 to induce neural differentiation in the adult hippocampal progenitor cell model system. It has yet to be shown whether this induced differentiation occurs via the retinoic acid receptor pathway. To address this, relative qRT-PCR was performed on two components of the retinoic acid receptor pathway, CYP26A1, a compound which is involved in the metabolism of ATRA within cells and which has been shown to be important in neural differentiation [173], and RAR-β, a receptor sub-type which is known to be involved in neuronal differentiation [174] (**Fig. 4.3.10**). RNA was harvested from each culture exposed to the three different concentrations of ATRA, EC19 and EC23 after 24hrs. All results obtained were set against the 24hr undifferentiated sample which was used as the relative control.

In the control ATRA samples, expression levels of the enzyme CYP26A1 increased as the retinoid concentration increased. This is as expected; if more ATRA is presented to cultures then the cells would have to work harder to keep cytostolic levels regulated to a

constant concentration. It is known that one of the mechanisms by which cells perform this is via the metabolism of ATRA to more polar compounds which are able to be excreted by the cell, a process which, as mentioned, is facilitated by the CYP26 compounds. The expression of this gene in EC23 samples showed a different pattern (**Fig. 4.3.10**). It appears that gene expression of CYP26A1 had reached maximal levels with the lower two concentrations of EC23, as an increase does not induce a subsequent increase in expression. The level of CYP26A1 in the highest EC23 concentration tested, 1μM, was significantly lower than the other EC23 samples, and the ATRA counterpart. Taken together with the morphological analysis to date, it suggests that this highest concentration of EC23 is an amount that cells are unable to deal with effectively; therefore it remains within the cells, possibly resulting in the death of cells. Expression profiles obtained in EC19 cultures also reflect the morphological data obtained (**Fig. 4.3.10**). No real differentiation is induced at the lowest concentration analysed, 0.01μM, and it appears as if the CYP26A1 is down regulated compared to undifferentiated sample. At 0.1μM, CYP26A1 is up-regulated in relation to the undifferentiated control, but not up to levels seen in ATRA or EC23, however, it is at this concentration that we see EC19-induced activity. As in the EC23 samples, the highest concentration studied only elicited a small increase in CYP26A1 expression, probably due to the fact the cells were dying with this level of retinoid. Therefore, at concentrations which elicited biological responses from the AHPCs a corresponding up-regulation of CYP26A1 expression was visualised. These data adds more weight to the argument that our synthetic retinoids, particularly EC23, are acting via the retinoic acid receptor pathway.

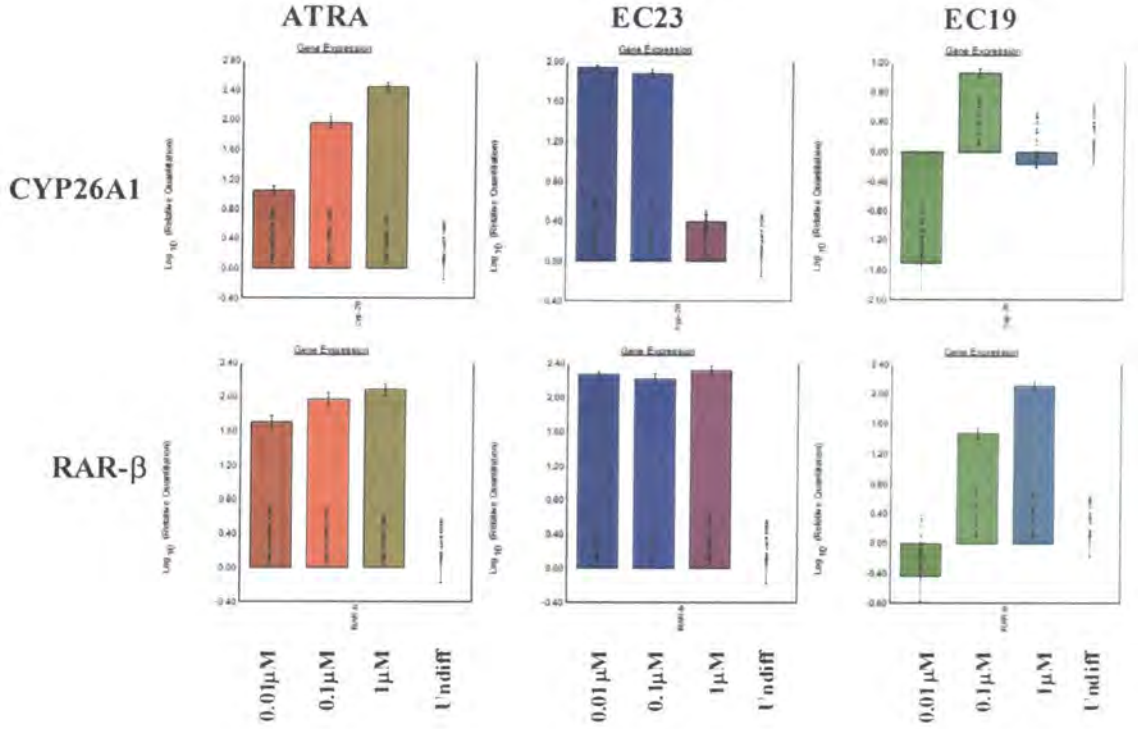


Figure 4.3.10: Quantitative analysis of CYP26A1 and RAR-β mRNA expression in AHPCs cultured with a range of concentrations of either ATRA, EC23 or EC19 for 24 hrs. Real time PCR analysis of the control ATRA concentration study revealed an increasing gradient of both genes, relative to an increase in ATRA concentration, 0.01, 0.1 and 1μM respectively. Both the 0.01 and 0.1μM EC23 cultures yielded the maximum amount of expression reached in ATRA cultures. The highest concentration of EC23 studied, 1μM, exhibited a decrease in expression compared to its ATRA counterpart for CYP26A1, this could be due to EC23 being toxic and killing cells at this concentration. Again, the highest concentration of EC19 was toxic to cells and no expression of the CYP26A1 was seen. The lowest concentration of EC19 showed a decrease in CYP26A1 expression relative to the undifferentiated control, with the 0.1μM culture showing some increase of expression of CYP26A1, but not to the same level as the other 2 retinoids. Relative quantification was carried out with the 24hr undifferentiated sample being set as the relative control for the coordinating retinoid samples.

As observed with the CYP26A1 gene, the expression profile for RAR-β within the control ATRA samples showed an increase in expression level as the concentration of the natural retinoid also increased (**Fig. 4.3.10**). Again, this indicated that expression of this retinoic acid receptor pathway component, RAR-β, is concentration dependant with regard to ATRA incubation of cultures. As also witnessed with CYP26A1 expression, this gene appeared to be already expressed at maximal levels at the lowest concentration of EC23 tested, as an increase in concentration (from 0.01μM to 0.1μM), elicited no further increase in expression level. This maximal level of expression is also observed in the 1μM sample, indicating that cells which were still viable were responding to the retinoid as expected, however, as already discussed, not many cells remained viable at this concentration due to its higher potency/toxicity than ATRA (**Fig. 4.3.10**). EC19 samples showed a concentration dependent expression of this receptor, however, the

levels observed were lower than those witnessed with the other two retinoids, ATRA and EC23 (**Fig. 4.3.10**). Expression of RAR-β at the lowest concentration tested was below the undifferentiated relative control, supporting evidence that at this concentration EC19 elicited no neural differentiation. In the 0.1μM EC19 condition an up-regulation of this gene was observed, again correlating with morphological data indicating neural differentiation had taken place. This level was significantly lower than that visualised with both ATRA and EC23 samples, which could be related to the differing proportions of neural cell-types seen in EC19 cultures compared to ATRA and EC23, however this would need further verification. There was an increase in expression of RAR-β in the highest concentration of EC19 tested compared to the lower EC19 conditions, suggesting that at this concentration, EC19 could be inducing an enhanced biological response, however, morphological analysis indicated that most cells were dying with this amount of retinoid and no differentiation analysis could be performed.

To conclude this section, it is inferred that as an increase in CYP26A1 and RARβ expression occurs in cultures incubated with either EC23 or EC19, they both are able to induce expression of components relating to the retinoic acid receptor pathway. Similar to morphological data, both EC23 and EC19 are more potent/toxic than the natural retinoid ATRA, reflected in their expression profiles obtained at the lowest and highest concentrations tested. These data backs up earlier findings on EC cells indicating that our synthetic retinoids, particularly EC23 induce differentiation via the retinoic acid receptor pathway. The synthetic retinoid EC23 appears to mimic ATRA, albeit at a lower potency threshold, and EC19 induces a different ratio of differentiation products.

4.3.2 Analysis of Natural and Synthetic Retinoid Induced Neural Differentiation of ReNcell VM Cultures:

ReNcell VM is a commercially available model of human embryonic neural progenitor cells. Published data dictates that these cells can be induced to differentiate with removal of the growth factors bFGF and epidermal growth factor (EGF) from the culture media. This section investigates the differentiation induced, if any, with the addition of the natural retinoid ATRA or the synthetic retinoids EC23 and EC19. As ATRA is a known embryonic morphogen it was hypothesised that it should elicit some effect from these cell types, however to the best of our knowledge, ATRA has not been used on this cell type before.

4.3.2.1 MTS cell viability analysis of ReNcell VM cultures exposed to the retinoids

Cultures of ReNcell VM cells were initially spiked with 1μM of each retinoid, ATRA, EC23 and EC19, for seven days then subjected to MTS cell viability/number analysis (**Fig. 4.3.11**). For retinoid-induced cultures, growth factors were removed from the media and retinoids were added at the correct concentration. Cell viability, therefore number, was compared against the undifferentiated control condition and cultures induced to differentiate with removal of growth factors alone, control differentiation. The sample inducing the least amount of colour change after addition of the MTS reagent, therefore containing the least number of cells was the ATRA sample. This was followed by both EC23 and EC19 samples, which showed similar levels of absorbance, then control differentiation, and lastly the undifferentiated control. These data suggests that all the retinoid conditions were attenuating the cell number in their respective cultures, therefore cell proliferation, in relation to the undifferentiated control, with the most enhanced effect being recorded in the ATRA culture. However, all cultures spiked with retinoids exhibited an enhanced effect compared to removal of growth factors alone, the differentiation protocol outlined for this cell type. It was not known at this stage whether the retinoids were inducing enhanced differentiation or were inducing cell death. This was verified in experiments outlined below.

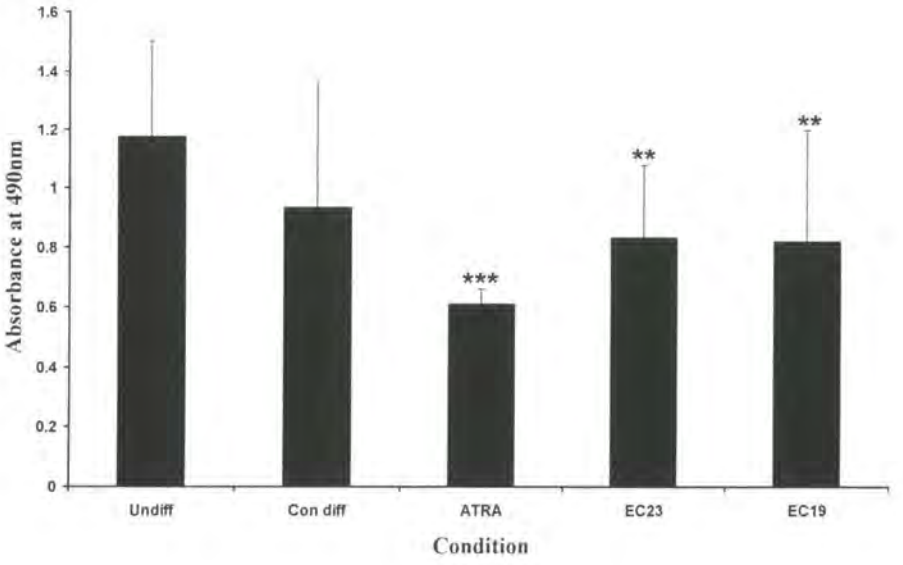


Figure 4.3.11: MTS cell number analysis carried out on 197VM human embryonic neural progenitor cells after 7 days in culture. Cells were either cultured in control proliferative media, control differentiation media (removal of growth factors), or exposed to 1μM retinoid (ATRA, EC23 or EC19) added to the control differentiation media. Under all differentiation conditions the cell number was attenuated compared to the proliferative control, with the most effect visualised in the ATRA condition. n=3, error bars represent ±standard deviation. ** $p \leq 0.005$, *** $p \leq 0.0005$, student's t-test compared to the undifferentiated control.

4.3.2.2 Morphological analysis of cultures exposed to retinoids

To address whether cultures spiked with the retinoids were being induced to differentiate or were undergoing cell death, morphological analysis was carried out. Initially, cultures were visualised under a light microscope after 7 days of culture and phase images were captured (**Fig. 4.3.12**). Undifferentiated control cells retained their typical round homogenous appearance, as outlined in technical information obtained with the cells from the company. In all differentiation-induced conditions the cells had significantly changed shape. Cells were longer and thinner and cultures contained a very large proportion of cells possessing visible processes, inferring differentiation had been induced. These data suggests that in all differentiation cultures, cells had dropped out of the cell cycle and had differentiated. It was therefore concluded that spiking cultures with retinoids were not inducing solely cell death.

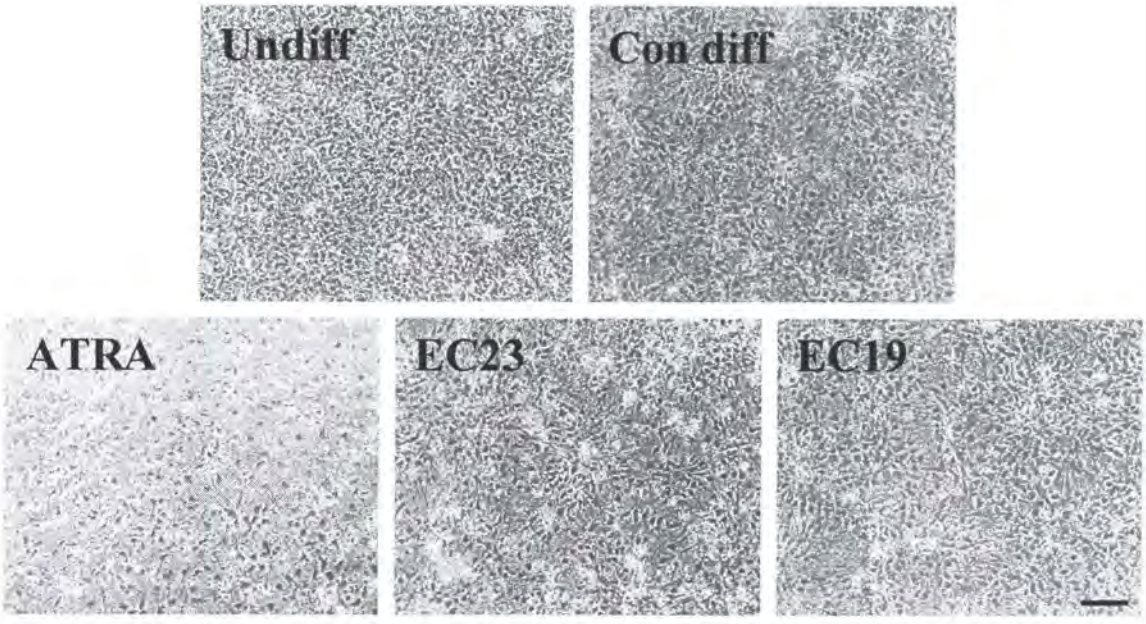


Figure 4.3.12: Phase images of 197VM human embryonal neural progenitor cells after 7 days in culture. Cells were either cultured in control proliferative media, control differentiation media (removal of growth factors), or exposed to 1μM retinoid (ATRA, EC23 or EC19) added to the control differentiation media. An abundance of neurite processes were seen in all differentiation conditions. n=3, scale bar represents 50μm.

4.3.2.3 Immunocytochemical analysis of cultures exposed to retinoids

To determine the level of neural differentiation induced, immunocytochemical analysis was performed. A panel of neural markers was used, namely the neural progenitor marker nestin, the glial marker GFAP, the neuronal specific β-III-tubulin marker TUJ1 and the late neuronal marker neurofilament 200 (NF-200). Nestin appeared to be ubiquitously expressed throughout all of the culture conditions tested, with an elevated expression level visualised in ATRA samples (**Fig. 4.3.13**). It was noted that the morphology of the cells expressing nestin changed upon removal of growth factors and with media supplementation with the retinoids. Cultures showed similar changes to those visualised under phase microscopy, suggesting that cells were undergoing some form of cytoskeletal re-organisation, inferring the induction of differentiation. Again, GFAP appears to be ubiquitously expressed throughout all cultures (**Fig 4.3.13**), however, the level of expression of this marker was up-regulated in cultures supplemented with the retinoids, possibly indicating more mature, differentiated neural cultures.

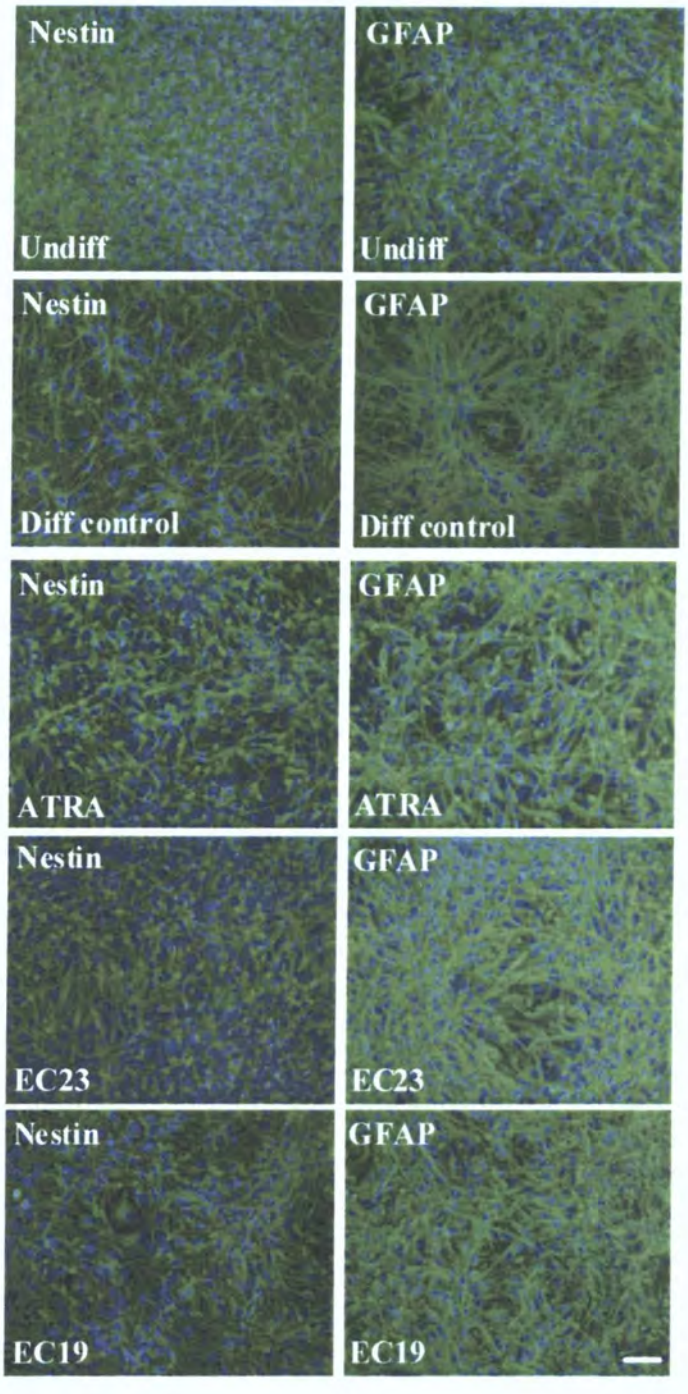


Figure 4.3.13: Immuno-cytochemically stained images of 197VM human embryonal neural progenitor cells after 7 days in culture. Cells were either cultured in control proliferative media, control differentiation media (removal of growth factors), or exposed to 1μM retinoid (ATRA, EC23 or EC19) added to the control differentiation media. An abundance of neurite processes were seen in all differentiation conditions. However, mature neuronal differentiation was only seen in ATRA and EC23 cultures (NF-200 staining) n=3, scale bar represents 50μm.

Next, neuronal differentiation was investigated. As mentioned, two neuronal specific markers were studied, TUJ1 and NF-200 (**Fig. 4.3.14**). Low level expression of TUJ1 was noted in both the undifferentiated sample and the differentiation control. While the morphology of cells had changed significantly between the two sample groups, expression levels remained mostly constant, with a small number of cells expressing a higher level of the marker in the control differentiation sample. However, when the culture conditions were spiked with either the natural retinoid ATRA or the synthetic retinoid EC23, an increase in TUJ1 expression was observed. Many cells throughout the cultures showed an enhanced expression level of this marker compared to the undifferentiated and control differentiated conditions. These data suggests that a more mature level of neural differentiation was being achieved with the supplementation of these retinoids into the culture system. TUJ1 expression levels in EC19 cultures remained comparable to control differentiated conditions, with a few individual cells exhibiting the up-regulated expression of this marker. To investigate the theory that spiking with retinoids elicited more mature neural cell types the late neuronal marker NF-200 was examined (**Fig. 4.3.14**). This marker was not expressed in undifferentiated cultures, nor was it expressed in cell induced to differentiate with removal of growth factors alone. These data backed up previous findings that removing only growth factors from media did appear to initiate neural differentiation, but was not conducive to obtaining mature neuronal phenotypes. After the addition of either ATRA or EC23 into the culture media, cells expressing the late neuronal marker were visualised. An indication that mature neuronal phenotypes were being stimulated under these defined conditions. No NF-200 positive cells were witnessed in EC19 cultures, again indicating the differing nature of this compound to the natural counterpart ATRA, and synthetic retinoid EC23. To quantify the amount of mature neuronal differentiation visualised in the sample groups, ImageJ software was used. The number of positive NF-200 perikaria/field of view in eight randomly selected photomicrographs/condition was calculated and results were shown graphically (**Fig. 4.3.15**). As expected, both the ATRA and EC23 conditions contained the most NF-200 expressing cells, with a higher level being visualised in the EC23 condition. The number of cells expressing NF-200 in EC19 was comparable with the differentiation control condition, which were both slightly higher than the undifferentiated control. These data verifies that media supplementation with either ATRA or EC23 induced neural differentiation to a more mature level than just following the standard protocol obtained with these commercially available embryonic neural

progenitor cells. It was noted that EC23 was able to mimic ATRA induced biological responses within this cell model system.

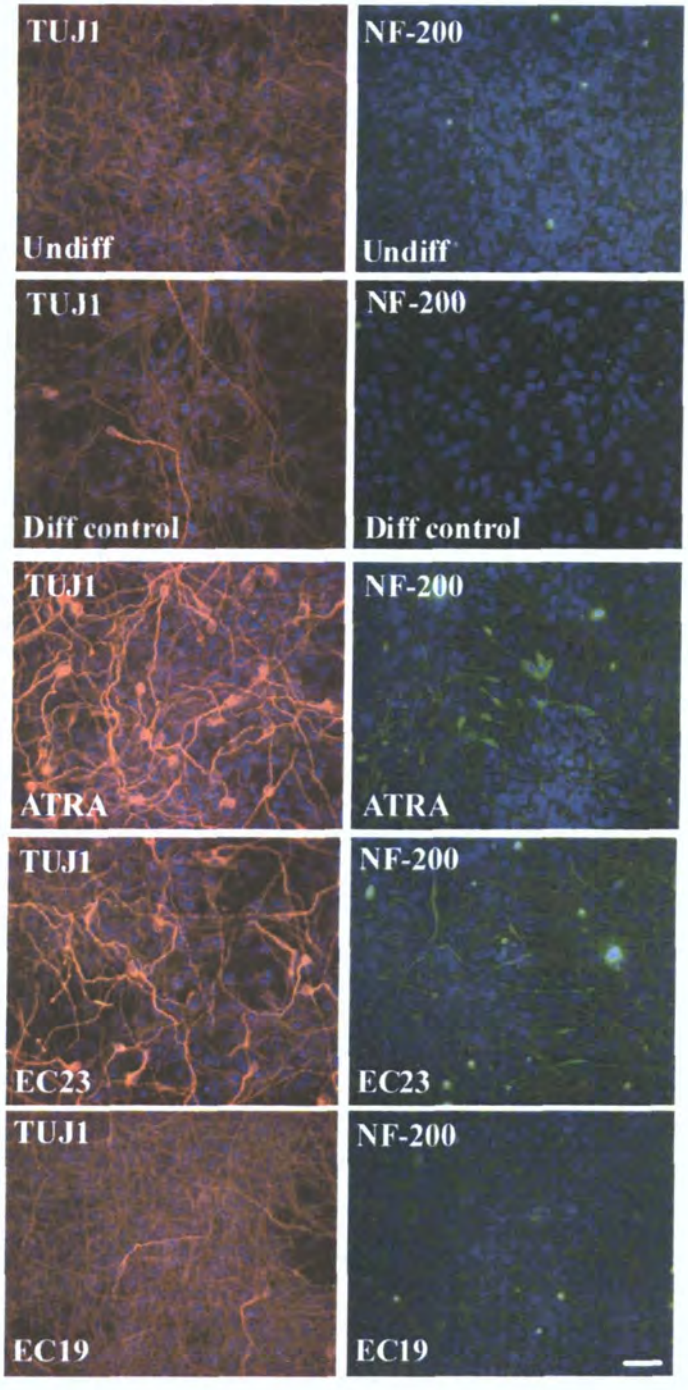


Figure 4.3.14: Immuno-cytochemically stained images of 197VM human embryonal neural progenitor cells after 7 days in culture. Cells were either cultured in control proliferative media, control differentiation media (removal of growth factors), or exposed to 1μM retinoid (ATRA, EC23 or EC19) added to the control differentiation media. An abundance of neurite processes were seen in all differentiation conditions. However, mature neuronal differentiation was only seen in ATRA and EC23 cultures (NF-200 staining) n=3, scale bar represents 50μm.

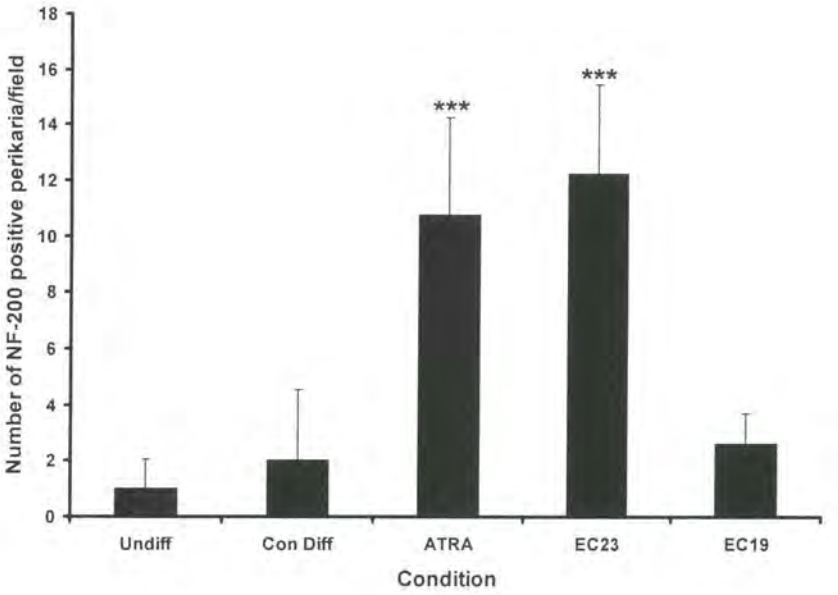


Figure 4.3.15: Induction of neural differentiation in cultures of 197VM embryonic neural progenitor cells with 1μM ATRA, EC23, and EC19. 7 day-differentiated cultures were immunocyto-stained for the late neuronal marker NF-200. Using ImageJ software the number of NF-200 positive cell bodies was counted on 8 random fields of view per condition. Results were averaged and represented graphically. When cultures were treated with either ATRA or EC23 the number of cells expressing the mature neuronal marker NF-200 increased. This was not the case with the EC19 retinoid condition. Error bars represent standard deviation of the mean, n=8. *** $p \leq 0.0005$, student's t-test compared to the undifferentiated control.

4.4 Discussion and Conclusions

4.4.1 Adult hippocampal neural progenitor cells

The initial aim of this body of work was to establish a suitable adult neural progenitor cell model system to investigate the activity of synthetic analogues of retinoic acid. To qualify as a suitable model system, the chosen cell line had to demonstrate differentiation toward a neural lineage in response to media supplementation with natural retinoids. The AHPC line in question has been shown to respond to ATRA in previous experiments, differentiating down a neural lineage [168]. Furthermore, hippocampal progenitor cells have been used for high-throughput screening to identify neuro-active synthetic small molecules [175].

Here, the addition of either ATRA or 13cisRA to cultures did indeed initiate differentiation, with expression of the neuronal marker TUJ1 and glial marker GFAP being up-regulated. In 13-cisRA cultures, cell number and immunological analysis confirmed there were consistently more TUJ1 positive cells than in ATRA counterparts, with also an increased total number of cells being present throughout the time course. One possible explanation for these findings could be the fact that 13-cisRA is thought to be a pro-form of ATRA and/or 9-cisRA [176], isomerising before it elicits an effect, as it is known that 13-cisRA is unable to bind effectively to either RARs or RXRs itself [177]. Potentially, this phenomena could instigate a time-lag before cells become exposed to the biologically active forms, accounting for the increase in cell number (proliferation) witnessed. Consequently 13cisRA cultures would contain more cells over-all, which ultimately commit to the neural lineage, leading to increased final numbers of TUJ1 positive cells (see Fig. 4.4.1 below). A similar phenomena was observed by Liu et al in 2008 [178]. They reported that at specific concentrations, 13-cisRA induced an increased amount of neuronal cell survival in their primary hippocampal neuron cultures compared to standard media controls, whereas, while ATRA didn't induce cell death, it didn't enhance cell survival above the controls either. An increase of up to 44% was observed in 13cisRA cultures compared to vehicle controls, with no reported increase in the amount of glial cells. These results can be compared to those presented here, with the only difference being the concentration at which the effect was elicited, 100 fold lower. This difference could be due to the fact that cultures of primary explants were used, which

may be more sensitive than the established immortalised cell line used here. These findings are of interest, since they provide evidence that adult hippocampal cells respond differently to different isomers of retinoic acid. They also re-enforce the need for stable non-isomerisable ATRA alternatives to allow tightly controlled concentration and isomer studies to be performed.

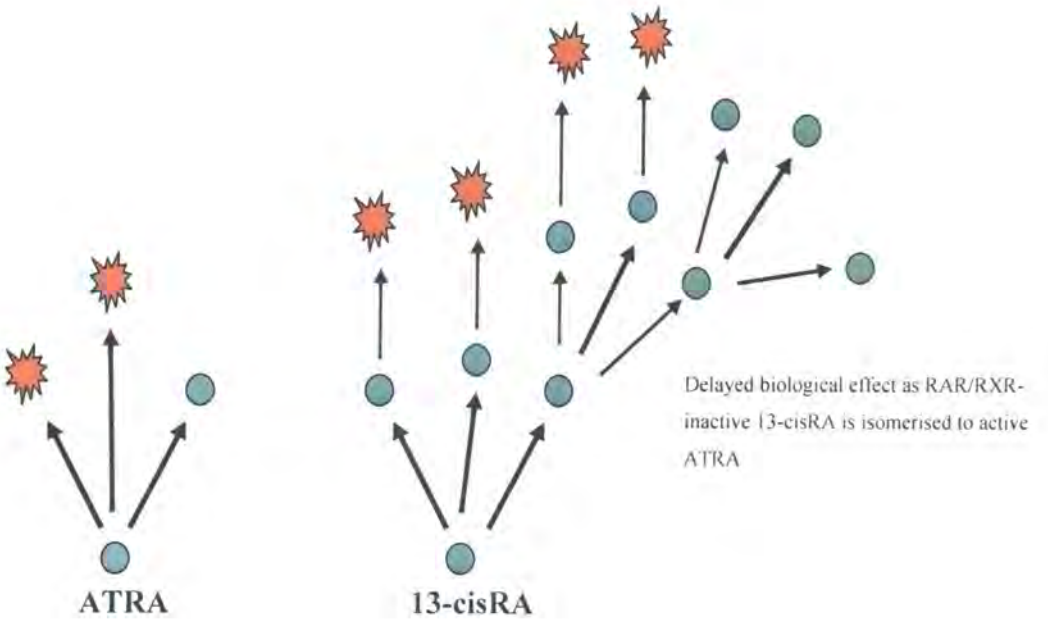


Figure 4.4.1 Diagram depicting hypothesised delay of 13-cisRA action and possible explanation for the observation that more TUJ1 positive cells are present in 13cisRA treated cultures compared to ATRA counterparts.

Having shown that the AHPC line was a suitable cell model system with which to evaluate retinoid activity, the next aim was to investigate the synthetic retinoid analogue, EC23, to determine whether it elicited any biological response. Different concentrations and cell densities were analysed to establish the optimal culture conditions for further analysis. This was an important part of the investigative process, similar to the first published studies investigating the *in vitro* culture survival of primary hippocampal neurons. In these early studies, the optimal bFGF culture concentration suitable for

prolonged cell proliferation and survival was discovered by using a similar trial and error approach [179]. After various EC23 concentrations and cell densities were evaluated, it was discovered that EC23 could elicit similar biological activity to ATRA providing the cells were seeded at a sufficiently high density. Cells exposed to EC23 appeared to differentiate down the neural lineage, with cells expressing the neuronal marker TUJ1 after 7 days in culture. The highest concentration of EC23 tested, 1μM, appeared to be toxic to cultures as revealed by decreased neuronal survival, whereas ATRA was not, indicating an increased potency/toxicity of EC23 on these cultures. The toxic effect of high concentrations of ATRA on *in vitro* hippocampal neuron survival is published, and is similar to that observed here with EC23 [178]. Published data states that after cultures were exposed to high levels of ATRA, neuronal survival was decreased and cultures became sub-optimal for on-going neural differentiation with apoptosis/cell death becoming prevalent. The data obtained after EC23 toxicity fits with the fact that at high doses ATRA is toxic and is known to induce apoptosis in some cells, being used as a chemotherapeutic agent either alone or in combination with other drugs because of this [180].

The apparent toxicity of EC23 at high concentrations may reflect its engineered stability. Not only is EC23 more chemically stable than ATRA, but it is possible that it is also more resistant to cellular metabolism. Both of these factors would contribute to EC23 remaining intact in the media/cells for relatively long periods compared to ATRA. This in turn may contribute to the toxic effects observed. Another point to consider would be that it may not be that EC23 is more potent/toxic than ATRA, it may just be that due to the instability of ATRA that although cultures are supposedly being exposed to equal concentrations of the retinoids, this may not be the case due to ATRA degradation, the problem which is trying to be resolved in this investigation. These preliminary findings indicate that it is possible to synthesise a retinoid that is more stable than its natural counterpart and can also induce similar cell differentiation under defined culture conditions. Hence, EC23 appears to be an attractive alternative to ATRA for reproducible neuronal differentiation of adult neural progenitor cells. EC23 is non-isomerisable therefore there is no problem of trying to decipher which isomer is eliciting which effect.

AHPCs were also cultured with the synthetic retinoid EC19. Again, a range of concentrations was tested with only one (0.1μM) resulting in any differentiation of cultures. At higher concentrations the retinoid appeared toxic, killing cultures, while at lower concentrations no differentiation was induced. Compared to the natural control retinoid ATRA, EC19 appears to have a much smaller concentration 'window' for inducing differentiation within this particular cell system. At the concentration which did induce differentiation, 0.1μM, as was witnessed with all other systems tested, EC19 induced differing proportions of specific cell sub-types compared to the ATRA control. A higher level of glial differentiation was visualised, with almost a two-fold increase in marker expression for this cell type in EC19 cultures. Therefore it was concluded that although EC19 was able to induce differentiation, given the right culture conditions, the patterns of differentiation were distinctly different to those achieved with the ATRA counterpart. This could potentially provide interesting insights into the study of glial development in adult neural progenitor cell model systems. Experiments carried out on adult neural progenitor cells have reported that glial differentiation is the predominant cell type induced after media supplementation with the bone morphogenetic protein 4 (BMP4) [181, 182]. During neural development, BMPs exert their action by inhibiting the default neuro-ectodermal differentiation, inducing the differentiation of epithelial lineages. These reports indicate that applying BMPs to committed adult neural precursors induced specific glial subtypes, possibly blocking the action of other neuronal specific pathways. It could therefore be hypothesised that EC19 is inducing similar glial effects, at the expense of neuronal differentiation. To confirm this hypothesis specific binding studies would need to be carried out to identify common EC19 and BMP4 downstream binding partners, followed by antagonism of those elements and recording of the induced cellular responses.

Real-time qPCR analysis of two specific components of the retinoic acid receptor pathway further revealed common functionality between ATRA and EC23. Genes studied were the ATRA metabolising agent CYP26A1, which has previously been shown to be involved in neural differentiation and regulation [173], and the nuclear receptor involved in neuronal differentiation, RAR-β [174]. Expression profiles obtained for both genes indicated that EC23 appeared to be inducing maximal levels of expression at the lowest concentration evaluated; backing up previous findings that EC23 appears to be

more potent than ATRA. Data also verifies that EC23 appears to be eliciting its effects via the retinoic receptor pathway. It is hypothesised that reducing the concentration of EC23 to amounts lower than tested here would eventually elicit a concentration dependent expression profile. Further work here would be useful to evaluate the optimal supplementation concentration of EC23 into AHPC media for maximal neuronal differentiation.

EC19-induced cultures also up-regulate the CYP26A1 and RAR-β genes, but only under very defined culture conditions, and the expression levels reached were lower than those obtained with the other retinoids tested. Again, this indicates that this analogue may elicit its effects predominantly via a different pathway or by activating other retinoid receptor complexes not evaluated here. It would be interesting for future work to investigate the expression profiles of a larger group of retinoid-activated compounds to discover any which EC19 is capable of inducing the expression of. It is important to note the vast difference in biological activity induced by EC23 and EC19, especially since this must be attributed to the only difference between these molecules, namely, the position of the carboxylic acid group in the *para-* versus *meta*-position respectively. Again, this finding prompts the need for further structure/activity-based investigation into how these analogues are eliciting their effects.

4.4.2 Embryonic neural progenitor cells

ReNcell VM is a commercially available embryonic neural progenitor cell line derived from the ventral mesencephalon of human foetuses. Cell lines and cells derived from this area of the embryonic brain are particularly important for therapeutic transplantation therapies for disorders associated with loss of dopaminergic neurons, such as Parkinson's disease [183] [184]. It is anticipated that the *in vitro* culture and subsequent controlled differentiation of neural progenitor cells derived from the ventral mesencephalon will limit the number of foetuses required per therapeutic transplantation into patients [185]. However, the *in vitro* differentiation of these neural progenitors still needs to be optimised to increase the yield of the neuronal sub-types required for such transplantation. One such compound which could have an effect on the differentiation products induced is ATRA. It is known that during neurulation in the early embryo, the

retinol dehydrogenase associated with the synthesis of retinoic acid has been localised to various areas of the developing CNS including cells within the ventral mesencephalon [186], thereby indicating the ability of these cells to respond to endogenous ATRA. Indeed, in an investigation into the differentiation potential of ventral mesencephalon progenitors *in vitro*, carried out in 2004 [187], the addition of ATRA into a defined dopamine-inducing differentiation protocol significantly increased the yield of dopamine neurons. Therefore, while it was predicted that media supplementation with ATRA should effect the differentiation of ReNcell VM cells, its effects had not been illustrated in the cell line before.

Experiments were carried out to investigate what effect, if any, the supplementation of ATRA would have when added to the standard differentiation media protocol. It was also investigated whether the addition of EC23 or EC19 would induce similar or differing effects from the ATRA control. Immunological analysis of a panel of neural-related proteins revealed very interesting findings. It was discovered that the addition of either ATRA or EC23 into the culture media induced the differentiation of more mature neuronal phenotypes compared to the standard differentiation protocol. NF-200, (a marker of late neuronal development), positive neurons were only visualised in these two culture conditions. The supplementation with EC19 did not induce these neuronal sub-types, but it did induce an up-regulation of the glial marker GFAP, also witnessed with the other two retinoids. Yet again, these results support the hypothesis that EC23 is a more stable alternative to ATRA for *in vitro* investigations into neuronal differentiation processes. These results are in-line with those reported above. As a result of these data, a revised differentiation protocol is suggested for the production of mature neuronal phenotypes from cultures of ReNcell VM cells:

Revised differentiation protocol:

ReNcell VM cultures are incubated with trypsin to obtain a single cell suspension, and then 100,000 cells/well were seeded into 12 well plates. Plates were allowed to get 75% confluent then were switched to differentiation media. Differentiation media consists of DMEM:F12 (1:1, Gibco) supplemented with 10mls B27 (Invitrogen), 2mM L-Glutamine (Sigma), 0.5ml Gentamycin (Gibco), 1ml 50mg/ml Heparin solution (Sigma) and either 1μM ATRA or the more stable alternative EC23. Cultures are incubated for 7 days, with

a complete media change being performed on day 3. Following this protocol should induce the production of NF-200 positive neurons which were not found following the standard differentiation protocol issued with the cells from the selling company.

4.4.3 Conclusions

The natural retinoid ATRA has been shown to be a vital component for both adult and embryonic neurogenesis (refer to Chapter 1). This chapter has dealt with evaluating the effects induced by supplementation of both adult and embryonic neural progenitor cultures with either the synthetic retinoid EC19 or EC23, and comparing results with those obtained from ATRA cultures. It was hoped that one of the synthetic compounds would mimic ATRA-induced differentiation, and therefore present itself as a potential attractive alternative for use in *in vitro* neurogenesis research. Previous data has illustrated that the synthetic compounds are more stable than ATRA under standard laboratory conditions, and that EC23 appears to mimic ATRA in embryonic stem cell model systems, inducing induction of neural differentiation. EC19 was also shown to induce differentiation in these cells, albeit down a different cell lineage.

Cultures of adult hippocampal neural progenitor cells differentiated down a predominantly neuronal lineage in response to both ATRA and EC23 supplementation. However, it was noted that for EC23 to induce this differentiation, cells were required to be seeded at a higher density. The higher potency of EC23 compared to ATRA was also revealed during real time qPCR analysis, as outlined above. EC19 was able to induce differentiation, albeit under very defined culture conditions. The differentiation induced was predominantly glial, different from control ATRA samples, and as such EC19 may be an attractive new candidate for use in understanding glial cell development in the adult hippocampal system. It was concluded that both synthetic retinoids were able to induce differentiation in this adult neural progenitor model system, with EC23 closely resembling ATRA cultures. The effects of all three retinoids appeared to be elicited via activation of the retinoic acid receptor pathway, although induction of the components analysed was at relatively low levels in EC19 cultures.

When cultures of ReNcell VM embryonic neural progenitors were supplemented with either ATRA or EC23, significantly more mature neuronal sub-types were visualised in culture, this was not seen with EC19 supplementation. It was therefore concluded that supplementation with either ATRA or EC23 greatly enhances the neuronal products obtained, therefore aiding future analysis of neurogenesis using this particular cell type.

In light of these findings an updated differentiation protocol is suggested for the induction of mature neuronal phenotypes.

In both cell model systems EC23 was shown to induce differentiation in a similar manner to ATRA. Combining these data, also with knowledge that EC23 is a much more stable compound when used under standard laboratory conditions, it is concluded that this compound appears to be an attractive alternative to ATRA for use in *in vitro* investigations into both embryonic and adult neurogenesis.

5 Chapter 5: Natural and Synthetic Retinoid Action on SH-SY5Y Neuroblastoma Cells

5.1 Introduction:

Retinoids have a close link to cancer biology. They are recognised as teratogenetic agents when found in excess (Chapter 1), but can also be incorporated into therapy regimes as chemotherapeutic agents for certain types of cancer, including neuroblastoma, with varying success. Retinoids appear to inhibit tumour growth by two main biological processes, either causing cell differentiation or apoptosis. For example, ATRA can be used to successfully treat some types of acute promyelocytic leukaemia [188], where they induce apoptosis in the aberrant cell population, or in the case of neuroblastoma, retinoids also induce neural differentiation in the responding cell population [189]. However, their use is associated with many debilitating serious side effects such as hepatotoxicity, headaches, nausea, vomiting, abdominal pain and mucocutaneous dryness, collectively known as retinoic acid syndrome [190]. Therefore, due to these limiting toxicities the maximum tolerated dose is often reduced to below therapeutically optimal levels. It also appears that cells become sensitised to ATRA very quickly during the treatment period due to an up-regulation of the CYP metabolising proteins [191], meaning that after time, a higher dose is required to elicit a comparable effect; however this dose increase is associated with the toxicity issues outlined above. Formation of synthetic retinoic acid metabolising blocking agents (RAMBAS) is an area of investigation which hopes to overcome this problem, as outlined in Chapter 1.

One type of cancer that responds in part to retinoid therapy is neuroblastoma, a childhood cancer [192] which accounts for 7-10% of all childhood cancers. As already mentioned, administration of retinoids appears to cause differentiation of the aberrant stem cell population. However, not all neuroblastomas respond to treatment in the same way, and as outlined above, retinoid sensitivity regularly occurs throughout the treatment process, with some neuroblastomas being totally resistant to retinoid treatment from the start. It appears that certain factors contribute to the tumour's response to retinoid therapy, for example, over-expression of the Bcl-2 gene is one of the key mechanisms which instil

resistance in these cells to retinoid treatment [193]. Several different approaches have been taken to try and optimise the use of retinoids for chemotherapeutic agents for neuroblastoma. For example the use of the ATRA isomer 13cisRA in clinical trials has proved more successful than ATRA therapy even though it is thought to isomerise back to ATRA before it exerts its biological effect [194, 195]. Two other interesting areas are the combining of ATRA and other compounds for joint treatment programmes, however so far this has had limited success in clinical trials [196], or the development of synthetic retinoid analogues which have enhanced therapeutic potential [197, 198]. It is this approach that we aimed to investigate here. The aim of this part of the investigation was to assess whether the synthetic retinoid compounds, EC19 and EC23, were able to inhibit the growth of a neuroblastoma cancer cell line to a comparable or greater degree than the established natural retinoids, ATRA and 13cisRA. If the synthetic retinoids were found to inhibit tumour growth it would then be important for subsequent studies to deduce whether they induced the kind of debilitating side effects seen with their natural counterparts. This would need to be investigated via animal models and would therefore only be carried out once the basic science was in place. Clearly, if these synthetic analogous caused reduced side effects then they might be considered potential therapeutic replacements for ATRA and 13cisRA. Future strategies specifically aimed at side effect reduction could include the tailoring of compounds to selectively activate receptors associated with cell differentiation, apoptosis and cell death.

5.1.1 SH-SY5Y Neuroblastoma cell line

The neural tumour cell line SH-SY5Y has been used in this study. The SH-SY5Y cell line is a thrice cloned sub-clone of the SK-N-SH cell line derived from neoplastic neural crest cells of a four-year old female child [199]. The aberrant cells were collected and cultured from a bone-marrow biopsy. In previous investigations, the SH-SY5Y neuroblastoma cell line has been shown to differentiate in response to both ATRA and 13cisRA [200], where it appears that 13cisRA is isomerised to ATRA before it elicits any effect. Although the SH-SY5Y cell line was initially cloned as a neuronal-specific N-type cell line, unless cultures undergo continual N-type selection, they have been shown to contain the three different cell types normally associated with proliferating neuroblastoma cultures, namely the neuronal N-type cells, which are the cell type that differentiate down the neural lineage, the substrate-adherent S-type cells, which appear

non-neural in shape and are possibly linked to Schwann cells, and the intermediate I-type cells, which are thought to be an intermediate phenotype cells go through before turning into either N- or S-type cells [201].

5.1.2 Aim and Objectives

Aim

The initial aim of this Chapter were to establish a valid neural tumour cell model system, and use this system to investigate retinoid action with a view to testing the biological activity of the synthetic retinoid compounds EC19 and EC23.

Objectives

- To represent a usable model, the cell line would be required to mimic a type of neural tumour that retinoid therapy is currently used on, and the cell line would need to show partial biological response to the natural retinoids. Biological activity of synthetic retinoids was investigated using a range of relevant biological techniques.
- The ability of the synthetic retinoids EC19 and EC23 to induce differentiation would be observed, and a range of concentrations studied to find a suitable 'optimal' concentration at which all other experiments would be carried out at.
- The ability of the synthetic retinoids EC19 and EC23 to inhibit cell proliferation would be evaluated and compared with results obtained with the natural retinoids ATRA and 13cisRA.

5.2 Methods

5.2.1 Tissue Culture

5.2.1.1 SH-SY5Y neuroblastoma cells

Cells were cultured under standard laboratory conditions as described in Chapter 2. SH-SY5Y cells were cultured in uncoated tissue culture T75 flasks (Nunc) or 12-well plates (Nunc). Proliferation media was MEM:F12 1:1 (Sigma), supplemented with 2mM L-Glutamine (Sigma), 1% non-essential amino acids (Gibco), 15% foetal calf serum (Sigma) and 1000 active units of penicillin/streptomycin solution (Sigma). To maintain optimum cultures, media was changed every 3 days, and cultures were passaged when they reached ~80% confluencey. To passage, media was aspirated, 2ml trypsin-versene solution (Cambrex) added and rocked over cells and incubated at 37°C for up to 5 mins. Cells were dislodged from the plasticware by tapping, and resuspended in 10mls MEM/F12 media. The suspension was pelleted by centrifugation at 800rpm for 2 mins. The supernatant was removed and the pellet resuspended in 1ml proliferation media and triturated to gain a single cell suspension. Cells then underwent a 1:3 to 1:6 split depending on cell numbers and were transferred to new plasticware.

5.2.1.2 Differentiation of SH-SY5Y cells

Cell cultures were induced to differentiate with the introduction of ATRA (Sigma, diluted in DMSO (Sigma)), 13-cisRA (Sigma, diluted in DMSO), EC19 (diluted in DMSO) or EC23 (diluted in DMSO) into the media. Unless stated, a concentration of 10μM retinoids was used throughout. In preparation for differentiation, single cell suspensions were prepared as outlined above, and cells were counted using a haemocytometer under a light microscope. Unless otherwise stated, cells were seeded at 20,000 cells/well in a 12-well plate. Cultures were maintained for up to 14 days with media being refreshed every 3 days.

5.2.2 Flow cytometry

For complete intracellular flow cytometry methods refer to Chapter 2, Section 2.4.

For complete cell cycle analysis methods refer to Chapter 2, Section 2.5.

Antibodies and stains used:

Antibody	Company	Dilution	Incubation time	Secondary
TUJ1	Covance	1:600	1hr	Cy3
Propidium Iodide	Applied Biosystems	1:100	10mins	N/A

5.2.3 Immunocytochemistry

For complete immunocytochemical methods refer to Chapter 2, Section 2.6

For complete BrdU pulsing and subsequent immunocytochemical staining methods refer to Chapter 2, Section 2.8.

Antibodies used:

Antibody	Company	Dilution	Incubation time	Secondary
Vimentin	Sigma	1:200	1hr	Alexafluor 488
TUJ1	Covance	1:500	1hr	Cy3
RAR-α	Santa Cruz	1:100	1hr	FITC
RAR-β	Santa Cruz	1:100	1hr	FITC
RAR-γ	Santa Cruz	1:100	1hr	FITC
BrdU	Molecular Probes	1:100	1hr	Alexafluor 488

5.3 Results

5.3.1 Concentration study of natural and synthetic retinoids

Initial experiments were carried out to establish a suitable concentration at which both synthetic retinoids appeared to be eliciting an effect in the SHSY-5Y cultures, at which all future analysis would be carried out. Initially a wide concentration range was tested incorporating 1μM, 10μM and 100μM concentrations of each retinoid tested, namely ATRA, EC19 and EC23. Cultures were set up and exposed to the retinoids for seven days before undergoing MTS viable cell number analysis (**Fig. 5.3.1**) and immunocytochemical analysis of markers of the two main cell sub-types found within the SH-SY5Y culture (**Fig. 5.3.2**). Two markers were used for immunocytochemical analysis, one for each of the specific cell types visualised within SH-SY5Y cultures. The β-III-tubulin antibody, TUJ1, was used as a marker of the neuronal N-type cells, and vimentin was used as a marker of the substrate-like S-type cells. All results were compared to both undifferentiated and vehicle-exposed (DMSO) controls. Cell number analysis indicated that exposure to the vehicle control, DMSO, alone did not inhibit cell number compared to the undifferentiated control, and can therefore be discounted as eliciting any effect on its' own.

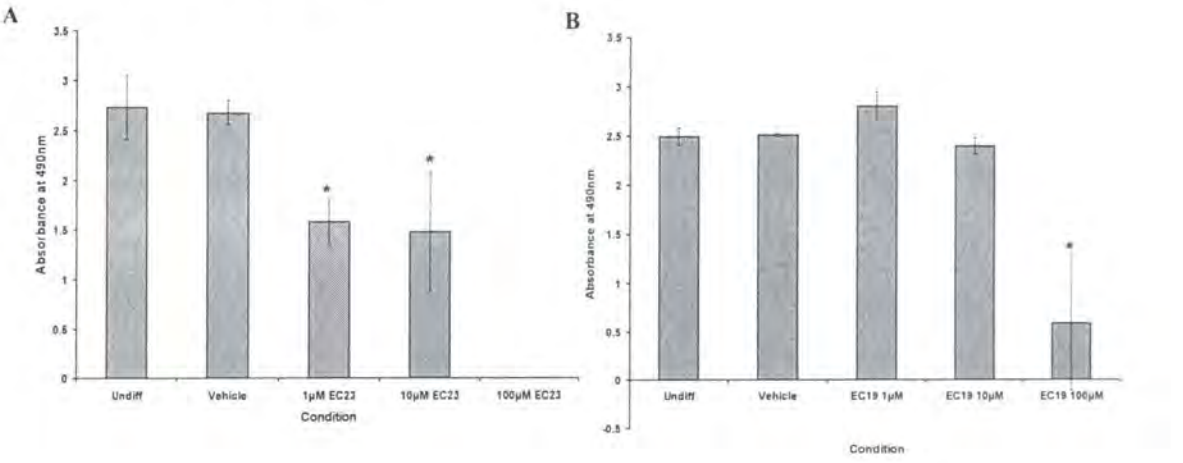


Figure 5.3.1: Concentration study of the synthetic retinoids EC23 and EC19 on SH-SY5Y cell viability. MTS cell number analysis was carried out on 7 days samples incubated with either 1μM, 10μM or 100μM retinoid. Undifferentiated and vehicle exposed cultures were treated as controls. 10μM concentration appears to be optimal for all retinoid compounds tested. n=2. * $p \leq 0.05$, student's t-test compared to the undifferentiated control.

Cell number analysis data revealed that as the concentration of the synthetic retinoid EC23 increased, the viable cell number decreased, with no viable cells visible in the highest concentration tested, 100μM (**Fig. 5.3.1A**). Cell numbers also decreased as the concentration of EC19 was increased (**Fig. 5.3.1B**), however, at the lowest EC19 concentration (1μM) the number of viable cells present was higher than that visualised in the undifferentiated control. 10μM EC19 did induce a decrease in cell number compared to 1μM, however numbers remained higher than those observed for EC23 at the same concentration. Even when cells were exposed to the highest concentration tested of EC19, 100μM, some viable cells were still present after 7 days in culture. Taken together these initial data suggested that EC23 is more potent/toxic to SH-SY5Y neuroblastoma cells than EC19. In addition, EC19 may act to induce an enhanced level of proliferation in these cancer model cells.

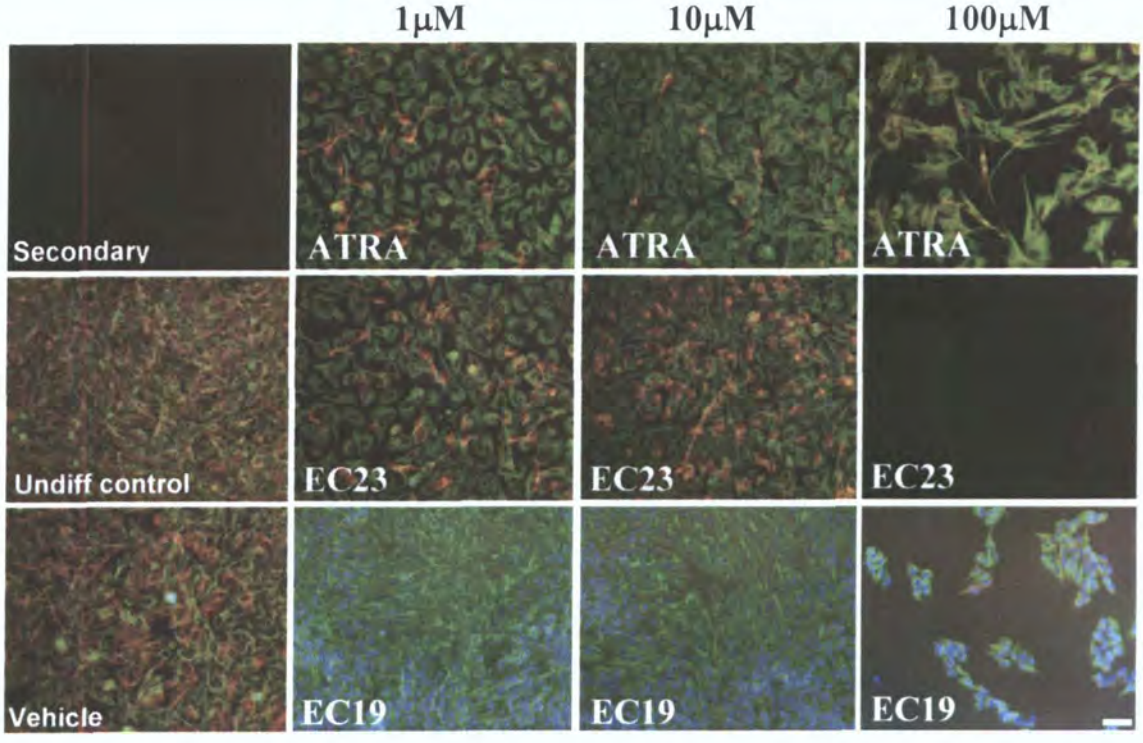


Figure 5.3.2: Concentration study of the synthetic retinoids on SH-SY5Y cell differentiation. Immunocytochemical staining of SH-SY5Y cells after incubation with either 1μM, 10μM, 100μM retinoid. Cells are stained for S-type marker vimentin (green), neuronal N-type marker TUJ1 (red). At the highest concentration tested cells were sub-optimal and dying. At all concentrations the synthetic retinoid EC19 appears to induce predominantly S-type cells. Scale bars: 50μm. n=3.

To examine what effect EC19 and EC23 were having on the individual SH-SY5Y cell sub-types, immunological analysis was performed and compared against the control retinoid ATRA (**Fig. 5.3.2**). Undifferentiated cultures contained a high number of both N-type and S-type cells, as did the vehicle control. After incubation with the control retinoid ATRA, the proportion of N- and S-type cells changed. The proportion of N-type cells decreased compared to control samples, and appeared to change shape, forming long processes indicating that differentiation into neuronal cell-types may have occurred, especially in the 10μM sample. EC23 also appeared to attenuate the number of N-type cells, but not to the same extent as ATRA. The over-all number of S-type cells was also reduced compared to the undifferentiated controls. EC19 appeared to inhibit N-type cell formation and increase S-type proliferation. To quantify the number of TUJ1-positive cells in ATRA and EC23 cultures, flow cytometry was performed (**Fig. 5.3.3**). It was noted that as the concentration of ATRA increased, from 1μM to 10μM, the number of cells solely expressing the TUJ1 N-type cell marker decreased, an indication that increased ATRA concentration increases the level of differentiation induced, therefore limiting proliferation of the N-type cells. The opposite was visualised with the synthetic retinoid EC23, in that an increase in concentration of the retinoid induced an increase in the number of cells solely expressing the N-type marker TUJ1. It was also noted that the vehicle control, DMSO alone, also appeared to be having an effect on the number of N-type cells compared to the undifferentiated control, increasing the amount of cells solely expressing TUJ1. Since EC23 cultures contained an intermediate number of N-type cells between control and ATRA counterpart cultures it was unclear whether this was due to either (a) increased neuronal effects or (b) general decreased potency associated with this retinoid. In order to try and resolve this point, it was decided to use a concentration of 10μM throughout the investigation, and experimental emphasis was placed on reviewing biological responses.

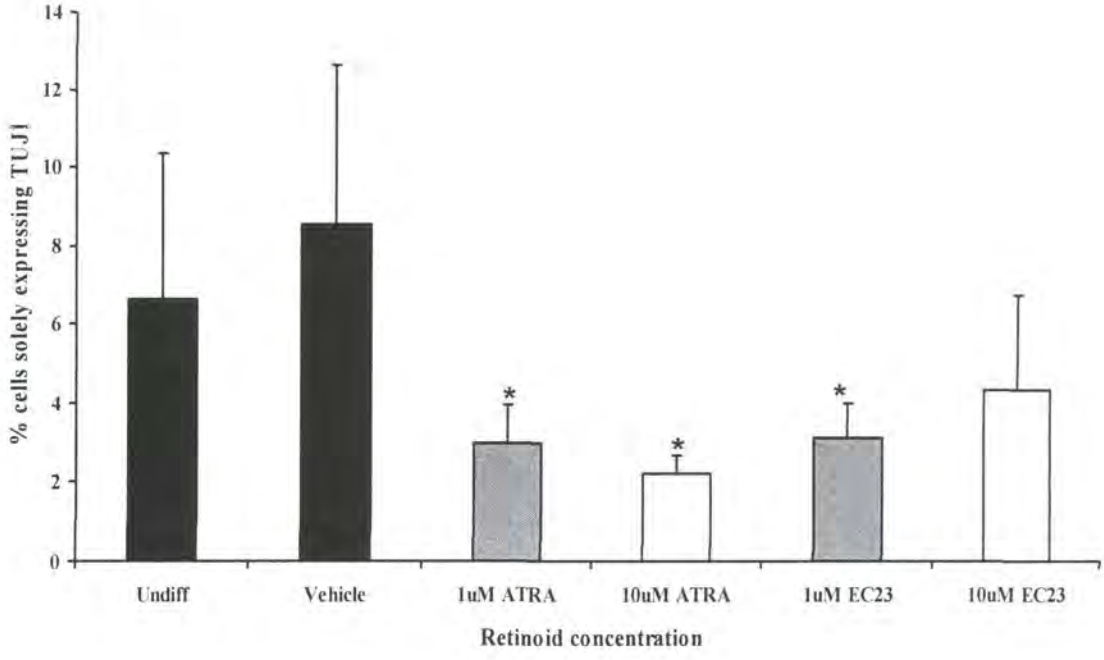


Figure 5.3.3: Flow cytometric analysis of the percent of SH-SY5Y cells solely expressing TUJ1 in cultures exposed to either 1μM or 10μM ATRA or EC23. Cells are stained for the S-type marker vimentin and the neuronal N-type marker TUJ1then analysed via flow cytometry. Using duel-gating the percent of cells exclusively expressing TUJ1 is obtained. As the concentration of ATRA increases the number of TUJ1 positive cells decreases. The opposite is seen with EC23. The most TUJ1 positive cells are observed in 10μM EC23 cultures. n=2. * $p \leq 0.05$, student's t-test compared to the undifferentiated control.

5.3.2 Retinoic acid receptor expression in SH-SY5Y cultures exposed to retinoids

Cultures were exposed to 10μM retinoid for seven days, then fixed and immuno-cytostained for the three different retinoic acid receptor (RAR) subtypes, alpha, beta and gamma (**Fig. 5.3.4**). All samples were counterstained with the nuclear dye Hoechst 33342 to help visualise whether receptor expression was localised to the nuclei. Hoechst counterstaining is not shown here as the receptor expression was at low levels and would be masked, however all receptor expression was seen to be nuclear. Expression of RAR-α appeared to be increased in EC19 cultures compared to the other retinoid-supplemented conditions and the undifferentiated control. RAR-β expression appeared to be up-regulated in cultures supplemented with ATRA and EC23 only. Non-specific staining was visualised in both undifferentiated and EC19 conditions and was disregarded. RAR-γ expression was slightly up-regulated in undifferentiated and EC19-suplemented cultures. These expression profiles reinforce the differences seen between EC19 and the other

retinoids, and indicate that the observed biological responses to EC19 are elicited, in part at least, by activation of different RAR-dependent biological pathways. It is known that RAR-β activation is associated with neural development, and this receptor may therefore be important during the retinoid-activated differentiation of the neuroblastoma cells into neuronal derivatives. However, before any solid conclusions can be drawn, the verification of these data would need to be confirmed via western blot analysis.

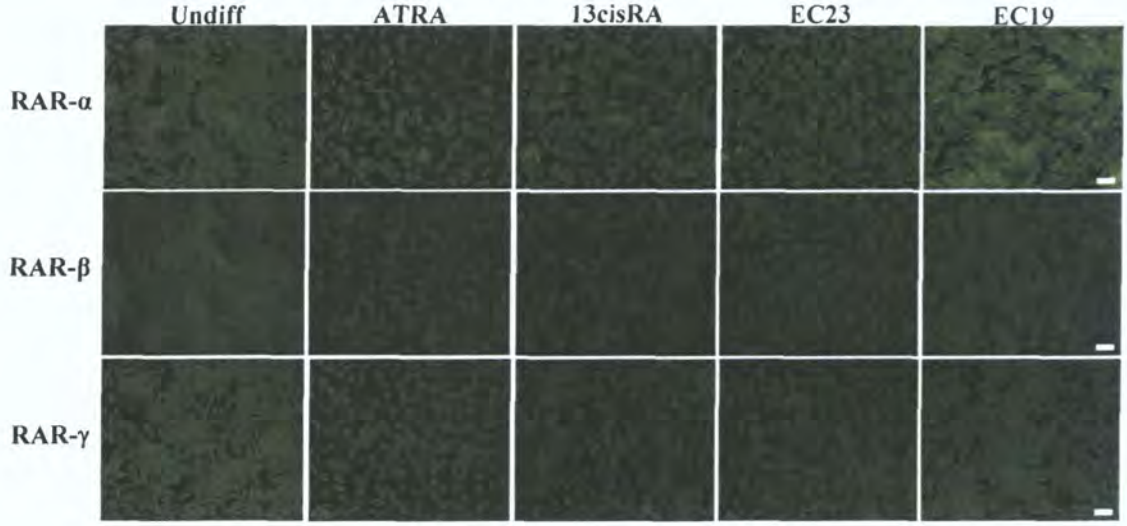


Figure 5.3.4: Regulation of the nuclear retinoic acid receptors during differentiation of SH-SY5Y cells. Immunocytochemical staining of SH-SY5Y neuroblastoma cells cultured with 10μM retinoid for 7 days. Retinoic acid receptor-α expression is increased in cultures exposed to EC19. Retinoic acid receptor-β expression is slightly increased in cultures exposed to ATRA, EC23 and EC19 after 7 days. The staining visible in the undifferentiated sample appears to be non-specific. Retinoic acid receptor-γ expression is slightly increased in undifferentiated cultures and those exposed to EC19 after 7 days. Scale bar: 50μm

5.3.3 Potency analysis of synthetic retinoids at 10μM concentration

Viable cell number analysis was carried out over a time course on cultures exposed to 10μM of either ATRA, 13cisRA, EC23 or EC19 (**Fig. 5.3.5**). This analysis was performed to see how cell numbers changed over the time course, i.e. was cell number maintained at a constant level after retinoid supplementation or did it increase over time? The control conditions, undifferentiated cultures and DMSO-treated cultures, both exhibited an increase in cell number as the time course progressed, indicative of healthy, proliferating cultures. Supplementation with either natural retinoid ATRA or 13cisRA showed an attenuation of cell number after the 7 day time point, suggesting that both retinoids are inhibiting cell proliferation beyond this point, possibly causing cells to drop out of the cell cycle and differentiate. The synthetic retinoid EC23 also appeared to limit

cell proliferation after the 7 day time point, even showing a slight decrease in cell number compared to ATRA and 13cisRA conditions. The synthetic retinoid EC19 was slightly more unpredictable in its induced responses. Cultures exposed to this retinoid did not show an attenuation of cell number over the time course, and the resulting cell number in the cultures was greatly variable compared to the other conditions tested, as revealed by the corresponding error bar in Figure 5.

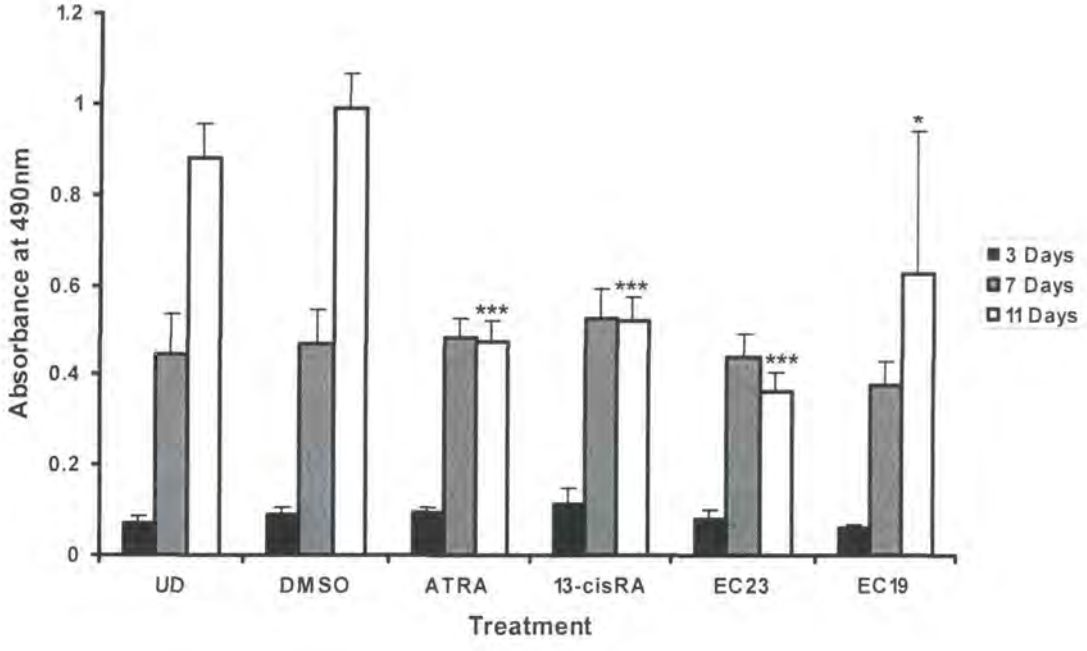


Figure 5.3.5: MTS cell analysis of SH-SY5Y cell number after exposure to 10μM ATRA, EC23 or EC19 over a specific time course. All retinoids are able to attenuate cell numbers over the time course in comparison to the control samples. EC19 is less predictable in its ability to inhibit cell numbers as indicated by the large error bar. n=6. * $p \leq 0.05$, *** $p \leq 0.0005$, student's t-test compared to the relevant undifferentiated sample.

Immunocytochemical analysis was carried out on cultures incubated with the retinoids after the 11 day time point (**Fig. 5.3.6**). Again, markers of the N- and S-type cell types were analysed by TUJ1 and vimentin immunostaining. As visualised in the concentration study, ATRA appeared to attenuate the number of N-type cells within the culture compared to control micrographs, and cells present appeared to have undergone differentiation with the presence of long neural processes. EC23 had also attenuated the number of N-type cells with regard to the undifferentiated control, but not to the same extent as ATRA, i.e. there was still a higher proportion of N-type cells to S-type cells compared to the ATRA samples. The N-type cells within this culture condition did not

appear to have undergone obvious neural differentiation; they did not appear to contain long neurites as seen in the ATRA condition. This could suggest that EC23 was not eliciting as potent an effect on these cancer cells as ATRA. The cultures exposed to EC23 appeared to resemble cultures exposed to the natural retinoid 13cisRA more closely. 13cisRA also did not induce neurite extension in the N-type component of its cultures, and the proportion of N- to S-type cells was also higher than in the ATRA control. EC19 cultures contained predominantly S-types cells, with very few cells solely expressing the N-type marker TUJ1. Figure 6 also appeared to indicate an apparent increase in the total cell number present within EC19 cultures compared with the undifferentiated control, however this would need to be verified via quantifiable methods. It appears that EC19 may be having some sort of enhanced proliferative effect on these model neuroblastoma cells, the opposite of the desired effect, but nonetheless an intriguing contrast to the closely related EC23 compound considering the only difference between the two analogues is the position of the carboxylic acid group.

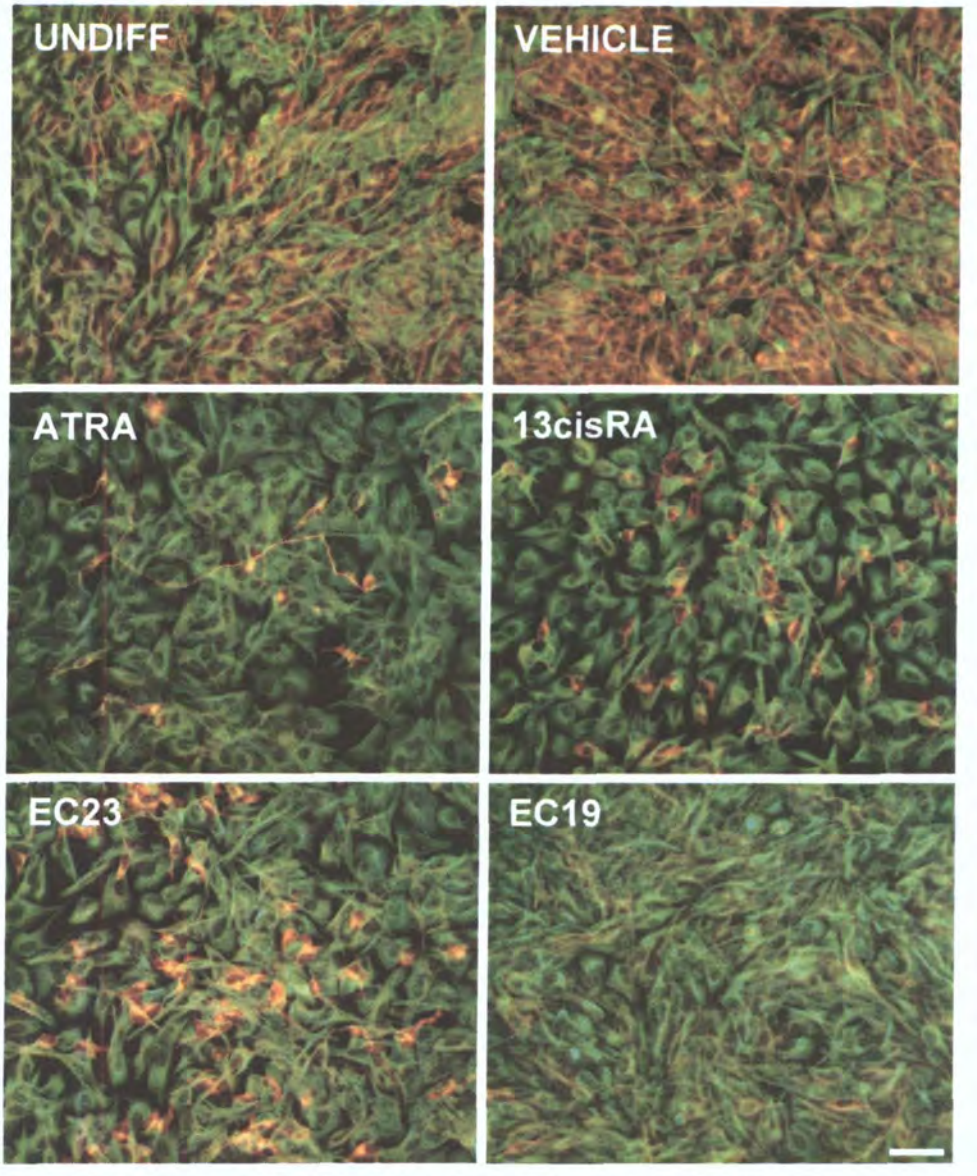


Figure 5.3.6: Immuno-cytochemical analysis of the biological effect of the synthetic retinoids on SH-SY5Y cell differentiation. Immunocytochemical staining of SH-SY5Y cells after incubation with 10μM retinoid for 11 days. Cells are stained for S-type marker vimentin (green), and neuronal N-type marker TUJ1 (red). EC23 cultures retain the most N-type cells followed by in turn 13cisRA, ATRA and EC19. The synthetic retinoid EC19 appears to induce predominantly S-type cells. Scale bars: 50μm. n=3.

5.3.4 Cell cycle analysis of SH-SY5Y cells incubated with retinoids

To investigate the effect of retinoids on the cell cycle of SH-SY5Y neuroblastoma cells, flow cytometric analysis of the cell cycle was performed (**Fig. 5.3.7**). Cells that were in S-phase and G_2/M-phase were considered to be undergoing cell division, and were therefore counted as dividing cells. Cell cycle analysis was performed at both three and seven day time points on cultures exposed to either ATRA, 13cisRA, EC19 or EC23. Dean-Jett analysis and statistics were followed and results were presented in graphical form (**Fig. 5.3.7**). Undifferentiated control cultures retained the highest percentages of

cells entering cell division at both 3 day (~40%) and 7 day (~30%) time points, with fewer dividing cells present at 7 days. This could be due to cultures becoming more confluent and less optimal for cell proliferation, possibly due to contact inhibition. ATRA cultures had far fewer dividing cells at both 3 and 7 days compared to the undifferentiated control. By 7 days, on average only 11% of cells within the ATRA cultures were still undergoing cell division/proliferation. 13cisRA cultures contained more proliferating cells than the ATRA differentiation control at both time points, with on average 17% of cells within the culture continuing to undergo cell division at the seven day time point. EC23 cultures contain less proliferating cells at both time points compared to the undifferentiated control, but the number of dividing cells was greater than those visualised in the ATRA counterpart. On average 17% of cells within the EC23-supplemented condition were undergoing cell division at the seven day time point, a figure which was comparable to 13cisRA cultures. EC19-supplemented cultures showed very little attenuation of proliferating cells over the time course investigated, and the number of dividing cells appeared to actually increase compared to the undifferentiated control by the 7 day time point, though the margin was only very slight.

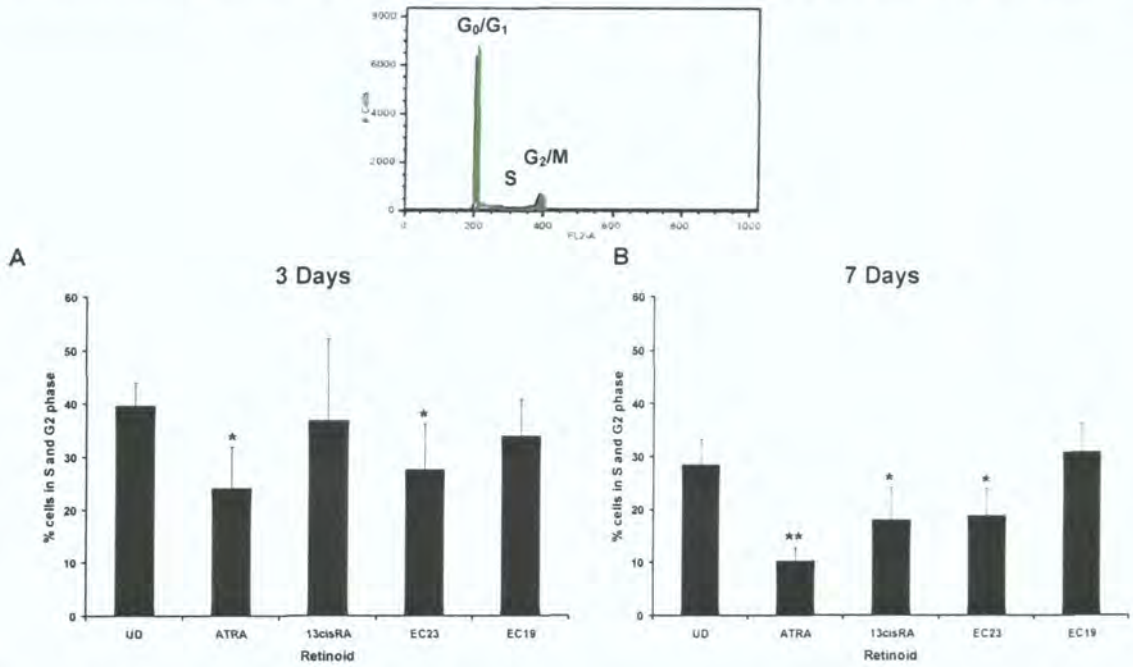


Figure 5.3.7: Cell cycle analysis in SH-SY5Y neuroblastoma cells exposed to either 10μM ATRA, 13cisRA, EC23 or EC19 for either 3 (**A**) or 7 days (**B**). Control undifferentiated cultures retain the highest percent of cells entering cell division at both 3 day and 7 day time points, with fewer dividing cells present at the 7 day time This could be due to cultures becoming more confluent and less optimal for cell proliferation. ATRA cultures had far fewer dividing cells at both 3 and 7 days. By 7 days not many proliferating cells are present at all. 13cisRA cultures contain more proliferating cells than ATRA controls

at both time points. EC23 cultures contain less proliferating cells at both time points than the controls, but the number of dividing cells is greater than the ATRA counterpart. EC19 cultures show very little attenuation of proliferating cells and the number appears to actually increase compared to the controls by the 7 day time point. n=3. * p $p \leq 0.05$, ** $p \leq 0.005$, student's t-test compared to the relevant undifferentiated sample.

5.3.5 BrdU analysis of SH-SY5Y cells incubated with retinoids

Now it had been established that supplementation of media with the retinoids did induce a decrease in cell proliferation in SH-SY5Y cells it was now investigated whether this attenuation of proliferation was linked to a particular cell type within the culture system. To analyse this, BrdU was spiked into cultures for 24 hours prior to fixation of cultures and immunocytochemical analysis. **Figure 5.3.8,** which shows cultures stained for BrdU expression, backs up previous cell cycle analysis data, indicating that after the seven day time course ATRA-supplemented cultures contained the lowest numbers of proliferating cells. EC23 cultures contained the next lowest number of proliferating cells, followed by the undifferentiated and vehicle controls, with EC19 supplemented cultures containing the highest level of cells which have continued to proliferate.

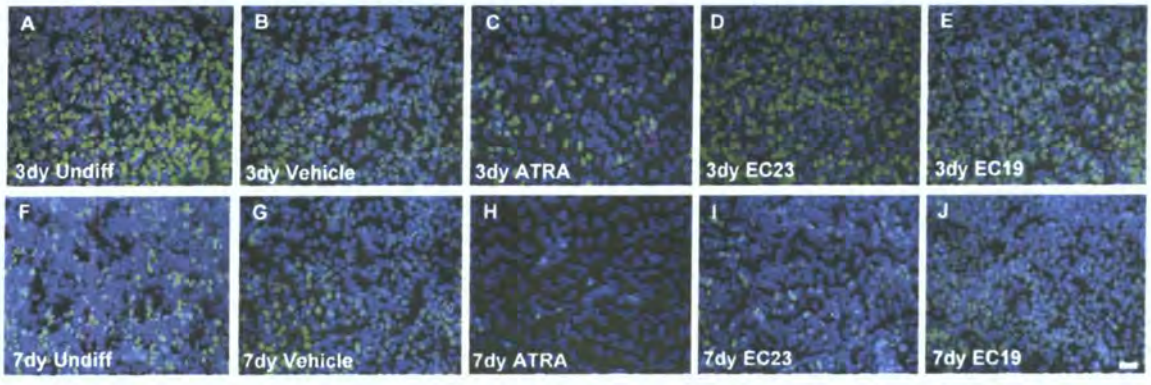


Figure 5.3.8: BrdU expression in SH-SY5Y neuroblastoma cells exposed to either 10μM ATRA, EC23 or EC19 for up to 7 days. Control undifferentiated and vehicle (DMSO) exposed cultures **(A,B,F,G)** exhibited a high number of BrdU positive cells at both 3 day and 7 day time points, with slightly fewer positive cells at the 7 day time point. This could be due to cultures becoming more confluent and less optimal for cell proliferation. ATRA cultures **(C, H)** had far fewer BrdU positive cells at both 3 and 7 days. By 7 days not many proliferating cells are present at all. EC23 cultures **(D,I)** contain less proliferating cells at both time points than the controls, but the number of BrdU positive cells is greater than the ATRA counterpart. EC19 cultures **(E, J)** show no attenuation of BrdU positive cells and the number appears to actually increase compared to the controls by the 7 day time point. All micrographs are counterstained with the nuclear marker Hoechst 33342. Scale bar: 50μm. n=3.

Samples were next dual stained with BrdU and the N-type cell marker TUJ1 (**Fig. 5.3.9**). In the undifferentiated and vehicle control cultures it was impossible to determine whether there was a particular cell type which had predominately undergone cell division, as so many cells were continuing to divide and therefore BrdU positive. There were many BrdU-positive cells within the EC19-supplemented samples also, however, as seen throughout this chapter, N-type cells appeared to be inhibited within this culture, and although TUJ1 positive cells were visualised, the level of expression was low, and no neurite extensions were seen. In both ATRA- and EC23 supplemented cultures, cells which were BrdU positive at the three day time point were predominantly TUJ1-negative (i.e. only had low level TUJ1 expression). Cells which were BrdU positive at the seven day time point in both conditions were also predominantly TUJ1 positive. These data suggests that both ATRA and EC23 inhibit the S-type cells from proliferating first, before they inhibit the N-type cell population. Once again EC23 was able to mimic ATRA-induced responses, albeit not to the same level. In fact EC23-induced biological responses visualised within this cancer cell model system appeared to more closely represent cultures supplemented with the natural retinoid 13cisRA, the retinoid of choice in therapeutic regimes for neuroblastoma. EC19 appeared to either elicit no response from this cell line, or at certain concentrations actually enhanced cell proliferation. These findings could potentially open the way for the use of these particular molecules as useful tools towards improving our understanding of neuroblastoma cancer biology.

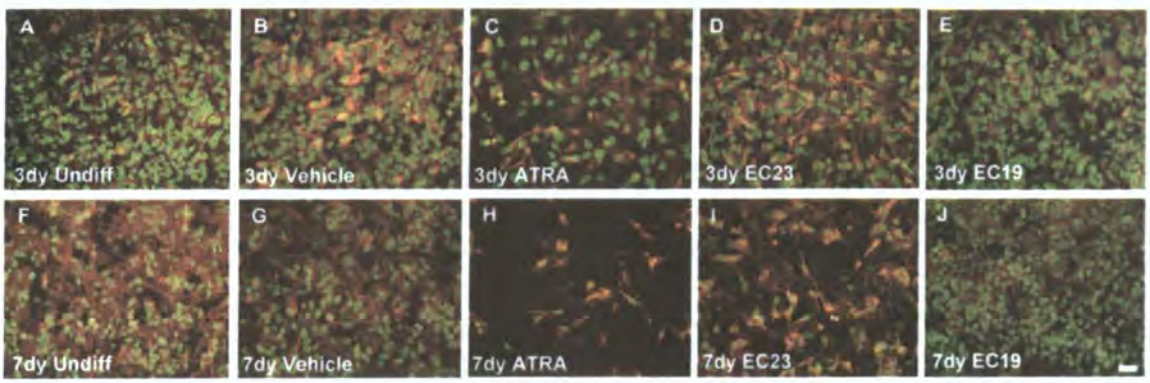


Figure 5.3.9: Co-localisation of BrdU and TUJ1 expression in SH-SY5Y neuroblastoma cells exposed to either 10μM ATRA, EC23 or EC19 for up to 7 days. It is difficult to distinguish at the 3 day time point if thee is any co-localisation of BrdU with a particular cell type, however, by the 7 day time point it becomes easier to see that BrdU predominantly appears to co-localise with the N-type cells. Scale bar: 50μm. n=3.

5.4Discussion and Conclusions:

The aims of this Chapter were to evaluate the activity of the synthetic retinoids EC19 and EC23 to inhibit cell proliferation in a model of neural cancer. The cell system used was a model of childhood neuroblastoma, a neural tumour which treatment programmes incorporating retinoids is already in clinical use. For the cell system to be considered a suitable model they were required to demonstrate a level of response to the addition of natural retinoids into the culture system. The cell line SH-SY5Y has previously been shown to fit this criteria, with a portion of the cells dropping out of the cell cycle and differentiating down a neural lineage, extending neural processes[199]. Experiments performed in this study are consistent with these observations. Concentration studies revealed that the synthetic retinoids EC19 and EC23 also induced a biological response from SH-SY5Y cells after media supplementation. EC19 appeared to inhibit N-type cell formation and enhance S-type proliferation, whereas EC23 appeared to mimic ATRA-induced responses and induce neural differentiation in the N-type cell component. EC23 appeared to be more potent than the synthetic retinoid EC19, which did not inhibit cell numbers to the same extent. In fact, EC19 appeared to actually increase cell numbers at the lowest concentration tested, 1μM. Further analysis was carried out at 10μM as this appeared to be optimal in terms of all retinoids clearly eliciting some level of biological response.

RAR expression analysis revealed that EC19 appeared to induce different RAR sub-sets compared to both ATRA and EC23. EC19 induced an up-regulation of the alpha receptor whereas ATRA and EC23 induced localised nuclear beta receptor expression. It has been observed with all cell types investigated throughout this thesis that EC19 elicits differing effects to ATRA and EC23, and these expression profiles may go part way to explaining these differences. The differential expression of the specific RAR sub-types has been the focus of many different neuroblastoma investigations. It is now understood that an up-regulation of RAR-β often occurs after successful retinoid supplementation and is associated with proliferation inhibition and differentiation of cells [202, 203]. For example, retinoid supplementation induces a RAR-β mediated activation of the HOX5A gene via a retinoic acid response element located in the 3' end of the gene, and has been shown to

induce corresponding apoptosis in breast cancer cells [204]. Indeed, it has also been reported that the level of RAR-β expression is an important indicator of retinoid sensitivity of primary neuroblastoma samples, the higher the expression level the better the prognosis [205]. Therefore the small increase in RAR-β expression after ATRA and EC23 exposure observed in this investigation could be interpreted as an indication that the cells were responding to the retinoid treatment, as a corresponding decrease in total cell number/culture and differentiation was observed in these samples. Therefore it can be concluded that EC23 appears to induce a similar response in SH-SY5Y cells as ATRA.

The increase in expression of RAR-α in cultures supplemented with EC19 is very interesting. While there are no papers specifically reporting the outcome of over-expression of RAR-α in neuroblastoma cultures, there are papers reporting the effects in other cancer models. For example, the over-expression of RAR-α fusion proteins inhibited cell differentiation of mouse bone marrow progenitor cells, leading to immortalisation of the myeloid progenitors, and creating cells similar to those seen in acute promyelocytic leukaemia [206]. It is possible to hypothesise that EC19 triggers enhanced proliferation in the SH-SY5Y cultures, somehow involving RAR-α-mediated activity, which would be of interest to investigate further from a cancer point of view. It would be interesting to investigate what effect treatment with an antagonist of RAR-α would have on neuroblastoma proliferation. It would be tempting to hypothesis that profileration would become decreased compared to controls. These are potentially important findings, and will contribute to the future development of more specific RAR orientated chemotherapeutic agents.

To investigate whether the synthetic compounds were more potently active than the natural retinoids on this neuroblastoma cell line, experiments were tailored to examining what effect media supplementation had on the cell cycle and markers of proliferation. Specifically this focussed on the two cell types found within healthy cultures, namely the N-type neuronal cells and S-type substrate-adherent like cells. Cell cycle analysis revealed that after the 7 day time point EC23 did not cause as many cells to stop dividing as ATRA. In fact, EC23 results were more in line with the other natural retinoid tested, 13-cisRA. As expected EC19 did not inhibit cell division to any extent compared with the

undifferentiated control. BrdU analysis was carried out to investigate which cell type was being affected by retinoid supplementation. It appeared that within ATRA and EC23 cultures S-type cell proliferation was being inhibited first, followed by the N-type cells. It was again noted that EC23 was found to be less effective at inhibiting cell proliferation as ATRA, although it did mimic its effects. EC19 appeared to be acting differently from the other retinoids tested which was consistent with its pattern of activity. The fact that EC23 did not appear as effective an inhibitor of proliferation as ATRA, indicates that it is not a viable alternative for further study in a chemotherapeutic context in the neuroblastoma cell line.

Other studies have also shown that synthetic arotinoids induce biological activity, however they have also shown that these compounds appear to have an increased level of toxicity [207], signifying them as inappropriate for use as therapeutic agents. This data ties in with findings reported in Chapter 2 concerning their use in adult progenitor cells, where they appeared toxic when used at concentrations recommended and commonly used for ATRA supplementation. However, the chemical stability achieved with these molecules remains very desirable, and reports of modifications to their basic structure have proved promising. For example, the incorporation of a heteroatom into one of the cyclic rings drastically reduced the toxicity observed, whilst retaining the ability to induce differentiation or apoptosis in cancer cell models [207]. One such compound, Tazarotene, which is already available as a treatment for psoriasis and acne, was subjected to phase 1 clinical trials on adult patients with advanced cancer [208]. It was shown to be tolerated well over a period of 12 weeks with fewer toxicity issues than associated with ATRA and 13cisRA, making it a promising compound requiring further investigation. Other chemical variations of arotinoids, namely flexible heteroarotinoids, also have potential as chemotherapeutic agents, by inducing the differentiation of cancer cells, Interestingly, however, these compounds do not activate any of the RARs or RXRs, prompting further investigation into their modes of action [209]. Novel retinoid compounds have also been evaluated with some success in the field of neuroblastoma research. The synthetic atypical retinoid ST1926 does not induce the differentiation of neuroblastoma cells but does induce apoptosis. ST1926 is able to function in this way in cell lines which have been shown to be ATRA-resistant, again making this a very interesting candidate requiring further evaluation [198]. All of these studies have indicated that while arotinoids

themselves are not conducive for use in chemotherapeutic therapies they have formed a solid base from which more viable chemical compounds have been created.

5.4.1 Conclusions

SH-SY5Y neuroblastoma cells are a suitable model for testing retinoid-induced inhibition of growth of cancer cells. The ability of the synthetic retinoids EC19 and EC23 to inhibit neural cancer cell growth was tested here. EC23 did induce neural differentiation within this cell model system and was able to limit cell proliferation in a manner similar to that witnessed with the natural retinoid ATRA; albeit not to the same extent. Therefore it was concluded that EC23 does not appear to be an effective alternative to ATRA for limiting neuroblastoma growth according to this particular cell model system.

EC19 did elicit biological responses after media supplementation. However, these were distinctly different to those witnessed with the control test retinoid ATRA. EC19 inhibited N-type cells, with very few being visible using immunocytochemical analysis. EC19 also appeared to enhance cell proliferation of the S-type component within the cultures. It can be concluded that while EC19 is not effective at inhibiting neuroblastoma cell growth it may be a valuable tool for use in the study of why cancer cells are proliferative. One tentative hypothesis may be that an up-regulation of the RAR-α sub-type is involved in this aberrant cell proliferation, as discussed above.

6 Chapter 6: Thesis Discussion and Final Concluding Comments

6.1 Summary and Discussion of Thesis Data

This thesis has investigated the biological activity of the retinoic acid analogues EC19 and EC23, specifically their ability to induce neural differentiation in a range of stem and progenitor cell model systems. EC19 and EC23 are arotinoids which have been shown to have enhanced chemical stability due to the insertion of a triple bond and an aromatic ring into the polyene linker region.

The main aim of this body of work was to establish whether either EC19 or EC23 would be viable as an attractive alternative to ATRA for *in vitro* experimental use. ATRA has been shown to be unstable when used under standard laboratory conditions, isomerising readily when exposed to heat and light. For example, when ATRA was exposed to UV light for 3 days only 37% of the compound remained in the intact un-degraded form [123]. This is likely to cause problems when an exact concentration of ATRA is required, i.e. for developmental studies, as outlined in Chapter 1. It has also been shown in this thesis how the different isomers of retinoic acid can elicit dramatically different results, again indicating the need for a non-isomerisable alternative to ATRA. Throughout the course of this investigation the biological activity of EC19 and EC23 was tested on stem cells, adult and embryonic neural progenitor cells and a model of neuroblastoma.

In all the cell model systems tested, the biological activity of EC23 closely mimicked that of ATRA, whereas EC19 did not. Work carried out in Chapter 3 illustrated the ability of both compounds to induce differentiation in stem cells systems. In both EC and ES cells, EC23 was able to induce neuronal differentiation in a similar manner to ATRA, up-regulating specific components of the retinoic acid receptor pathway. EC19 also induced cellular differentiation under specific conditions, but induced epithelial-like 'plaques' of cells at the expense of neural differentiation. Similar findings were reported in Chapter 4, where the two compounds were cultured with adult and embryonic neural progenitor cells. In the adult model system, EC23 was able to induce mainly neuronal differentiation, similar to ATRA, whereas EC19 induced an increased amount of glial differentiation. Interestingly, in the embryonic neural progenitor system retinoids had not previously been used as part of the differentiation protocol. It was noted that after either

ATRA or EC23 media supplementation more mature, NF-200 positive, neuronal sub-types were observed compared with the established differentiation protocol. Again this was not the case with EC19. Chapter 5 dealt with the ability of the synthetic retinoids to inhibit proliferation of a neuroblastoma cell model system. While EC23 was able to induce a degree of neural differentiation, the ability of this compound to inhibit proliferation was less than that of ATRA. It appeared that low level supplementation of media with EC19 actually enhanced the proliferation of this cancer model. This phenomenon could be exploited to aid in the understanding of the mechanisms behind cancer cell proliferation, if the downstream activation partners were identified.

It is important to relate how these data fit in with other published retinoid biological data. As outlined in the introduction to this thesis, there have been many novel synthetic retinoids synthesised and their associated RAR/RXR binding capacities reported. Indeed, EC23 has been shown to activate all of the retinoic acid receptors [122], and data in this thesis suggests that the biological activity elicited by both EC19, and to a greater extent EC23 are mediated via the nuclear retinoic acid receptor pathway. However, it must be noted that some retinoids appear to act in non-nuclear modes. Retinoids which have this ability include a sub-group of arotinoids, the heteroarotinoids. As these compounds have a similar chemical structure to the arotinoids tested in this thesis it is relevant to explore these findings. For example, the synthetic heteroarotinoid SHetA2 induces apoptosis in head and neck squamous carcinoma cells through a receptor-independent and mitrochondria-dependent pathway [210]. The retinoid does not activate any of the retinoic acid receptors and appears to induce apoptosis by increasing the production of reactive oxygen species within the mitochondria. This RAR/RXR independent retinoid action has been demonstrated to overcome much of the toxicity and teratogenicity normally associated with the clinical use of retinoids [207]. As a result of this, non-receptor mediated retinoid activity could prove very beneficial for treatments of cancers. Another example showed that synthetic heteroarotinoids could induce apoptosis in ATRA-resistant HL60 myeloid leukemia cells [211]. As far as this thesis is concerned, although it is known that EC23 activates the RARs, it cannot be ruled out that some of its activity may be associated with non-receptor mediated events. To better understand this, more experimentation would have to be performed, perhaps looking at the levels of reactive oxygen species released by the mitochondria, and also blocking the activity of the receptors via an antagonist and investigating whether any biological activity still occurs

after EC19/23 supplementation. As it is not known which receptors EC19 activates it would also be interesting to evaluate the possibility that some of the results observed could be induced via non-receptor mediated pathways.

Proteomic analysis of retinoid-induced proteins revealed that after exposure of EC cells to EC23 for 7 days, an increase in CRABP-I was observed compared to the ATRA differentiation control. This could indicate that EC23 is either more biologically potent, and/or the cell finds it harder to break down this more stable synthetic compound. However, it must also be noted that differences in activity between EC23 and ATRA are also liable to arise from alternative active breakdown products. For example it has been shown that the breakdown products resulting from ATRA metabolism also elicit their own biological activity. In 1993 a paper published in Nature revealed that 4-oxo-retinoic acid is a highly active modulator of positional specification during xenopus development, and not just an excretable bi-product of ATRA metabolism [212]. However, they did also note that the activity of this compound on ES cells was less than ATRA. It is not known what, if any, active cellular breakdown products derive from EC23. Nonetheless the apparent resistance to cellular breakdown suggests that such molecules would be generated at lower rates and hence be present at lower concentrations than those arising from ATRA. Furthermore any breakdown products of EC23 would be structurally, and by inference, functionally different from their natural counterparts. Again, this is an area which requires further investigation.

Finally it must also be acknowledged that the retinoic acid receptor pathway is by no means the only pathway influencing the neuronal differentiation of cells *in vitro* or *in vivo*. Many of the well known and studied signalling pathways are involved in neural development and CNS axis orientation, both working independently and interacting with the retinoic acid receptor pathway. Recent published data has indicated distinct roles for FGF, Wnt and retinoic acid in posteriorizing the neural ectoderm [213]. In this study of the anterior/postior patterning of the late blastula to gastrula embryo of the zebrafish, they revealed how FGF and Wnt signals suppress expression of anterior genes, such as the retinoid activated *cyp26* gene. This suppression allowed posteriorization of the neural tissue to occur, regulated by retinoic acid. Therefore it was discovered that the *cyp26* gene played an important role in linking FGF, Wnt and retinoic acid signals. Other papers have also revealed links between the retinoic acid receptor pathway and the nerve growth

factor and sonic hedgehog signalling pathways during neurite outgrowth (extensively reviewed in [214]). It has been shown that sonic hedgehog is one of the downstream targets of the RARβ2 signalling pathway [215], and it is expressed in the injured dorsal root ganglia neuronal subtypes that extend neurites in response to retinoic acid [216]. Therefore, while studying the biological effects of our synthetic retinoids on neural differentiation it is important to aknowledge that other pathways may be being influenced, and better yields of neuronal differentiation could be obtained if these pathways were manipulated also.

6.2 Possible future directions

While the findings reported here, answered the main questions set out at the beginning of this piece of work, new questions have arisen which are deserving of attention. Three main questions and areas for future work are outlined below.

6.2.1 Structure of synthetic retinoids relates to function

The importance of retinoid structure and how this can have a great influence on biological outcome has been clearly demonstrated throughout this investigation. The only chemical difference between the two synthetic retinoid compounds EC19 and EC23 is the position of the carboxyl group either in the *meta-* (EC19) or *para*-position (EC23). This seemingly small chemical modification appears to alter the structure of the molecule enough to interrupt interaction with specific receptor subsets. EC19 does not appear to be as biologically active as EC23 when tested on any of the cell model systems. Its effects tend to be different from those induced by ATRA or EC23, again an indication that different retinoic acid receptors, or indeed other receptors, are being activated. The importance of structure/activity relationships between retinoids have already been realised, for example, 9cisRA, which differs from ATRA only by a *cis* configuration at the 9 carbon position is able to induce both differentiation and apoptosis in the N-type cells of neuroblastoma, whereas ATRA is only able to induce differentiation [217]. This outcome is obviously desirable from a therapeutic standing and as such, has been exploited for therapeutic applications. Research and development has focussed on creating novel structures containing the *cis-* configuration with some success. For example, a novel synthetic heterocycle retinoid based on the structure of TTNBP, contains a *cis*-bond isomerisation and has been shown to induce enhanced levels of apoptotic activity in a culture model of APL. Furthermore, the compound has further

been shown to induce apoptosis in culture models of APL which are apparently non-responsive to ATRA treatment [218]. These studies exemplify the importance of understanding structure/activity relationships, and how to use these for therapeutic/research gain. In the context of this thesis, understanding how structure ultimately effects cellular differentiation is very important, and will build on the existing knowledge base for use in the future synthesis of more selective retinoid structures.

6.2.2 Preliminary analysis of a novel β-selective synthetic retinoid

EC19 and EC23 were designed to activate all of the RAR sub-types; however it is known that specific receptors are important for different biological developmental processes, and some are linked to the side-effects observed when these compounds are used therapeutically. As outlined in Chapter 1, it is well known that the beta retinoic acid receptor is involved in neuronal differentiation [174, 219], specifically the RAR-β2 subtype [174]. As part of an on-going research initiative, receptor-specific synthetic retinoids are being generated which need to be tested biologically. A novel synthetic compound was synthesised in the Chemistry Department, Durham University, modelled on published data pertaining to a selective β-retinoic acid receptor agonist compound [220]. In terms of neuronal development, the β-receptor plays an important role. Therefore it is hypothesised that a β-selective receptor agonist may enhance the neuronal differentiation induced in stem cell models. Experiments could be carried out as before to verify this biological activity of the β-agonist.

6.2.3 Manipulation of EC23 synthesis and storage for commercialisation

One of the main aims of this investigation was to evaluate whether synthetic retinoid compounds synthesised in the Durham University Chemistry Department, would act as an attractive alternative to ATRA for *in vitro* use. Specifically, ATRA alternatives are required which are stable since ATRA is extremely heat- and photo-labile, isomerising readily under standard laboratory conditions (see Chapter 1). One of the main conclusions resulting from this thesis was that EC23 appeared to mimic ATRA-induced activity, inducing neuronal differentiation within the various cell model systems tested. This molecule therefore possesses potential as a commercial product, and as the work obtained for this thesis is linked to the commercial entity ReInnervate Ltd, commercialisation of the product needs to be evaluated. Before EC23 could be

considered for validation by prospective commercial partners, further investigative experimentation would be required relating to the possibility for bulk production and accurate dispensing into commercial vials. Subsequently, biological activity of this product would need to be assessed before the product can be put forward for the next stage of the commercialisation process.

6.3 Final concluding comments

In conclusion, all results obtained throughout this thesis indicate the feasibility of the synthetic arotinoid, EC23, being used as a more stable alternative to ATRA in *in vitro* investigations into retinoid modes of action and neurogenesis. EC23 has been able to induce neuronal differentiation in a diverse range of cell model systems, covering embryonic stem, and neural progenitor cells through to models of adult neural progenitor cells. Up-regulation of key retinoic acid receptor components has strengthened arguments that EC23 elicits its effects via this retinoic acid receptor pathway. Novel findings have been established along the way, including new methods to induce more mature neuronal phenotypes with retinoid media supplementation in the embryonic neural progenitor cell line ReNcell VM. Links between profilin and stathmin regulation and retinoid-induced differentiation have been discovered during proteomic analysis of Tera2.sp12 EC cells cultured with either ATRA or EC23 for seven days. Finally, it was shown that EC23 was able to induce motor neuron phenotypes when used under defined culture conditions in human embryonic stem cell cultures. The bulk production and dispensing of EC23 in commercially viable quantities was shown to not affect its biological activity, making this a very attractive commercial product which is now being taken to the next step of the commercialisation process via ReInnervate Ltd, a Durham University Spin-out biotechnology company.

EC19 was not as biologically effective as EC23 in inducing differentiation in the cell model systems tested. Structural analysis revealed that the conformation of this synthetic retinoid did not favour activation of the retinoic acid receptors, however when it did elicit a response it was invariably different from that witnessed with either ATRA or EC23. In the neuroblastoma model system, EC19 appeared to actually enhance cell proliferation, potentially making this retinoid an interesting compound for use in cancer research. The inclusion of a methyl group into this compound greatly enhanced its biological activity. However this subtle modification did not alter the structural alignment of the compound, and must therefore influence its activity in some other way, possibly by altering its lipophilic nature, or by enhancing its selective protein binding capacity by some other means. This was an interesting finding and requires further experimentation to address these possibilities.

Work obtained throughout this thesis can be used as a base for future synthesis and structural modelling of novel synthetic retinoids. It has been established that proteomic analysis can be performed after only seven days of culture with the retinoids and gives a good indication of retinoid action. Work carried out on the novel beta-agonist and the 3-Me-EC19 compound indicated that chemical modelling has a valuable contribution to make to the process of novel retinoid synthesis. However, modelling alone is not sufficient to fully predict the induced biological outcome, due to the complicated nature of retinoid modes of action.

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