Synthesis and evaluation of synthetic retinoid derivatives as inducers of stem cell differentiation†

Victoria B. Christie,[a,b] Jonathan H. Barnard,[c] Andrei S. Batsanov,[c] Caroline E. Bridgens,[a,c] Emily B. Cartmell,[c] Jonathan C. Collings,[c] Daniel J. Maltman,[a,b] Christopher P. F. Redfern,[d] Todd B. Marder,*‡[c] Stefan Przyborski§[a,b] and Andrew Whiting*[c]

Received 21st May 2008, Accepted 7th July 2008
First published as an Advance Article on the web 8th August 2008
DOI: 10.1039/b808574a

All-*trans*-retinoic acid (ATRA) and its associated analogues are important mediators of cell differentiation and function during the development of the nervous system. It is well known that ATRA can induce the differentiation of neural tissues from human pluripotent stem cells. However, it is not always appreciated that ATRA is highly susceptible to isomerisation when in solution, which can influence the effective concentration of ATRA and subsequently its biological activity. To address this source of variability, synthetic retinoid analogues have been designed and synthesised that retain stability during use and maintain biological function in comparison to ATRA. It is also shown that subtle modifications to the structure of the synthetic retinoid compound impacts significantly on biological activity, as when exposed to cultured human pluripotent stem cells, synthetic retinoid 4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid, **4a** (*para*-isomer), induces neural differentiation similarly to ATRA. In contrast, stem cells exposed to synthetic retinoid 3-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid, **4b** (*meta*-isomer), produce very few neurons and large numbers of epithelial-like cells. This type of structure–activity-relationship information for such synthetic retinoid compounds will further the ability to design more targeted systems capable of mediating robust and reproducible tissue differentiation.

Introduction

Retinoids are a class of natural and synthetic analogues of all-*trans*-retinoic acid (ATRA), which is a metabolite of vitamin A (retinol), a fat-soluble vitamin required by all mammals. ATRA, and its two naturally occurring isomers, 9-*cis*-retinoic acid (9CRA) and 13-*cis*-retinoic acid (13CRA) (Fig. 1), are capable of mediating many biological processes both during embryonic development and in adult life, particularly in the nervous system.[1] They are vital in the mediation of cell proliferation, differentiation and apoptosis, and they maintain these processes in both normal and tumour cells, *in vivo* and *in vitro*. Due to the ability of different retinoids to control differentiation and apoptosis of tumour cells, they have potential therapeutic uses in the treatment and prevention of cancer.[2] Indeed, ATRA and other commercially available retinoids are currently used in a variety of cell differentiation therapies,[3] rather than the naturally occurring retinoids, which contain a polyene chain comprising five conjugated double bonds, and are thus excellent chromophores which efficiently absorb light in the region of 300–400 nm (depending on the solvent). This makes these molecules particularly susceptible to photoisomerisation, leading to degradation into a mixture of retinoic acid isomers.[4]

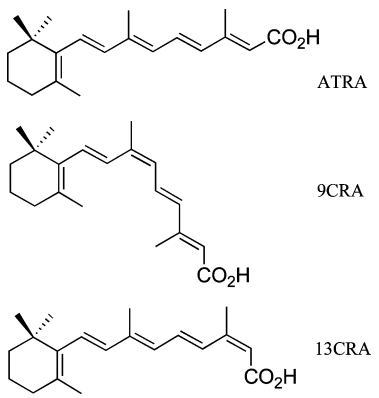


Fig. 1 Structures for ATRA, 9CRA and 13CRA.

Compound stability is an important feature when reagents are routinely used for cell culture applications. During the preparation and maintenance of cell cultures, the concentration levels of retinoids have been shown to decrease markedly over time, and

[a]*Department of Biological Sciences, Durham University, Science Laboratories, South Road, Durham, DH1 3LE, UK*
[b]*ReInnervate Ltd., Department of Biological Sciences, Durham University, Science Laboratories, South Road, Durham, DH1 3LE, UK*
[c]*Department of Chemistry, Durham University, Science Laboratories, South Road, Durham, DH1 3LE, UK*
[d]*Northern Institute for Cancer Research, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK*

† Electronic supplementary information (ESI) available: X-Ray crystallographic information. CCDC reference numbers 688676–688681. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b808574a

‡ Corresponding authors concerning Chemistry.
§ Corresponding author concerning Biology.

this could be a direct consequence of both their degradation and metabolism.[5] Isomerisation of ATRA is understood to be an important part of its metabolic pathways within cells, since the resulting isomers have different mechanisms of action.[5,6] Indeed, this is an important point that is all too often over-looked by users of natural retinoids in the cell culture laboratory. A warning to this effect has been issued to cell biologists using retinoic acids as inducers of cell differentiation by Murayama *et al.*,[4] who reported that the isomers of ATRA differentially affect the ability of mammalian stem cells to differentiate along alternative lineages, and they stated that care should be taken to protect retinoic acids from isomerisation. This is particularly relevant when the cellular response is determined by the concentration(s) of the isomer(s) present in solution. For example, the induction of the differentiation of pluripotent stem cells using retinoids is variable, resulting in the differential activation of key molecular pathways involved in tissue development in a concentration-dependent manner.[7] In turn, this variation has the potential to result in mixed proportions of alternative differentiating cell types which leads to increased culture heterogeneity. To reduce such variability in differentiation response and improve reproducibility, it is essential that whatever is used to induce cell differentiation is in the same form and concentration every time it is used. Currently, this cannot be guaranteed, especially when using reagents such as ATRA and its stereoisomers because they are highly susceptible to isomerisation under sample preparation conditions, storage of stock solution conditions and in culture.

It is because the different isomers have diverse effects on cells that some attempts have been made to control ATRA's sensitivity and tendency to isomerise. For example, a number of additives which inhibit either *cis–trans* interconversion or oxidation have been evaluated, including bovine serum albumin (BSA), fibrogen, lysozyme, phosphatidylcholine *N*-ethylmaleimide and vitamin C.[8] However, the addition of such molecules to cell culture media is often not viable because such additives may affect cell behaviour in their own way. In addition, none of these additives can completely prevent isomerisation and, for example, the use of BSA is not possible in serum-free culture media.

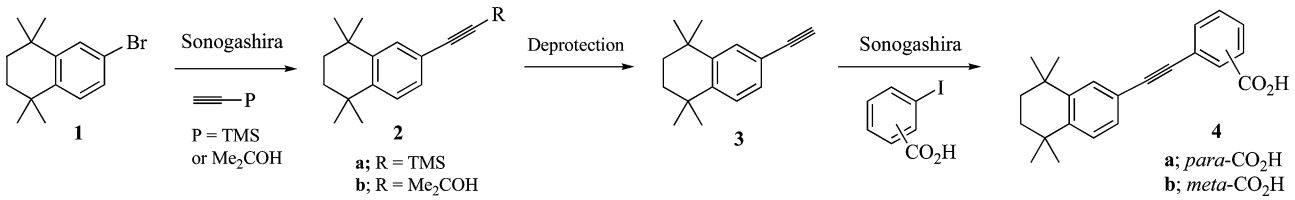
In this paper, we describe our recent work on the design, preparation and preliminary biological evaluation of synthetic retinoids which remain stable to degradation for extended periods under normal laboratory light. The ability of these types of compounds to modulate tissue development; notably, the induction of cell differentiation in a recognised pluripotent stem cell model of human embryogenesis,[9] is demonstrated.

Results and discussion

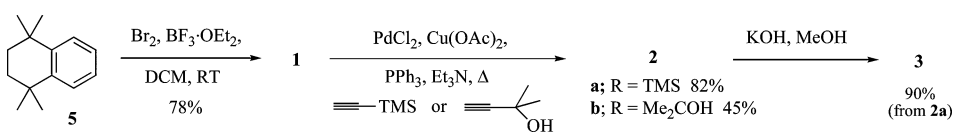
Synthetic retinoid synthesis

Retinoids have three structurally important regions: a hydrophobic section; a variable linker; and an acidic function, and extensive chemical modifications to these motifs are possible. For example, incorporating an aromatic ring to replace part of the polyene chain forms a new class of retinoids, sometimes called arotinoids. The major consequence of this replacement is an increase in chemical and physical stability of the resulting retinoids compared to natural retinoic acids, which are unstable under a wide variety of conditions, including exposure to heat and light.[4] A common replacement for the trimethylcyclohexenyl ring and part of the polyene chain in the retinoic acids is the 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene unit,[10] and hence, we decided to incorporate such a function. In addition, it was expected that an acetylene moiety would not only provide a suitable linear structure, but it would also act as a non-isomerisable linker unit. Hence, it was decided to access isomeric compounds of type **4**, in which the isomeric placement of the carboxylate function at either the *para*- or *meta*-positions might mimic some of the different natural retinoic acid isomers, as has been reinforced by competitive binding studies with nuclear receptor proteins.[11] It was envisaged that this series would be readily accessible, as outlined in Scheme 1, using serial cross-coupling reactions starting from bromide **1**.

Although bromide **1** is known,[12] an improved preparation was developed (Scheme 2) involving boron trifluoride-catalysed bromination. Subsequent Sonagashira cross-coupling of bromide **1** with trimethylsilylacetylene (TMSA) or 2-methylbut-3-yn-2-ol, according to literature methods[13] (with careful degassing to remove oxygen, and thus avoid diyne formation) initially gave no conversion to either the TMSA derivative **2a** or dimethyl carbinol product **2b**. However, a combination of $PdCl_2$ and PPh_3 in conjunction with a Cu(II) co-catalyst gave improved results, with the reaction of bromide **1** with TMSA yielding **2a** in 82% yield. Larger scale reactions generally had small amounts



Scheme 1 Proposed synthetic sequence for the synthesis of isomeric retinoid analogues **4**.



Scheme 2 Synthesis of acetylene derivative **3** *via* bromide **5**.

of 1,4-bis(trimethylsilyl)butadiyne present as by-product, which were readily removed later in the synthesis (Scheme 1). The corresponding dimethyl carbinol **2b** could also be accessed using the same protocol, though in lower conversion (45%), and hence the TMSA derivative **2a**, which could be readily de-silylated using KOH to give acetylene **3** (Scheme 2), was carried forward for the synthesis of synthetic retinoids **4**. It is clear that **1** has unusually low reactivity for an aryl bromide, suggesting a strong deactivation by the inductively donating alkyl groups of the tetrahydrotetramethylnaphthalene unit.

As the coupling of bromide **1** with either TMSA or 2-methylbut-3-yn-2-ol required relatively high palladium-catalyst loadings, a more reactive coupling partner was considered, and iodide **6** appeared to be an ideal alternative reagent to bromide **1**. However, a suitable, efficient method for the synthesis of iodide **6** was required.

Iodide **6** was prepared efficiently by an interesting, adapted literature method[14] in 71% yield (Scheme 1). Cross-coupling of iodide **6** with TMSA under Sonogashira conditions occurred readily at room temperature with only 1% Pd catalyst resulting in an 88% yield of acetylene **2a**. Subsequent deprotection of **2a** with hydroxide gave the alkyne **3**, which was subjected to a second Sonogashira coupling with both *para*- and *meta*-iodobenzoate methyl esters to provide each of the isomeric esters **7a** and **7b**, in 87% and 59% yields respectively (Scheme 3). Saponification of the methyl esters **7** produced the corresponding carboxylic acids **4** in 72 and 78% yields, for the *para*- and *meta*-isomers respectively.

Stability of synthetic retinoids *versus* ATRA

The photostability of the synthetic retinoids **4** was tested and compared with that of ATRA. The susceptibility of ATRA to undergo photoisomerisation and degradation when exposed to ordinary laboratory (fumehood) fluorescent light (see Experimental section) in the visible to near-UV range was demonstrated first using ^{1}H NMR spectroscopy. This showed that prolonged exposure to fluorescent light led to progressive degradation of ATRA, and after 3 days there was substantial isomerisation/degradation of the ATRA (*ca.* 37% remaining), as shown in Fig. 3 (light-exposed) compared with Fig. 2 (no light exposure). In contrast, ATRA was found to be stable for at least 3 days when kept in the dark. When

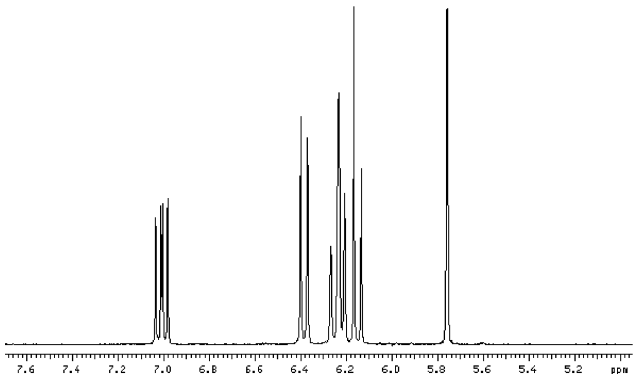


Fig. 2 ^{1}H NMR at 400 MHz of ATRA in DMSO-d_6 (5.0–8.0 ppm region) in the absence of light.

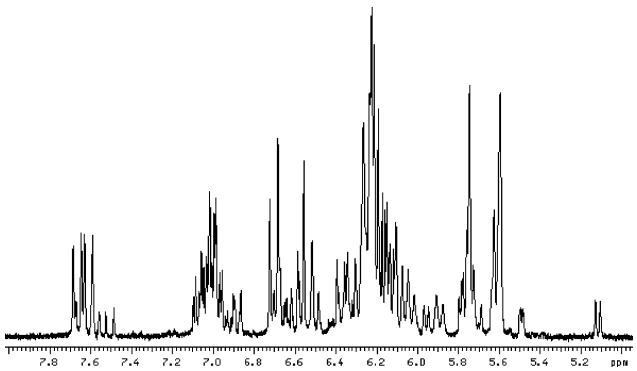
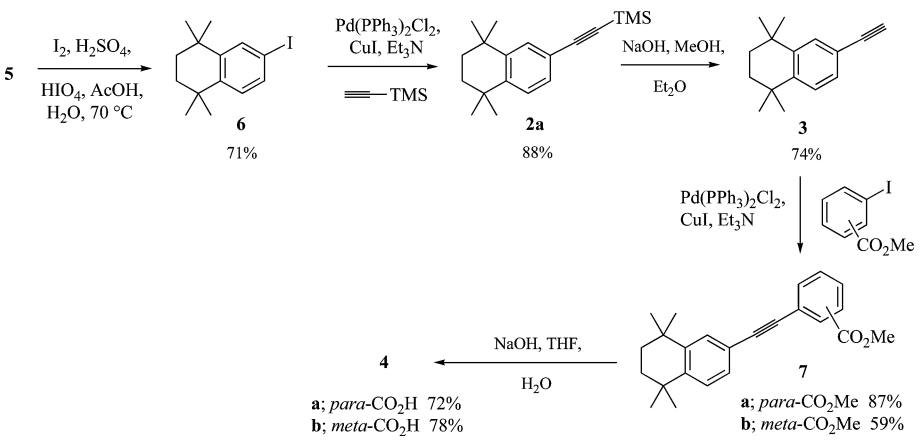


Fig. 3 ^{1}H NMR at 400 MHz of ATRA in DMSO-d_6 (5.0–8.0 ppm region) after 3 weeks exposure to white fluorescent light at a distance of 40 cm.

the synthetic retinoids **4** were exposed to the same wavelength of fluorescent light for 3 days, and the ^{1}H NMR spectra compared with control samples which had been left in the dark for the same length of time, it was found that both **4a** and **4b** were completely unaltered (Figs. 4 and 5). These results clearly demonstrate that the replacement of the polyene chain in ATRA by a diphenylene acetylene motif does result in a dramatic increase in photostability compared with ATRA.



Scheme 3 Synthesis of synthetic retinoids **4** starting with iodide **6**.

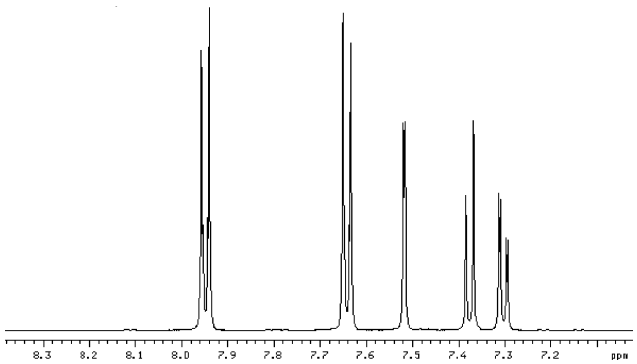


Fig. 4 ^{1}H NMR at 400 MHz of **4a** in DMSO-d_6 (5.0–8.0 ppm region) after 3 weeks exposure to white fluorescent light at a distance of 40 cm.

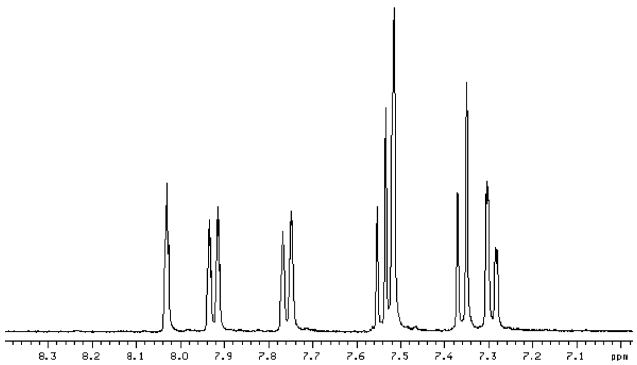


Fig. 5 ^{1}H NMR at 400 MHz of **4b** in DMSO-d_6 (5.0–8.0 ppm region) after 3 weeks exposure to white fluorescent light at a distance of 40 cm.

Effect of synthetic retinoids on the differentiation of human pluripotent stem cells

Having prepared synthetic retinoids **4**, they were assessed for their ability to induce the differentiation of the human embryonal carcinoma stem cell line, TERA2.cl.SP12. Cell lines of the TERA2 lineage are proven models of human embryonic development[9] and have frequently been used to study neural differentiation in particular.[15] To compare the effect of natural *versus* synthetic retinoids on stem cell differentiation, cultures of TERA2.cl.SP12 cells were incubated with 1 μM and 10 μM concentrations of each type of compound for up to 21 days. Control cultures consisted of cells treated with the organic solvent (DMSO) alone (1 : 1000 dilution in tissue culture medium), which was used as the vehicle to dissolve each synthetic retinoids tested. The number of viable cells was determined during the first 7 days of treatment with each test compound by using a standard MTS assay to monitor the rate of cell proliferation (data not shown). Cell numbers were compared to those of the control. Note that in all of our experiments using ATRA, careful precautions were taken to ensure that only pure ATRA was used, and that no degradation could occur during the period of the experiments, by exclusion of light. On this basis, accurate comparisons could be made on the effects all compounds studied.

Cultures treated with DMSO alone (control) continued to proliferate, became over-confluent and were terminated after 7 days. In contrast, cell proliferation in cultures exposed to 1 μM and 10 μM solutions of ATRA and the *para*-isomer synthetic retinoid **4a** slowed significantly, indicative of cells exiting the cell cycle and committing to differentiation. However, the number of viable cells in cultures treated with a 1 μM solution of the *meta*-isomer synthetic retinoid **4b** were not different to those of the control and such cultures became over-confluent and were terminated, indicating the inability of this compound to arrest cell proliferation at this concentration. Cultures exposed to the higher concentration of **4b** (10 μM) did remain viable though with reduced proliferation. Furthermore, the rate at which cells committed to differentiate in response to **4b** (10 μM) was slower compared to that induced by either ATRA or **4a**.

TERA2.cl.SP12 cells treated with natural or synthetic retinoids responded positively and showed marked differentiation (Fig. 6). The morphology of cultures exposed to **4a** were similar to those treated with ATRA, with both containing heterogeneous cell populations and, in particular, containing structures resembling neural rosettes typically seen in neural cell cultures (Fig. 6B and C).[15] Alternatively, 21 days of treatment with 10 μM **4b** resulted in highly confluent cultures containing distinct regions of large flat epithelial-like cells (referred to as 'plaques') (see Fig. 6D). Plaques formed by the large flat cells were distributed throughout the culture and were large enough to be seen with the naked eye against the bottom of the culture flask. Furthermore, the large flat cells within the plaques showed strong staining for epithelial cytokeratin-8 (Fig. 7). Such structures were observed to a lesser extent in cultures treated with ATRA and **4a** and occupied a significantly smaller area compared to cultures treated with **4b** (Fig. 7D, E). Indeed, the proportion of the cell population that

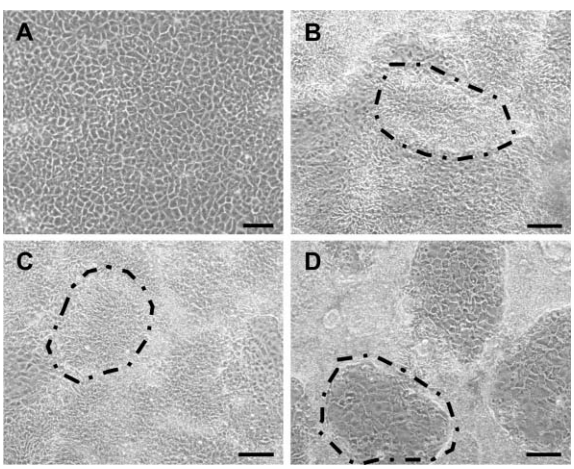


Fig. 6 Morphological appearance of TERA2.cl.SP12 human pluripotent stem cells in the presence and absence of retinoid. (**A**) TERA2.cl.SP12 stem cells (control). (**B**) TERA2.cl.SP12 cells exposed to 10 μM ATRA for 21 days. (**C**) TERA2.cl.SP12 cells exposed to 10 μM **4a** (EC23) for 21 days. (**D**) TERA2.cl.SP12 cells exposed to 10 μM **4b** (EC19) for 21 days. TERA2.cl.SP12 stem cells retained a homogeneous 'cobblestone' appearance throughout the culture (**A**). Cultures exposed to ATRA differentiate and become heterogeneous in appearance consisting of neural rosettes [bordered area, (**B**)] and regions of flat epithelial-like cells (referred to as 'plaques'). Similarly, cultures exposed to **4a** formed neural rosettes [bordered area, (**C**)], but fewer plaques compared to ATRA. In contrast, **4b** induced the formation of multiple, large plaques of flat epithelial-like cells [bordered area, (**D**)] throughout the culture with no evidence of neural rosettes. Scale bars: 100 μm.

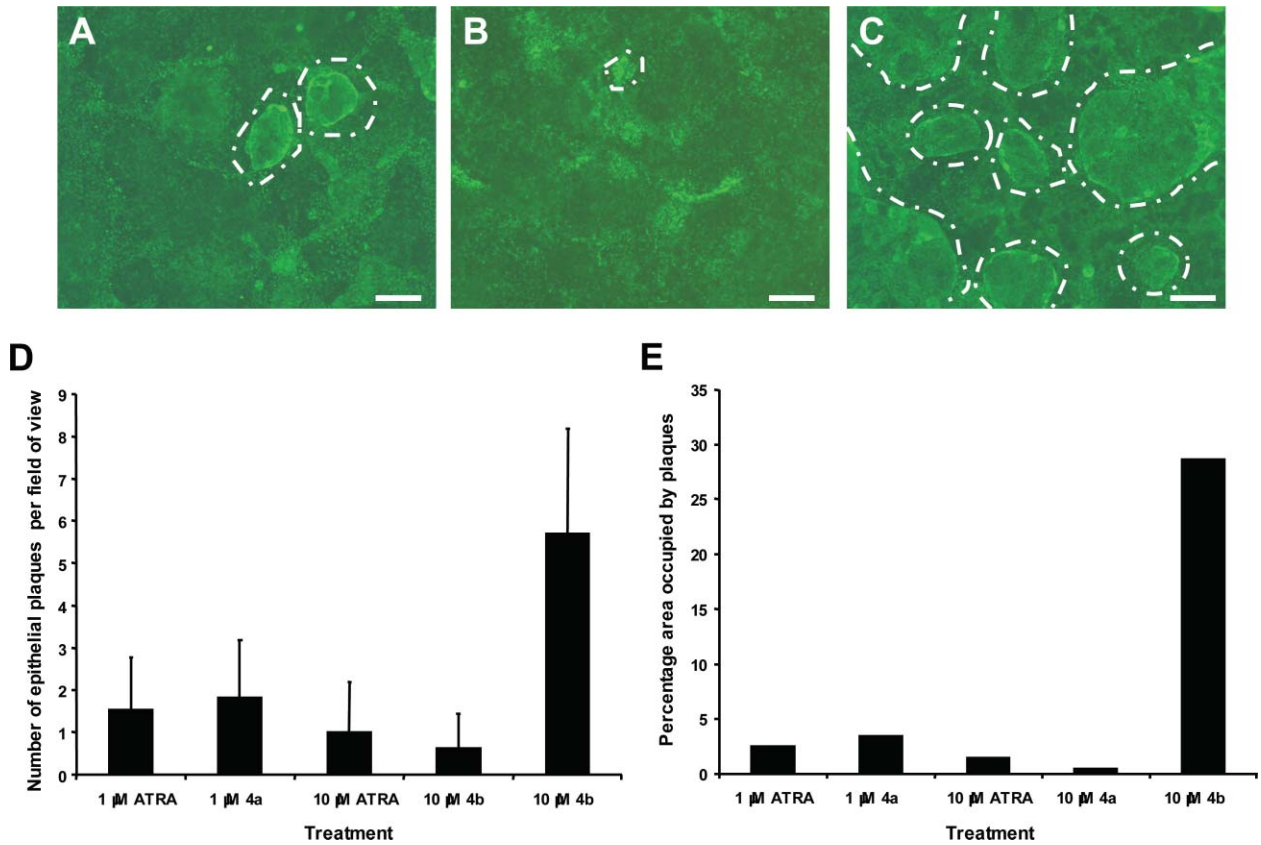


Fig. 7 Induction of cytokeratin-8-positive epithelial plaques in response to retinoids. Immunofluorescence micrographs show cytokeratin-8 expression in TERA2.cl.SP12 cultures exposed to 10 μM of either ATRA, **4a** (EC23) or **4b** (EC19) (**A**–**C** respectively). Plaque areas are outlined in each image. For each condition, 12 randomly selected fields of view were collected. The number of plaques (**D**) and the area which they occupied (**E**) was determined per field of view in cultures exposed to ATRA, **4a** and **4b** at a concentration of either 1 μM or 10 μM for 21 days. The number and average area covered by epithelial plaques increased significantly in **4b** cultures. As the concentration of ATRA or **4a** increased, the number of plaques per field and their average area occupancy per field of view decreased, being almost totally absent in cultures exposed to 10 μM **4a**. Data represent mean ± SD, $n = 12$.

formed plaques was particularly low in cultures treated with a 10 μM solution of **4a**. Cultures treated with solutions of either 10 μM ATRA or **4a** resulted in slightly fewer cytokeratin-8-positive plaques compared to those treated with a 1 μM dose of either ATRA or **4a**, but this was not significant.

Induction of cellular differentiation in response to retinoids was further evaluated by monitoring the expression profile of known markers for stem cell and differentiated cell phenotypes (Fig. 8). Flow cytometry was performed on samples of cells tested for the expression of the stem cell antigens, SSEA-3 (globoseries stage specific embryonic antigen-3) and TRA-1-60 (keratin-sulfate-associated glycoprotein stem surface marker). The reduction in the expression level of SSEA-3 and TRA-1-60 indicated cells committing to differentiate in response to both ATRA and **4a**. The activity of ATRA and **4a** was similar at both the 1 μM and 10 μM concentrations. After 7 days exposure to either ATRA or **4a**, the stem cell antigens were heavily down-regulated; however, SSEA-3 and TRA-1-60 expression levels remained comparatively high in cultures treated with **4b** up to 7 days after addition. Nonetheless, the decrease in stem cell antigen expression relative to control cultures was marked, indicating that cellular differentiation had been triggered in a proportion of cells. After 7 days, cultures treated with 1 μM **4b** were discarded due to overgrowth, as recorded above. However, in cultures treated with 10 μM **4b**, SSEA-3 and TRA-1-60 continued to decline to levels comparable to those cultures treated with either ATRA or **4a** for 14 days. These data indicate that **4b** can induce the differentiation of TERA2.cl. SP12 cells, albeit at a slower rate than either ATRA or **4a**. Commitment of cells to differentiate in response to each of the retinoids tested herein correlated with the regulated levels of cell proliferation as determined by the MTS assay (as described above).

TERA2.cl.SP12 cells are well known for their ability to form neurons in response to ATRA.[15] Accordingly, flow cytometry was used to evaluate the expression of antigens associated with differentiating neural cell types, VINIS-53 (neuronal cell adhesion molecule (NCAM)) and A2B5 (ganglioseries antigen marking early-stage neural cells) (Fig. 8), markers which are expressed during the early stages of neuronal development. Each retinoid tested induced the expression of VINIS-53 and A2B5, although there were some differences between the different compounds in the rate of expression and the maximum levels attained. Notably, cells exposed to 10 μM **4a** showed the highest levels of VINIS-53 and A2B5 during the period tested, whilst those treated with **4b** were considerably slower at inducing the expression of neural antigens, which correlated with the delayed decrease in stem cell markers.

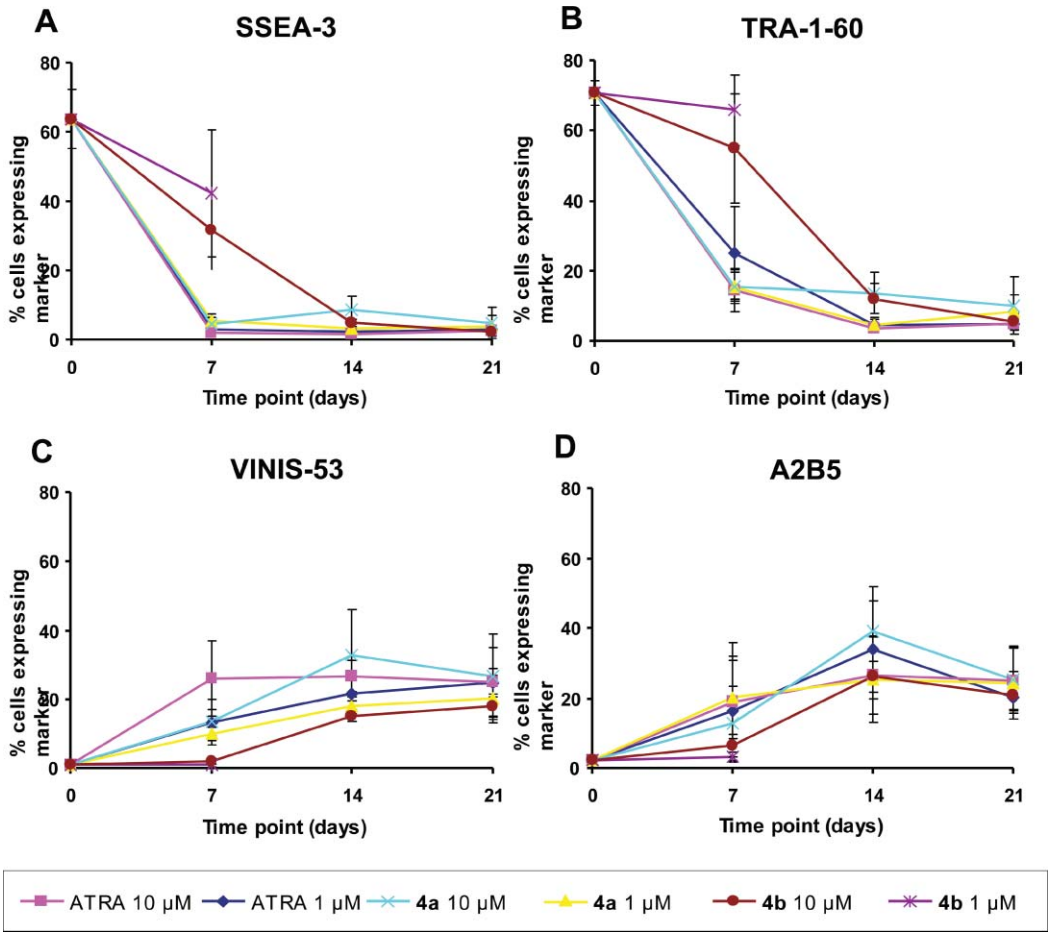


Fig. 8 Flow cytometric analysis of the induction of TERA2.cl.SP12 cell differentiation in response to retinoids. Markers of stem cells (SSEA-3 and TRA-1-60, **A**, **B** respectively) and neural differentiation (VINIS-53 and A2B5, **C**, **D** respectively) were differentially regulated during the 21 day culture period in response to 1 μM or 10 μM ATRA, **4a** or **4b**. Both concentrations of ATRA and **4a** resulted in a rapid decrease in expression of the stem cell markers and reciprocal increase in neural marker expression. Similarly, 10 μM **4b** induced differentiation as evidenced by the absence of the stem cell markers after 14 days, although this down-regulation occurred at a slower rate than that induced by **4a** and **4b**. Treatment with 1 μM **4b** did not induce differentiation; cells continued to proliferate and the experiment was terminated after 7 days as a consequence of becoming over-confluent. Data represent mean ± SEM, $n = 3$.

Confirmation of neural development of TERA2.cl.SP12 cells was demonstrated by immunocytochemical staining of cultures treated with each of the retinoid compounds over 21 days (see Figs. 9 and 10). Nestin, a protein marker expressed by neuroprogenitor cells, showed low levels of expression in TERA2.cl.SP12 stem cells which was significantly up-regulated throughout cultures treated with either ATRA or **4a** at either 1 μM or 10 μM concentrations (Fig. 9). Treatment with both ATRA and **4a** induced large numbers of nestin-positive structures resembling 'neural-rosettes' which are characteristic of aggregations of cultured neuroprogenitor cells. In contrast, cells exposed to solutions of **4b** resulted in lower levels of nestin expression which was localised to discrete areas, and nestin expression was absent in the large plaques consisting of flat epithelial-like cells. The expression of neurofilament 200 (NF200) was also examined as a cytoskeletal marker of mature neurons (Fig. 10). As expected, TERA2.cl.SP12 stem cells did not express NF200. Large numbers of NF200-positive neurons were identified in cultures treated with either ATRA or **4a** for both concentrations tested. In contrast, cultures exposed to **4b** produced few mature neurons and the large epithelial plaques stained negative for NF200. Collectively, these data indicate that the retinoids ATRA and **4a** behave in a similar manner and induce the differentiation of human pluripotent stem cells to form mature neural derivatives. Quantification of the number of NF200-positive perykarya in each culture treatment was calculated as a mean from six randomly selected cultures (Fig. 10). In rank order, the highest number of NF200-positive perykarya (and by inference number of mature neurons), was seen in cultures treated with 10 μM **4a** > 10 μM ATRA > 1 μM **4a** > 1 μM ATRA >> 10 μM **4b**. Indeed exposure to 10 μM **4a** resulted in approximately 38% more NF200-positive cells compared to 10 μM ATRA. According to these data, the stable synthetic retinoid **4a** appears to be a more potent inducer of neuronal differentiation compared to naturally occurring ATRA. Although the results also indicate a degree of neuronal differentiation in cultures exposed to 10 μM **4b**, the levels were very much lower than those observed following either ATRA or **4a** treatment.

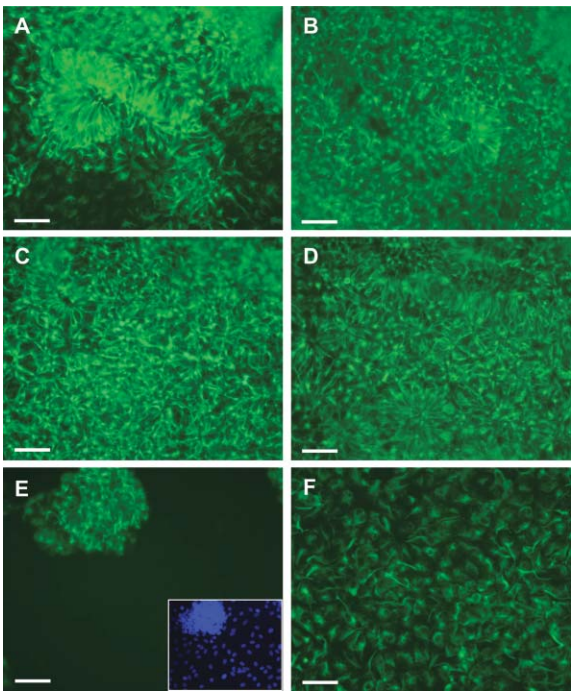


Fig. 9 Differential regulation of nestin, a protein marker of neuroprogenitor cells, in response to retinoids. Immunofluorescence micrographs show nestin expression in TERA2.cl.SP12 cultures exposed to (**A**) 1 μM or (**B**) 10 μM ATRA, (**C**) 1 μM or (**D**) 10 μM **4a** and (**E**) 10 μM **4b** for 21 days. Undifferentiated cells displayed relatively low levels of nestin expression (**F**). Both concentrations of ATRA and **4a** elicited a strong nestin expression response (**A–D**), although unlike 1 μM ATRA (**A**), 1 μM **4a** (**C**) repeatedly showed consistently high levels homogenously distributed throughout the culture. In cultures exposed to **4b** (**E**), nestin expression was restricted to discrete areas and the large flat cells within the plaques which dominated the **4b** cultures stained negative for nestin. Panel (**E**) includes an inset to show Hoescht 33342 counterstaining indicating the location of all cell nuclei in the culture. Scale bars: 50 μm.

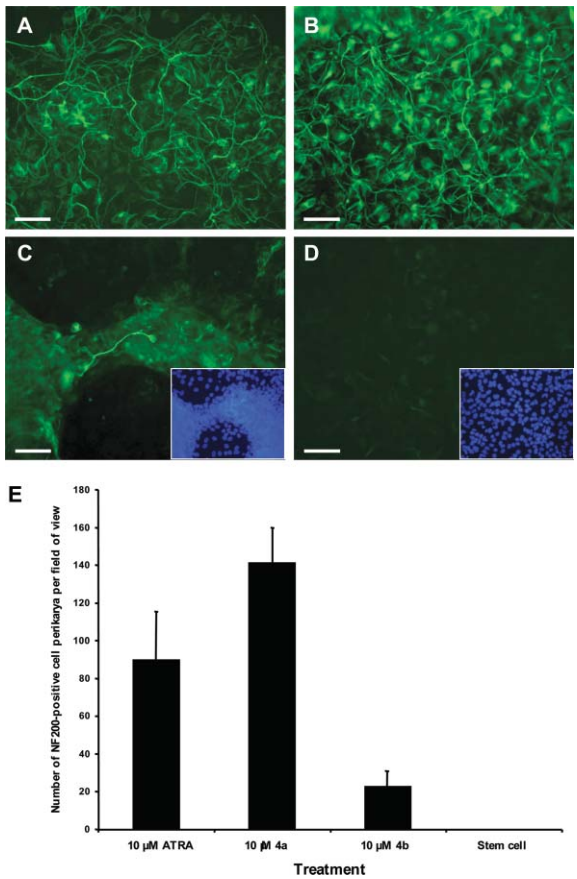


Fig. 10 Induction of neural differentiation in cultures of TERA2.cl.SP12 cells exposed to retinoids. Immunofluorescence micrographs show expression of neurofilament-200 (NF200), a marker of mature neurons, in TERA2.cl.SP12 cultures exposed to (**A**) 10 μM ATRA, (**B**) 10 μM **4a** or (**C**) 10 μM **4b** for 21 days. TERA2.cl.SP12 stem cells did not express NF200 (**D**). Cultures treated with ATRA and **4a** formed large numbers of NF200-positive neurons (**A**, **B** respectively) whereas those exposed to **4b** developed very few neurons and such cells were located between the epithelial plaques (**C**). Inset images to show Hoescht 33342 counterstaining indicating the location of all cell nuclei in the culture (**C**, **D**). Scale bars: 50 μm. Quantification of the numbers of NF-200 positive cell perikarya induced in response to 10 μM ATRA, **4a**, or **4b**, was determined from 6 randomly selected fields of view per condition (**E**). Data represent mean ± SEM, $n = 6$.

Conclusions

In this study, we have designed, synthesised and tested the stability of two synthetic retinoids, **4a** and **4b**, and directly compared their stability and biological activity to that of naturally occurring ATRA. We have shown that both **4a** and **4b** remain stable and are resistant to light under conditions which cause isomerisation and degradation of ATRA. Our studies suggest that there are some significant advantages to using synthetic retinoids such as **4a** and **4b** over using ATRA in the cell culture laboratory since this avoids potential problems associated with solutions of ATRA, which are very susceptible to photoisomerisation and which may subsequently influence their function to modulate cell activity.[4] It is important to note that as well as observing normal handling procedures, light needs to be excluded particularly when carrying out *in vitro* studies with RAs, whereas **4a** and **b** can be handled under normal ambient light conditions. At present, we can not be certain that all of the biological effects of ATRA are replaced by **4**; however, ongoing studies may reveal situations in which there are discernable differences. In the meantime, cell biologists can be confident of retinoid stability when using the synthetic compounds tested herein, and that many of the observable effects of ATRA examined in these studies are reproduced. Testing the biological effects of the synthetic retinoids showed that they possess the ability to induce the differentiation of human pluripotent stem cells. However, **4a** in particular displayed the ability to induce neural tissues to a degree that was at least as effective, or better than that observed for ATRA. In contrast, **4b** induced cell differentiation, resulting in distinctly different cultures which were composed primarily of epithelial cells with few neural phenotypes. These data indicate that subtle differences in the molecular structure can have a dramatic effect upon the biological function, exemplified in this study by the isomeric synthetic retinoids **4b** and **4a**. Such information regarding structure–activity-relationships will be important in the future design and application of new synthetic compounds aimed at controlling cellular development processes. The observed similarity in the function of **4a** to ATRA highlights that the *para*-isomer synthetic retinoid **4a** is

an exciting compound for use in stem cell science since it has the added benefit of stability. It is noteworthy that **4a** and ATRA both show competitive binding towards RAR receptors, and not to RXR receptors,[11] but it is premature to be certain that the observed biological effects reported here are directly linked to this. Further studies in this area will be reported in due course.

Experimental

General synthetic chemistry

All cross-couplings were carried out under an N_2 atmosphere using standard Schenk techniques. Triethylamine was distilled over CaH_2 under N_2. Other reactions were performed in the air in reagent-grade solvents. The compound 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene was obtained from Maybridge and used without further purification. Trimethylsilylacetylene was obtained from Fluorochem, 3-iodobenzoic acid and methyl 4-iodobenzoate from Aldrich, and 2-methylbut-3-yn-2-ol from Alfa Aesar.

Melting points were obtained using a Gallenkamp melting point apparatus. NMR spectra were performed on Varian Mercury (^{1}H) and Bruker Avance (^{13}C) spectrometers with chemical shifts referenced to solvent resonances and reported relative to tetramethylsilane. EI-MS analyses were performed on an Agilent 6890 GC with a 5973 Inert MSD, using UHP helium as the carrier gas. ES-MS analyses were performed on a Thermo-Electron Corp. Finnagan LTQ-FT mass spectrometer. Elemental analyses were obtained using an Exeter Analytical CE-440 analyzer. UV-vis spectra were recorded in $CHCl_3$ on a Hewlett-Packard 8453 diode array spectrophotometer using standard 1 cm quartz cells.

The crystal and molecular structures of compounds **1**, **2a**, **3**, **6**, **7a**, and **7b** were determined by single-crystal X-ray diffraction, details of which are provided in the ESI†.

6-Bromo-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene 1[12]

To a solution of 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene **5** (10.0 g, 53.0 mmol) in DCM (60 ml) at 0 °C under N_2 was added Br_2 (15.58 g, 97.5 mmol). $BF_3{\cdot}Et_2O$ (8.27 g, 58.3 mmol) in DCM (10 ml) was added dropwise over 2 h. The reaction mixture was diluted with 40 : 60 EtOAc–hexane (150 ml) and washed with saturated Na_2SO_3 solution (100 ml), saturated $NaHCO_3$ solution (100 ml), and H_2O (100 ml). The organic layer was dried ($MgSO_4$), filtered and evaporated to give a dark brown oil. Kugelrohr distillation (120 °C, 8×10^{-3} mbar) gave **1** as pale yellow crystals (11.02 g, 78%); mp 43–45 °C; *m/z* (EI) 267 (M^+); Anal. Calcd. for $C_{14}H_{19}Br$: C, 62.93; H, 7.17; Found: C, 62.81; H, 7.16. All ^{13}C and ^{1}H NMR data were identical to those reported in the literature.[12]

Trimethyl(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)silane 2a

$PdCl_2$ (75 mg, 0.43 mmol), $Cu(OAc)_2$ (77 mg, 0.43 mmol), **1** (1.14 g, 4.30 mmol) and PPh_3 (0.56 g, 2.14 mmol) were placed in a 250 ml Schlenk flask under N_2. Dry, degassed triethylamine (100 ml) was added *via* cannula and TMSA (0.7 mL 5.14 mmol) added *via* syringe. After 18 h at 70 °C, triethylamine was evaporated and the residue was passed through a short silica gel column (hexane as eluent) to give the crude product as a viscous, pale yellow oil after evaporation which slowly solidified to give an off-white solid which was recrystallised from ethanol to give the trimethylsilylacetylene adduct **2a** (1.0 g, 81%); mp 51–52 °C. δ_H (400 MHz; $CDCl_3$) 7.40 (1H, m, Ar), 7.21 (2H, m, Ar), 1.66 (4H, s, CH_2), 1.26 (6H, s, CH_3), 1.25 (6H, s, CH_3), 0.24 (9H, s, CH_3); δ_C (126 MHz, $CDCl_3$) 145.7, 144.8, 130.2, 129.0, 126.4, 120.0, 105.8, 92.6, 34.9, 34.8, 34.2, 34.1, 31.7, 31.6, 0.0; *m/z* (EI) 284 (M^+); Anal. Calcd. for $C_{19}H_{28}Si$ C, 80.21; H, 9.92; found C, 80.04: H, 9.90.

(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)dimethylcarbinol 2b

$PdCl_2$ (0.331 g, 1.87 mmol), $Cu(OAc)_2$ (0.274 g, 1.87 mmol), **1** (5.0 g, 18.71 mmol) and PPh_3 (2.45 g, 9.35 mmol) were placed in a 500 ml Schlenk flask, and the flask was evacuated and back-filled with N_2 gas (3 ×). Triethylamine (150 ml) was added *via* cannula, followed by 2-methylbut-3-yn-2-ol (4.72 g, 56.13 mmol). The solution was stirred under N_2 at 70 °C for 3 days. Triethylamine was evaporated and the residue was passed through a short silica gel column (hexane, then 10% EtOAc–hexane as eluent). The EtOAc–hexane solution was washed with 1 M HCl solution (100 ml), dried ($MgSO_4$) and evaporated to give **2b** as an off-white solid (2.25 g, 45%); mp 107–109 °C; δ_H (500 MHz, $CDCl_3$) 7.36 (1H, s, Ar), 7.24 (1H, d, *J* 7.8 Hz, Ar), 7.21 (1H, d, *J* 7.8 Hz, Ar), 2.05 (1H, s, OH), 1.67 (4H, s, CH_2), 1.62 (6H, s, CH_3), 1,27 (6H, s, CH_3), 1.26 (6H, s, CH_3); δ_C (126 MHz, $CDCl_3$) 145.6, 145.1, 130.1, 128.9, 126.8, 119.8, 92.8, 82.8, 65.8, 35.2, 35.1, 34.5, 34.4, 31.9, 31.9, 31.8; *m/z* (EI) 270 (M^+); HRMS calcd. for $C_{19}H_{26}ONa$ ([MNa]$^+$) 293.18759, found 293.18776, and HRMS calcd. for $C_{19}H_{25}$ ([M − OH]$^+$) 253.19508, found 253.19522.

6-Iodo-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene 6

To a mixture of 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene (3.76 g, 20 mmol), iodine (2.04 g, 8 mmol) and periodic acid (0.92 g, 4 mmol) was added glacial acetic acid (20 ml), water (4 ml) and concentrated H_2SO_4 (98%, 1 ml). The reaction mixture was heated to 70 °C for 4 h. A precipitate formed upon cooling, which was filtered off, dissolved in hexane and passed through a short silica gel column (hexane as eluent). The hexane was evaporated and the residue was re-crystallised from ethanol to give iodide **6** as a white crystalline solid (4.45 g, 71%); mp 69–70 °C; δ_H (200 MHz, $CDCl_3$) δ 7.59–7.62 (1H, d, J 2 Hz, Ar), 7.40–7.47 (1H, dd, *J* 8 and 2 Hz, Ar), 7.01–7.07 (1H, d, J = 8 Hz, Ar), 1.66 (4H, s, 2 × CH_2), 1.28 (6H, s, 2 × Me), 1.26 (6H, s, 2 × Me); δ_C (100 MHz, $CDCl_3$) 147.7, 144.6, 135.6, 134.6, 128.7, 91.1, 34.9, 34.8, 34.3, 34.1, 31.8, 31.7; MS (EI): 314 (M+); Anal. Calcd. for $C_{14}H_{19}I$ C, 53.52; H, 6.10; found C, 53.66; H, 6.13.

6-Trimethylsilylethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene 2a

6-Iodo-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene (3.14 g, 10 mmol), $Pd(PPh_3)_2Cl_2$ (0.07 g, 0.1 mmol) and CuI (0.02 g, 0.1 mmol) were added to a 250 ml Schlenk flask which was evacuated and purged with N_2 (3 ×). Triethylamine (150 ml) was added *via* cannula under N_2, followed by trimethylsilylacetylene

(1.18 g, 12 mmol). After 12 h at RT, triethylamine was evaporated and the residue was passed through a short silica gel column (hexane as eluent) to give the crude product as a viscous, pale yellow oil after evaporation which slowly solidified to give an off-white solid, which was recrystallised from ethanol to give the trimethylsilylacetylene adduct **2a** (2.50 g, 88%). All spectroscopic and analytical properties were identical to those reported above.

6-Ethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene 3

To a solution of 6-trimethylsilylethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene (1.42 g, 5 mmol) in methanol (50 ml) and diethyl ether (50 ml), was added NaOH (0.14 g, 3.5 mmol) in water (2 ml). After 4 h, the mixture was extracted with diethyl ether, washed with water (3 ×), dried ($MgSO_4$) and evaporated to give 6-ethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene **3** as a pale yellow oil which slowly solidified to give a white solid (0.78 g, 74%); mp 48–49 °C; υ_{max} (KBr disc, cm^{-1}) *inter alia* 2105 (C≡C); δ_H (200 MHz, $CDCl_3$) 7.47 (1H, s, Ar), 7.27 (2H, s, Ar), 3.03 (1H, s, CH), 1.70 (4H, s, CH_2), 1.29 (6H, s, 2 × Me), 1.26 (6H, s, 2 × Me); δ_C (100 MHz, $CDCl_3$) 146.1, 145.1, 130.5, 129.2, 126.6, 119.1, 84.3, 75.9, 34.9, 34.8, 34.3, 34.2, 31.8, 31.7; *m/z* (EI): 212 (M+); Anal. calcd. for $C_{16}H_{20}$ C, 90.51; H, 9.49; found C, 90.27; H, 9.57.

Methyl 3-iodobenzoate

A solution of 3-iodobenzoic acid (24.8 g, 100 mmol) in methanol (300 ml) was treated with sulfuric acid (98%, 5.0 g). After heating at 70 °C for 5 h, the mixture was extracted with diethyl ether, washed with water (3 ×), dried ($MgSO_4$) and evaporated. The residue was passed through a short silica gel column (hexane as eluent), to give the product as a white crystalline solid (21.1 g, 81%); mp 53–54 °C (lit. 54–55 °C[1]). All spectroscopic and analytical properties were identical to those reported in the literature.[16]

4-(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)-benzoic acid methyl ester 7a

Methyl 4-iodobenzoate (3.59 g, 13.7 mmol), $Pd(PPh_3)_2Cl_2$ (0.09 g, 0.14 mmol) and CuI (0.03 g, 0.14 mmol) were added with stirring to a 500 ml Schlenk flask. After evacuation and purging with N_2 (3 ×), triethylamine (200 ml) was added *via* cannula under N_2, followed by 6-ethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene **3** (3.50 g, 16.5 mmol). After 18 h at RT, the triethylamine was evaporated and the residue was passed through a short silica gel column (hexane, followed by hexane–DCM, 4 : 1 as eluent) to give the crude product, which was re-crystallised from ethanol to give the product **7a** as a white crystalline solid (4.13 g, 87%); mp 128–129 °C; υ_{max} (KBr disc, cm^{-1}) *inter alia* 2207 (C≡C), 1712 (C=O); δ_H (400 MHz, $CDCl_3$) 8.03 (2H, d, *J* 9 Hz, Ar), 7.59 (2H, d, *J* 9 Hz, Ar), 7.51 (1H, s, Ar), 7.31 (2H, s, Ar), 3.94 (3H, s, MeO), 1.71 (4H, s, 2 × CH_2), 1.28 (12H, s, Me); δ_C (100 MHz, $CDCl_3$) 166.8, 146.3, 145.4, 131.7, 130.3, 129.7, 129.4, 129.0, 128.6, 127.0, 119.8, 93.3, 87.2, 52.4, 35.2, 35.1, 34.6, 34.5, 32.0, 31.9; λ_{max} ($CHCl_3$) 310 nm (ε 26400 M^{-1} cm^{-1}); Anal. calcd. for $C_{24}H_{26}O_2$ C, 83.20; H, 7.56; found C, 83.03; H, 7.59.

3-(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid methyl ester 7b

Methyl 3-iodobenzoate (0.52 g, 2 mmol), $Pd(PPh_3)_2Cl_2$ (0.014 g, 0.02 mmol) and CuI (0.004 g, 0.02 mmol) were added to a 250 ml Schlenk flask which was evacuated and purged with N_2 (3 ×). Triethylamine (50 ml) was added *via* cannula under N_2, followed by 6-ethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene **3** (0.44 g, 2.1 mmol). The reaction mixture was stirred overnight at RT, then heated at 70 °C for 1 h. After cooling, the triethylamine was evaporated and the residue was passed through a short silica gel column (hexane, followed by hexane–DCM, 4 : 1 as eluent) to give the crude product, which was re-crystallised from ethanol, to give **7b** as a white crystalline solid (0.41 g, 59%); mp 100–101 °C; υ_{max} (KBr disc, cm^{-1}) *inter alia* (C≡C), 1717 (C=O); δ_H (200 MHz, $CDCl_3$) 8.23 (1H, s, Ar), 7.97–8.01 (1H, d, *J* 5 Hz, Ar), 7.72–7.68 (1H, d, *J* 5 Hz, Ar), 7.50 (1H, s, Ar), 7.38–7.45 (1H, t, *J* 5 Hz, Ar), 7.28 (2H, s, Ar), 3.95 (3H, s, MeO), 1.70 (4H, s, 2 × CH_2), 1.30 (6H, s, 2 × Me), 1.28 (6H, s, 2 × Me); δ_C (100 MHz, $CDCl_3$) 166.5, 145.9, 145.2, 135.6, 132.7, 130.4, 130.0, 128.9, 128.7, 128.4, 126.7, 124.1, 119.8, 90.9, 87.2, 52.0, 35.0, 34.9, 34.4, 34.2, 31.8, 31.7; MS (EI) 346 (M^+); λ_{max} ($CHCl_3$) 289 nm (ε 23000 M^{-1} cm^{-1}); Anal. calcd. for $C_{24}H_{26}O_2$: C, 83.20; H, 7.56; found C, 82.99; H, 7.56.

4-(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)-benzoic acid 4a

A solution of 4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid methyl ester **7a** (0.35 g, 1 mmol) in THF (20 ml) was treated with aqueous 20% NaOH (20 ml). After heating at 70 °C for 20 h, the reaction mixture was diluted with diethyl ether (150 ml) and water (150 ml), then 1 M HCl solution was added until mixture reached pH 1. The ether layer was separated, dried ($MgSO_4$) and evaporated to give an off-white powder, which was re-crystallised from acetonitrile to give the product **4a** as a white crystalline solid (0.24 g, 72%); mp 254–256 °C; υ_{max} (KBr disc, cm^{-1}) *inter alia* 2205 (C≡C), 1681 (C=O); δ_H (400 MHz, $CDCl_3$) 8.09 (2H, d, *J* 8.5 Hz, Ar), 7.62 (2H, d, *J* 8.5 Hz, Ar), 7.51 (1H, s, Ar), 7.31 (2H, s, Ar), 1.68 (4H, s, CH_2), 1.31 (12H, s, Me); δ_C (125 MHz, $CDCl_3$) 171.5, 146.5, 145.4, 131.7, 130.4, 130.3, 129.6, 129.1, 128.4, 127.0, 119.7, 93.9, 87.8, 35.1, 35.0, 34.6, 34.5, 32.0, 31.9; *m/z* (ESI) 377 (M + 2Na), 331 (M − H); λ_{max} ($CHCl_3$) 310 nm (ε 26900 M^{-1} cm^{-1}); HRMS calcd. for $C_{23}H_{23}O_2$ 331.16926 (M − H), found 331.16949.

3-(5,5,8,8-Tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)-benzoic acid 4b

A solution of 3-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid methyl ester **7b** (0.35 g, 1 mmol) in THF (20 ml) was treated with aqueous 20% NaOH (20 ml). After heating at 70 °C for 20 h, the reaction mixture was diluted with diethyl ether (150 ml) and water (150 ml), then 1M HCl solution was added until mixture reached pH 1. The ether layer was separated, dried ($MgSO_4$) and evaporated to give an off-white powder. The product was re-crystallised from acetonitrile to give **4b** as a white crystalline solid (0.26 g, 78%); mp 223–224 °C; υ_{max} (KBr disc, cm^{-1}) *inter alia* 2210 (C≡C), 1690 (C=O); δ_H (200 MHz, $CDCl_3$) 8.25 (1H, s, Ar), 8.00–8.04 1H, (d, *J* 5 Hz, Ar), 7.76–7.80 (1H, d, *J* 5 Hz, Ar), 7.50 (1H, s, Ar), 7.42–7.50 (1H, t, *J* 5 Hz,

Ar), 7.28 (2H, s, Ar), 1.70 (4H, s, CH_2), 1.32 (6H, s, 2 × Me), 1.30 (6H, s, 2 × Me); δ_C (100 MHz, d_6-DMSO) 167.0, 146.3, 145.5, 135.7, 132.4, 131.9, 130.1, 129.7, 129.0, 127.4, 123.4, 119.5, 91.1, 87.8, 34.9, 34.8, 34.5, 34.4, 31.9, 31.8; λ_{max} ($CHCl_3$) 289 nm (ε 29 000 $M^{-1}cm^{-1}$); Anal. calcd. for $C_{23}H_{24}O_2$ C, 83.10; H, 7.28; found: C, 82.38; H, 7.24; HRMS calcd. for $C_{23}H_{23}O_2$ 331.16926 (M − H); found 331.17050.

Exposure to light

ATRA and the synthetic retinoids NMR samples were prepared and left to stand in ordinary laboratory light, with additional irradiation from a standard fluorescent light *ca.* 30 cm above the sample. The spectral characteristics of the light were measured using a spectrophotometer and are shown in Fig. 11.

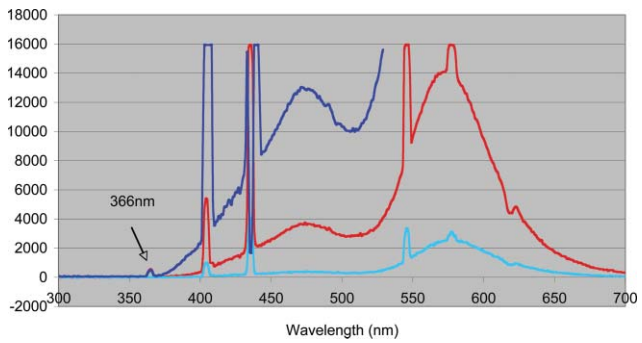


Fig. 11 Red line = room lights (note shortest wavelength feature 366 nm). Light blue = lamps used for irradiation of the samples, with a short integration period. Dark blue = lamps used for the sample irradiation, with a longer integration period (note shortest wavelength feature 366 nm, similar to room light).

Tissue culture

Human pluripotent TERA2.cl.SP12 embryonal carcinoma stem cells were maintained under standard laboratory conditions as described by Przyborski.[9] In brief, cells were cultured in DMEM (Sigma) supplemented with 10% 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 a 0.25% trypsin EDTA (Cambrex) solution was used. Cultures intended for flow cytometric analysis were set up in T25 flasks (Nunc) while 12-well plates (Nunc) were used for MTS assays and immunocytochemical studies. In preparation for use in cell culture experiments, stock solutions of synthetic retinoids and ATRA (Sigma) were prepared in DMSO (Sigma) to concentrations of 10mM. Aliquotted stock solutions were stored at −80 °C in the dark.

Flow cytometry

Flow cytometric analysis was carried out on live cells using antibodies recognising cell surface markers. The expression of markers indicative of the stem cell (SSEA-3 and TRA-1-60 (generous gift from Prof. P. Andrews, University of Sheffield)) or neural cell (A2B5 (Abcam) and VINIS-53 (generous gift from Prof. P. Andrews, University of Sheffield)) phenotype was determined to indicate the status of cellular differentiation by TERA2.SP12 cells.[9] Suspensions of single EC cells or their differentiated derivatives were formed by the addition of 1 ml 0.25% trypsin/EDTA solution and cell numbers determined using a haemocytometer. Cells were added to a 96-well plate (0.2×10^6 cells per well) for incubation with primary (1 : 20) and FITC-conjugated secondary antibody (Cappell, 1 : 100) as previously described.[9,15] Labelled cells were analysed in a BD Biosystems FACSFlow cytometer. Thresholds determining the numbers of positively expressing cells were set against the negative control antibody P3X.

Immunocytochemistry

Cells were grown in 12-well culture plates for immunocytochemical analysis. Confluent cultures of stem cells and their differentiated derivatives were fixed in ice-cold 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) for 30 min at room temperature (RT), followed by 3 washes in PBS. Cell membranes were permeabilised by treatment with 1% Triton-X-100 (Sigma) in PBS for 10 min at room temperature. 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 for 30 min at room temperature. Primary antibodies: nestin (Chemicon, 1 : 100); NF-200 (Sigma 1 : 100); cytokeratin-8 (Sigma, 1 : 100) were diluted in blocking solution and incubated with the cells for 1 h at RT. After washing, cells were incubated in FITC-conjugated secondary antibody (Alexafluor 488 1 : 600) for 60 min. After washing in PBS, samples were mounted under coverslips using Vectashield, (Vector Labs) containing 1 μg ml^{-1} Hoechst 33342 nuclear staining dye (Molecular Probes). Imaging was performed on a fluorescent microscope under restricted light conditions (Nikon Diaphot 300). Fluorescence photomicrographs, including Hoecsht 33342, were acquired using the appropriate filter sets and an adapted digital camera (Nikon).

Acknowledgements

We thank the MRC for a studentship to C. E. B., the EPSRC for a DTA studentship to J. H. B., Durham County Council for a studentship to V. B. C., ReInnervate Ltd, North East England Stem Cell Institute (NESCI), One NorthEast and Durham University for support, J. Dostal for carrying out elemental analyses, and the EPSRC Mass Spectrometry Service at the University of Wales, Swansea. We also thank Dr A. Beeby (DU) for spectrophotometric measurements.

References

1 (*a*) V. B. Christie, T. B. Marder, A. Whiting and S. A. Przyborski, *Mini-Rev. Med. Chem.*, 2008, **8**, 601–608; (*b*) M. Maden, *Nat. Rev. Neurosci.*, 2007, **8**, 755–765.
2 L. Altucci and H. Gronemyer, *Nat. Rev. Cancer*, 2001, **1**, 181–193.
3 N. P. Mongan and L. J. Gudas, *Differentiation*, 2007, **75**, 853–870.
4 (*a*) A. Murayama, T. Suzuki and M. Matsui, *J. Nutr. Sci. Vitaminol.*, 1997, **43**, 167–176; (*b*) D. K. Bempong, I. L. Honigberg and M. N. Meltzer, *J. Pharm. Biomed. Anal.*, 1995, **13**, 285–291; (*c*) T. Suzuki, S. Rao Kunchala, M. Matsui and A. Murayama, *J. Nutr. Sci. Vitaminol.*, 1998, **43**, 729–736; (*d*) S. Rao Kunchala, T. Suzuki and A. Murayama, *Ind. J. Biochem. Biophys.*, 2000, **37**, 71–76.

5 (*a*) G. Y. Han, B. S. Chang, M. J. Connor and N. Sidell, *Differentiation*, 1995, **59**, 61–69; (*b*) M. Lansink, A. M. van Bennekum, W. S. Blaner and T. Kooistra, *Eur. J. Biochem.*, 1997, **247**, 596–604; (*c*) H. Han, J. Kwon, M. Park, S. Park, S. K. Cho, Y. Rho, J. Kim, H. Sin and S. Um, *Bioorg. Med. Chem.*, 2003, **11**, 3839–3845.
6 R. Alvarez, M. J. Vega, S. Kammerer, A. Rossin, P. Germain, H. Gronemeyerb and A. R. Lera, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 6117–6122.
7 (*a*) F. Mavilio, A. Simeone, E. Boncinelli and P. W. Andrews, *Differentiation*, 1988, **37**, 73–79; (*b*) A. Simeone, D. Acampora, L. Arcioni, P. W. Andrews, E. Boncinelli and F. Mavilio, *Nature*, 1990, **346**, 763–766.
8 (*a*) P. Venepally, L. G. Reddy and B. P. Sani, *Arch. Biochem. Biophys.*, 1997, **343**, 234–248; (*b*) R. W. Curley and J. W. Fowble, *Photochem. Photobiol.*, 1988, **47**, 831–835.
9 (*a*) S. A. Przyborski, *Stem Cells*, 2001, **19**, 500–504; (*b*) S. A. Przyborski, V. B. Christie, M. W. Hayman, R. Stewart and G. M. Horrocks, *Stem Cells Dev.*, 2004, **13**, 400–408.
10 H. A. Bruson and J. W. Kroeger, *J. Am. Chem. Soc.*, 1940, **62**, 36–44.
11 C. J. Gambone, J. M. Hutcheson, J. L. Gabriel, R. L. Beard, R. A. S. Chandraratna, K. J. Soprano and D. R. Soprano, *Mol. Pharmacol.*, 2002, **61**, 334–342.
12 (*a*) G. Garipova, A. Gautier and S. R. Piettre, *Tetrahedron Lett.*, 2005, **61**, 4755–4759; (*b*) H. Kagechika, E. Kawachi, Y. Hashimoto, T. Himi and K. Shudo, *J. Med. Chem.*, 1988, **31**, 2182–2192.
13 (*a*) M. Ebisawa, K. Ohta, E. Kawachi, H. Fukasawa, Y. Hashimoto and H. Kagechika, *Chem. Pharm. Bull.*, 2001, **49**, 501–503; (*b*) V. Vuligonda, S. M. Thacher and R. A. S. Chandraratna, *J. Med. Chem.*, 2001, **44**, 2298–2303.
14 H. Suzuki, *Org. Synth.*, 1971, **51**, 94–96.
15 (*a*) S. A. Przyborski, I. E. Morton, A. Wood and P. W. Andrews, *Eur. J. Neurosci.*, 2000, **12**, 3521–3528; (*b*) R. Stewart, V. Christie and S. A. Przyborski, *Stem Cells*, 2003, **21**, 248–256.
16 H. H. Wuest, F. F. Frickel and A. Nuerrenbach (BASF A.-G., Fed. Rep. Ger.), *Ger. Offen.* CODEN: GWXXBX DE 3434946 A1 19860403, 1986, 23 pp.; *Pat. Appl.* DE 84-3434946 19840922; *Chem. Abstr.*, **105**, 172069.