Journal Name

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Cite this: DOI: 10.1039/c0xx00000x

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Synthesis and applications of 2,4-disubstituted thiazole derivatives as small molecule modulators of cellular development

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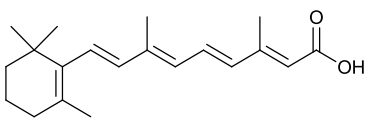
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Understanding how the structure of molecules relates to their function and biological activity is essential in the development of new analogues with targeted activity. This is especially relevant in mediating developmental processes in mammalian cells and the regulation of stem cell differentiation. In this study, thiazole-containing small molecules were synthesised and investigated for their ability to induce the differentiation of human pluripotent stem cells and their derivatives. Analyses of cell morphology, cell viability, expression of cell surface markers and ability to induce cell differentiation and regulate neurite formation identified the analogue with the longest and most bulky hydrophobic side chain as possessing comparable or enhanced activity to all-*trans*-retinoic acid (ATRA). Interestingly, a shorter, less bulky, known thiazole compound reported to be isoform selective for the retinoic acid receptor β2 (RARβ2) agonist did not mediate differentiation under the conditions tested, however, activity could be restored by adjusting the structure to a longer, more bulky molecule. These data provide further insight into the complexity of compound design in terms of developing small molecules with specific biological activities to control the development and differentiation of mammalian cells.

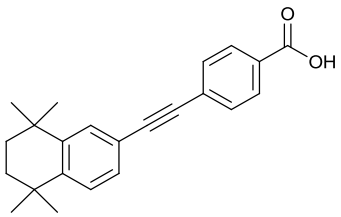
Introduction

Retinoids are a class of signalling molecules that include vitamin A along with its natural and synthetic analogues. These small molecules are involved in regulating important biological pathways from embryogenesis through to adult homeostasis, and influence the proliferation and differentiation of a diverse range of cell types, including those of the nervous system.[1] All-*trans*-retinoic acid (ATRA) in particular is the major metabolite of vitamin A; however, due to the presence of five conjugated double bonds, it is susceptible to photo-isomerisation into different retinoic acid isomers. In cell culture studies such isomers were found to induce a variety of different cellular effects compared to the use of ATRA.[2] The propensity for natural retinoids to isomerise or be removed by degradation and/or metabolism potentially makes routine handling of these small molecules difficult in the laboratory.

Synthetic retinoids have been utilised with great success in probing the cellular effects of retinoids while avoiding these issues.[3] In particular, synthetic retinoid EC23, and its sila-analogue, contain a triple bond linker region that prevents isomerisation and has been shown to be more stable and hence, more potent compared with ATRA.[4] Indeed, enhanced differentiation of human pluripotent TERA2.cl.SP12 embryonal carcinoma (EC) stem cells and ReNcell 197VM neural progenitor cells has been observed during treatment with EC23.[4c]



ATRA



EC23

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† Electronic Supplementary Information (ESI) available, see DOI: 10.1039/b000000x/

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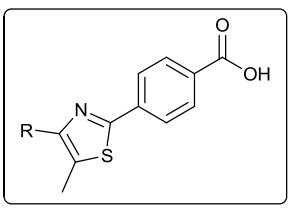
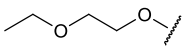
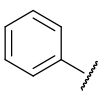
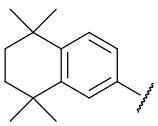
The effects of retinoids are mediated primarily through binding to, and activation of, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) which are members of the ligand-dependent transcription factor superfamily of nuclear receptors.[5] Three subtypes of RARs exist: α, β and γ; all of which have been studied extensively in the F9 murine embryonal carcinoma stem cell line, and have provided important insights into the different roles of each subtype.[6] While ATRA and related synthetic retinoids such as EC23 are pan-agonists for the RARs and are useful general inducers of stem cell differentiation, more attention is now being focused on the synthesis of subtype-selective ligands that would facilitate further studies into the function of these receptors. However, this is a complicated endeavour due to the highly similar nature of the RAR ligand binding domains (LBDs): the domain of RARβ differs from that of RARα and RARγ by one and two residues respectively. Nevertheless, small molecules exhibiting subtype-selective activities have been discovered via high throughput screening assays.[7] A 3D comparison of the RAR LBDs found that the RARβ binding site was significantly larger due to the presence of an additional cavity between H5 and H10 caused by the position of the I_{263} side chain.[8] It may be postulated, therefore, that ligands with larger side-chains are able to occupy the additional space within the RARβ retinoid binding site and hence, may acquire selectivity for this subtype.



1 R = 

2 R = 

3 R = 

Differential promoter usage and alternative splicing produce the different isoforms observed with each RAR subtype; the RARβ gene has four isoforms: β1-4. RARβ2 is the most abundant isoform and is of particular interest in the area of neuroscience research. In one study, up regulation of RARβ2 was observed in both embryonic and adult mouse dorsal root ganglia (DRG) neurons exposed to ATRA, along with a corresponding stimulation of neurite outgrowth.[9] In models of nervous system injury, overexpression of RARβ2 by lentiviral vectors in adult DRG or corticospinal tract neurons resulted in axonal outgrowth and functional recovery.[10] It would appear, therefore, that RARβ2 is the crucial transducer of the retinoic acid signal in neurons, and ligands selectively targeting this isoform would greatly benefit studies exploring conditions associated with an absence or lack of function of this receptor. However, the design of isoform-selective agonists represents an even more challenging prospect since isoforms of a particular RAR subtype possess identical ligand-binding domains, and differences lie entirely within the ligand independent *N*-activation domain.[11]

Despite these difficulties, agonists for RARβ2 have been identified using a high throughput screening assay of a chemical library, namely receptor selection and amplification technology (R-SAT).[12] Alkoxythiazole **1** was reported to be such a compound, displaying 78% of the activity for RARβ2 compared to the reference compound, AM-580. Thus, in order to explore any relationship between isoform-selectively and the inducement of cellular effects, **1** was synthesised along with two novel derivatives containing more bulky phenyl (**2**) and 1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthyl (**3**) side-chains with the aim of performing in vitro assays on cell differentiation and neural development to observe any changes in cell growth and morphology.

Results and Discussion

Synthesis of small molecules

Synthesis of the reported RARβ2-selective agonist **1** was based on the literature procedure,[12] broadly as outlined in Scheme 1. Ethyl 4-cyanobenzoate was heated with equimolar equivalents of 2-mercaptopropionic acid and pyridine under microwave conditions to provide the desired hydroxythiazole **5** after recrystallisation. However, despite TLC and MS analyses indicating the presence of only one product, two species were observed by ^{1}H NMR spectroscopy and were found to be inseparable. The overlapping ethyl signals and an additional doublet and quartet peak in the alkyl region of the spectrum were deduced to be consistent with the keto-tautomer **5b** (Scheme 2), the occurrence of which was not reported previously and constituted a significant proportion of the mixture (approximately 15%).

The thiazole moiety is present in naturally occurring molecules possessing important antibiotic,[13] antitumour[14] and immunosuppressive[15] properties. In particular, 4-hydroxy-1,3-thiazoles are known as active inhibitors of 5-lipoxygenase[16] and CDK5[17] and exist in different tautomeric forms. The enol-form is favoured in polar solvents and the keto-form in nonpolar solvents.[18] The lower energy of the aromatic 4-hydroxythiazole structure, as well as the presence of an aromatic substituent at the 2-position, is likely to account for the preferred enol form in this case.[19] Alkylation of the mixture of **5a** and **5b** would be expected to 'lock' the product in the aromatic form; indeed, after reaction with 2-bromoethyl ethyl ether to give **6**, loss of signals associated with the keto-tautomer was observed by ^{1}H NMR analysis of the product. Following a basic hydrolysis, alkoxythiazole **1** was obtained in 56% yield after recrystallisation.

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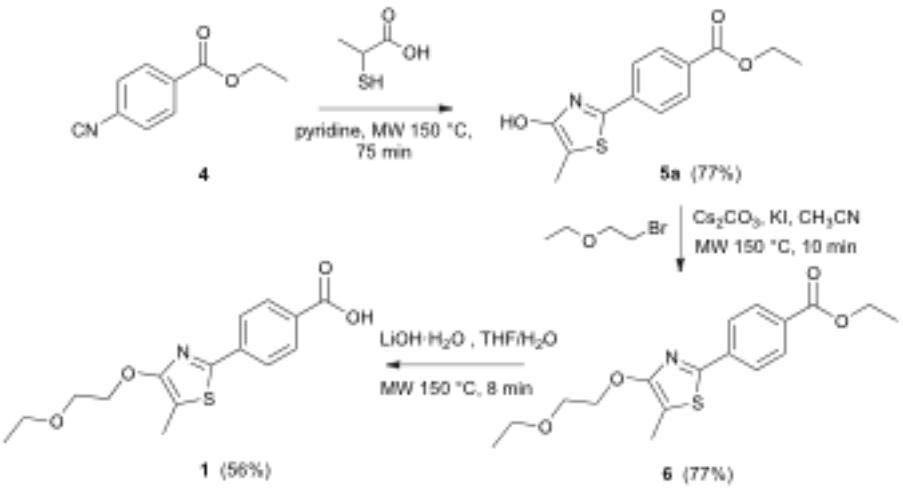

Scheme 1 Synthesis of alkoxythiazole **1**.

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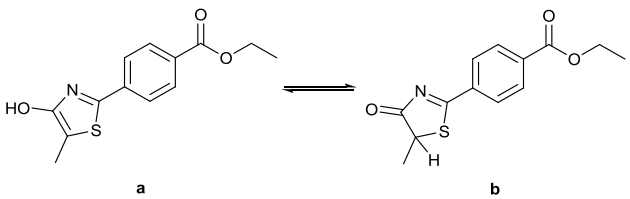
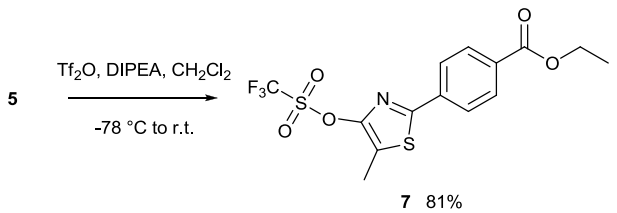


Scheme 2 Keto-enol tautomerism of **5a**.



Equation 1 Synthesis of triflate **7**.

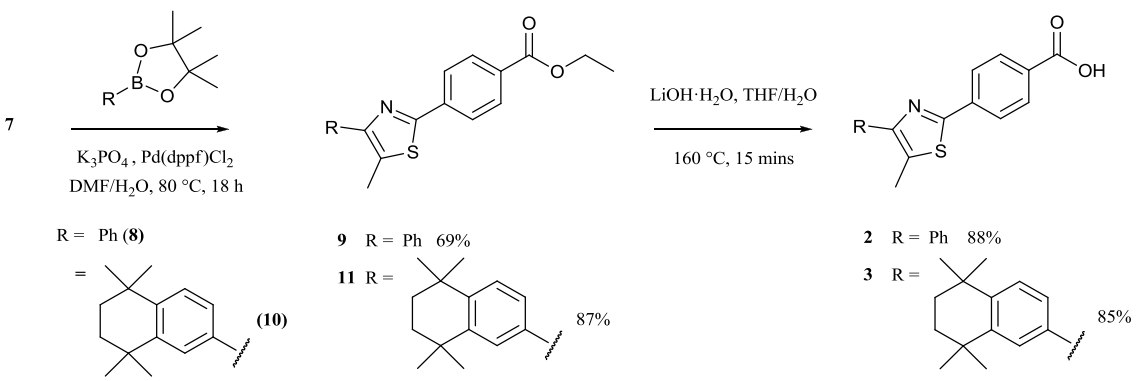
In order to enable further functionalisation at the 4-position by means of cross-coupling reactions, and hence, access different analogues with more bulky side-chains and of different overall lengths, a triflation reaction was performed with the isolated keto-enol mixture **5**. Procedures for the conversion of hydroxythiazoles to triflates are known[20] and have been employed in the derivatisation of these compounds into biologically useful agents.[21]

Initially, the Comins' reagent was used as the triflating agent; however, after stirring with **5** for 6 hours in tetrahydrofuran, TLC analysis showed predominately a mixture of starting materials. The use of triflic anhydride with pyridine also did not provide the desired triflate; however, when pyridine was replaced by triethylamine, **7** was obtained in 20% yield after silica gel chromatography. The yield was improved by use of a more hindered base, diisopropylethylamine (DIPEA), which provided **7** in 81% yield after purification (Eqn. 1).

Suzuki-Miyaura cross-couplings (Scheme 3) were then performed with triflate **7** to provide different small molecules with retinoid-like structures. Commercially available 4,4,5,5-tetramethyl-2-phenyl-[1,3,2]-dioxaborolane **8** was reacted with triflate **7**, using K_3PO_4 and 3 mol% $Pd(dppf)Cl_2$ as catalyst in DMF/H_2O at 80 °C. After 18 hours reaction and purification by silica gel chromatography, 4-(5-methyl-4-phenyl-thiazol-2-yl)-benzoic acid ethyl ester **9** was isolated in 69% yield. Subsequent base hydrolysis of ester **9** by stirring with 3 equivalents of $LiOH{\cdot}H_2O$ in THF proved sluggish and low-yielding, despite the addition of excess base and applying gentle heating over several days. However, a microwave-assisted hydrolysis proved more effective, providing the desired acid **2** in 88% yield after recrystallisation (Scheme 2). Carboxylic acid **3** was prepared in an analogous manner using 4,4,5,5-tetramethyl-2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)-[1,3,2]-dioxaborolane **10** in 85% isolated yield.

Effect of thiazole retinoid-like small molecules on the differentiation of human pluripotent stem cells

Having prepared alkoxythiazole analogues **1**, **2** and **3**, they were assessed for their ability to induce the differentiation of the pluripotent human EC stem cell line, TERA2.cl.SP12. Cells of this lineage are proven models of human embryonic



Scheme 3 Preparation of thiazole derivatives **2** and **3** via Suzuki-Miyaura cross coupling reactions.

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development[22] and have been used to study neural differentiation.[23] Cultures of TERA2.cl.SP12 cells were incubated with compounds **1**, **2** and **3,** supplemented in the culture media to a final concentration of 10 µM for up to 14 days.

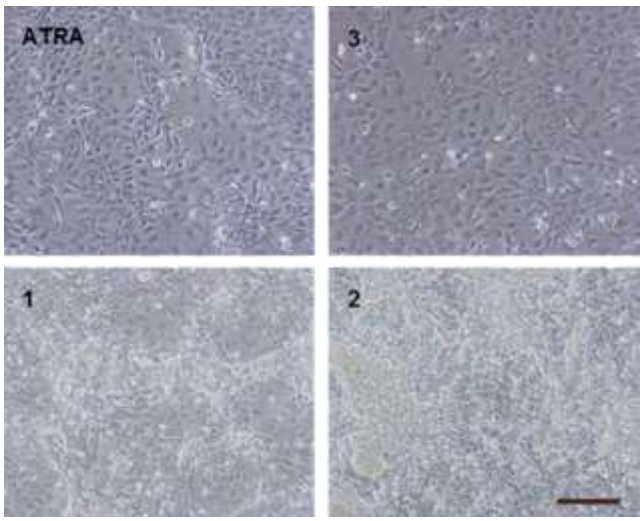

Fig. 1 Morphological appearance of TERA2.cl.SP12 human pluripotent stem cells exposed to 10 µM of **ATRA**, **1**, **2** and **3** for 7 days. Note the similar appearance of cells treated with ATRA and **3**, and the high cell numbers in cultures treated with **2** and **3**, indicating cells continuing to proliferate rather than committing to differentiation. Scale bar represents 500 µm.

The effects of the test compounds on stem cell differentiation were compared with the effect of the natural retinoid ATRA, which acted as a positive control. Hence, cultures consisting of cells treated with 10 µM ATRA in the culture media were also established and incubated alongside each other. Further cultures were set up and supplemented with the loading vehicle DMSO to act as a negative differentiation control. The latter were processed for analysis after 3 days. In order to minimise ATRA degradation and isomerisation, all culture flasks were handled under reduced light conditions. For reproducibility of results, cell cultures for each compound were set up in triplicate for each time point and incubated for over 3, 7 and 14 days.

After 7 days, it became apparent that cell proliferation in cultures exposed to compound **3** had slowed considerably, which is consistent with cells committing to differentiate in response to ATRA. In contrast, cells treated with compounds **1** and **2** continued to proliferate, and very high cell numbers were observed (Fig. 1), which is a clear indication that these molecules were unable to induce cell differentiation and arrest cell proliferation at the concentration used. The appearance of the cells after 14 days followed a similar trend, i.e. cell proliferation slowed in response to ATRA and compound **3**, whereas cultures exposed to compounds **1** and **2** became over-confluent due to continued cell proliferation.

After incubating for 7 days in compound-supplemented media, cells were prepared for an assay of cell viability by combining culture media containing any potentially detached or dead cells with trypsinised live cells from each experimental condition. The resulting cell solution from each flask was centrifuged then re-suspended in 0.1% BSA solution to enable cell counting. Cells from cultures exposed to ATRA, **1**, **2** and **3** were diluted in a staining solution and the proportion of live (intact cytoplasmic membranes excluded the dye) to dead (compromised cell membranes stained positive for the dye) cells was determined using a Viacount assay on a Guava EasyCyte cytometer. The number of viable cells, percentage viability, and total cell counts were then recorded (Fig. 2).

Continued cell proliferation in cultures treated with compounds **1** and **2** was reflected in the high cell count and number of viable cells, whereas with ATRA and compound **3** these values were lower as cells exit the cell cycle and commit to differentiation. However, cells exposed to compounds **1** and **2** showed lower percentage viability than with cultures treated with ATRA and compound **3** which is consistent with sub-optimal culture conditions due to high cell numbers and over cell proliferation. The similarity of cell viability values obtained for the ATRA and compound **3** treated cultures supports the earlier observation from cell morphology that these compounds act in a comparable fashion when exposed to this cell line.

In order to quantify the effects of the thiazole-containing compounds on inducing differentiation of the TERA2.cl.SP12 cell line, cells were analysed for expression of known markers for stem cell and differentiated cell phenotypes. Cells from each experimental condition were incubated with antibodies for the stem cell antigens SSEA-3 (globoseries stage specific embryonic antigen-3) and TRA-1-60 (a keratin-sulphate-associated glycoprotein stem surface marker). Following this, incubation with a secondary fluorescent antibody allowed the proportion of cells expressing the marker to be determined through flow cytometry (Fig. 3).

After 3 days, expression of SSEA-3 and TRA-1-60 in cultures

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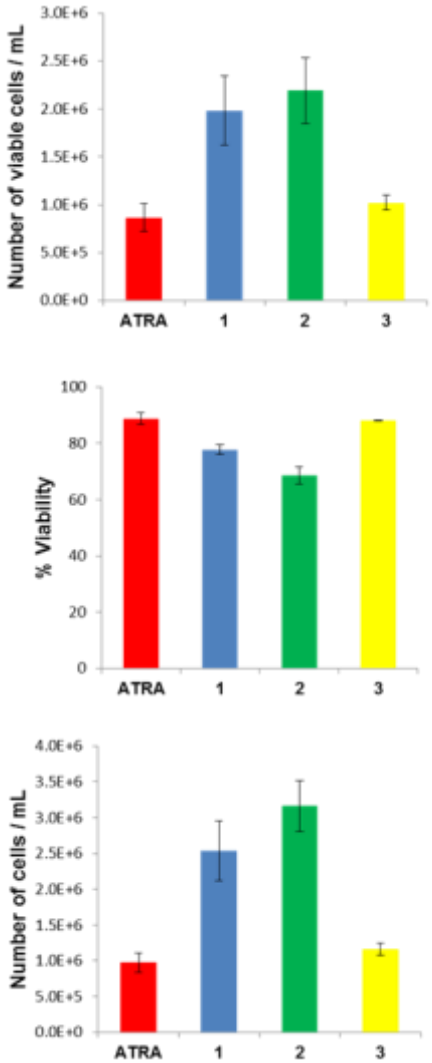

Fig. 2 TERA2.cl.SP12 EC stem cells incubated for 7 days in culture media supplemented with 10 μM of **ATRA**, **1**, **2** or **3**. The number of viable cells, percentage viability and total cell counts were determined using a Viacount assay and represented in graphical format (for reproducibility, results are the average of triplicate assays ± SEM for each culture condition, n=3).

treated with ATRA and compound **3** began to decrease compared to the DMSO negative control. This was especially clear for SSEA-3 where levels decreased by approximately 55% and 42% in the ATRA and compound **3** cultures, respectively. These data provide a strong indication of TERA2.cl.SP12 stem cells committing to differentiate in response to these compounds. However, no significant change in expression of these stem cell markers was observed for cells exposed to compounds **1** and **2**, suggesting that such cells retain a more stem cell-like phenotype. After 7 days, expression

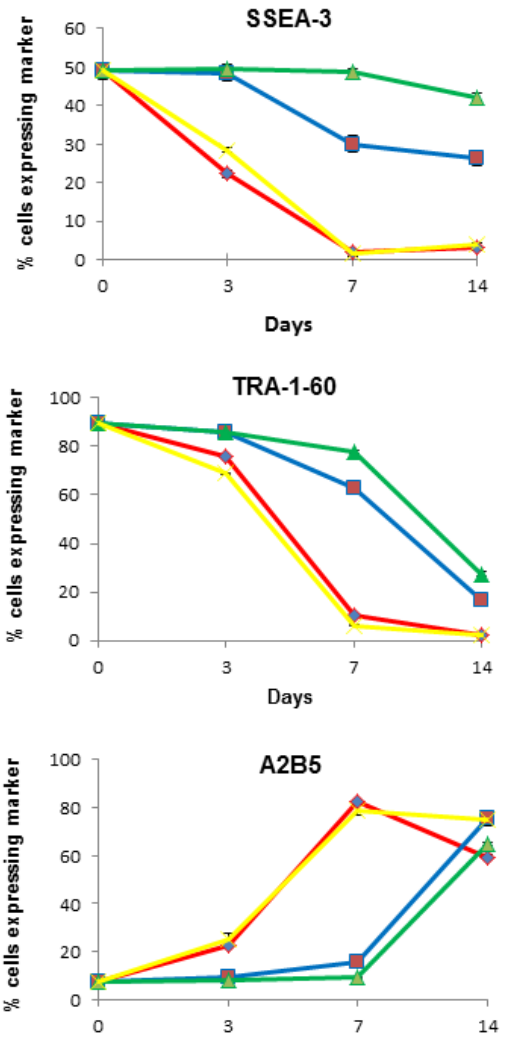

Fig. 3 TERA2.cl.SP12 EC cells exposed to 10 μM of **ATRA**, **1**, **2** and **3** for 14 days and analysed for expression of stem cell markers (SSEA-3 and TRA-1-60) and a neural marker (A2B5). Expression of SSEA-3 and TRA-1-60 decreased significantly with ATRA and **3**, with a corresponding increase in A2B5 expression. Conversely, a high level of stem cell character was observed for **1** and **2**, with minimal A2B5 expression, indicating that these analogues do not induce differentiation. After 14 days, spontaneous differentiation of cell cultures exposed to **1** and **2** may account for the decrease in SSEA-3 and TRA-1-60 levels, along with the high expression of A2B5. (For reproducibility, results are the average of triplicate assays ± SEM for each culture condition, n=3)

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of SSEA-3 and TRA-1-60 in ATRA and compound **3** treated cultures was greatly reduced (Fig. 3), while a significant decrease in expression was also observed for **1** (approximately 40% and 30% reduction in expression of SSEA-3 and TRA-1-60, respectively). After 14 days, expression of the stem cell antigens in the ATRA and compound **3** treated cultures remained minimal; however, it was surprising to note at this point the dramatic reduction in TRA-1-60 expression for cultures treated with compounds **1** and **2**, indicating that differentiating cells may be present.

Since TERA2.cl.SP12 cells are known to form neurons in response to exposure to ATRA,[20] expression of the antigen A2B5 (ganglioseries antigen marking early-stage neural cells), which is associated with differentiating neural cell types, was also monitored by flow cytometry. As expected, A2B5 expression increased in response to ATRA over the course of the experiment. A very similar pattern of expression was recorded in cultures treated with compound **3**. This suggests that compound **3** is capable of inducing differentiation of TERA2.cl.SP12 cells, and has a comparable level of activity to ATRA. After 7 days, no significant increase in antigen expression was observed in response to compounds **1** and **2**, yet by day 14, an elevated A2B5 expression was recorded that was similar to the levels observed with ATRA and compound **3**. It would appear that differentiated cell types were present in these cultures; however, further investigation is required to determine if this was the result of spontaneous differentiation in the cultures or sub-optimal growth conditions rather than the effects induced by these compounds. Nevertheless, given the length of time required before a change in cell phenotype was observed, it is unlikely that compounds **1** and **2** behave as true inducers of differentiation.

Effect of a thiazole retinoid-like small molecule on neurite outgrowth from differentiated human pluripotent stem cells

As the thiazole retinoid-like small molecule, compound **3** was shown to induce neural commitment by flow cytometric analysis, it was hypothesised that these differentiated stem cells would subsequently form neurites. To assess neurite outgrowth a human model of neurite formation was used. Suspension aggregates of the human pluripotent stem cell TERA2.cl.SP12 were differentiated with 0.1 µM EC23, ATRA or compound **3** for 21 days by media supplementation, to induce neural commitment. EC23 is a photo stable synthetic retinoid that has been found to exhibit a higher level of activity towards the induction of neural differentiation than ATRA.[4,25] After 21 days, neurospheres from each treatment group were placed on laminin and poly-D-lysine coated substrates to induce neurite formation. Neurospheres were maintained for 10 days prior to analysis.

To visualise and quantify neurite outgrowth from each cell aggregate, immunocytochemical analysis of the neuronal marker β-III-tubulin was performed. β-III-tubulin staining showed the formation of many individual neurites projecting from the central neurosphere differentiated either with compound **3** or EC23. Quantification of neurite number showed a significant enhancement of neuritogenesis in stem cells differentiated by compound **3** or EC23 compared to ATRA or the undifferentiated control (Fig. 4).

The ability of EC23 or compound **3** to enhance neurite outgrowth in this model is likely due to the stability of these compounds. Increased stability may increase retinoic acid receptor activation and the reduction in metabolite formation can result in loss of heterogeneous biological effects. While no neurites formed in the undifferentiated group, the stem cells stopped proliferating and TUJ-1 negative cells migrated from the neurosphere, indicating that retinoic acid receptor activation is essential and sufficient for neural differentiation in this model.

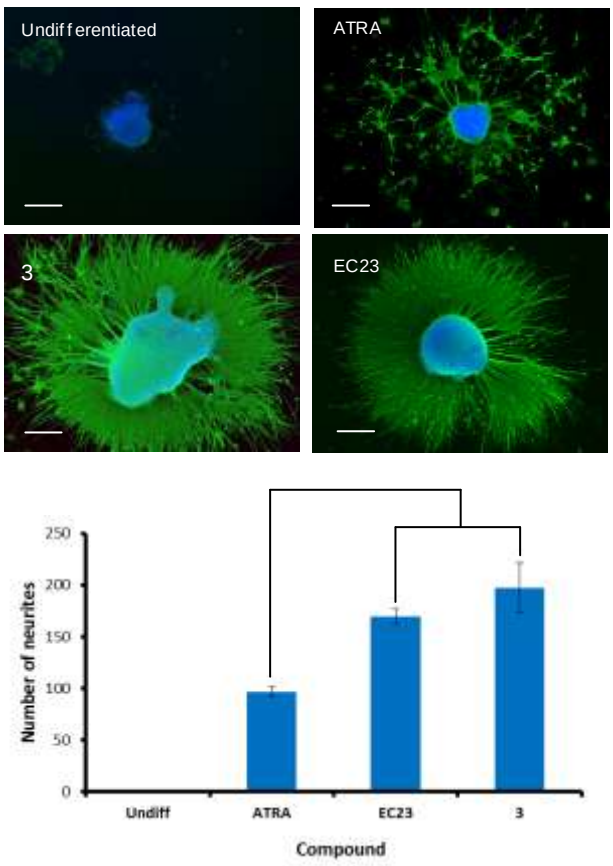

Fig. 4 Neurite outgrowth from differentiated TERA2.cl.SP12 EC stem cells. Cell aggregates were differentiated with 0.1 µM EC23, ATRA or **3** for 21 days. Differentiated aggregates were placed onto a laminin and poly-D-lysine substrate for 10 days to allow neurites to form. Neurites were stained with the pan-neuronal marker β-III-tubulin (green) and cell bodies were stained with DAPI (blue). Results are triplicate. Error bars ± SEM *p<0.05

Effect of an RARβ2 agonist on the differentiation of human neuroprogenitor stem cells

As reported above, the RARβ2 agonist, compound **1**, was found to be largely ineffective in its ability to induce the differentiation of EC stem cells. We therefore also assessed its ability to modulate the differentiation of the neuroprogenitor cell line, ReNcell 197VM. Stock cultures of these progenitor cells were grown and expanded according to previously described methods.[24] Once the cells had reached approximately 75% confluency, experimental conditions were set up involving the withdrawal of growth factors from the culture media and the incorporation of either compound **1**, ATRA or EC23 at a final concentration of 1 μM. Undifferentiated (growth media supplemented with FGF and EGF) and differentiated (without the addition of growth factors) control cultures were also established to enable comparison of results. Cultures were maintained for 7 days after which phase contrast micrographs were taken and showed that as expected, the undifferentiated control cultures continued to proliferate and became over-confluent. In contrast, the control differentiation cultures ceased to proliferate and by day 7 appeared as zones of proliferative cells surrounded by less populated areas of neural-type cells. Cells exposed to compound **1** appeared similar to the control differentiation cultures, while cells treated with ATRA or EC23 displayed predominately neuronal morphology compared to the control cultures.

Immunocytochemical staining was then performed to record the expression of the pan-neuronal marker β-III-tubulin and the marker of mature neurons, NF-200 (Fig. 5). Quantification of positively stained cells for β-III-tubulin and NF-200 showed that, compared to the differentiated control cultures, compound **1** did not induce significant neuronal differentiation (Fig. 6). Control differentiation cultures without retinoid supplementation stained for the general neuronal marker β-III-tubulin and showed minimal levels of NF-200 expression, which is indicative of an immature neuronal phenotype. In contrast, cultures exposed to ATRA displayed an approximately 1.5-fold increase in cells staining positive for β-III-tubulin along with a 3-fold increase in those staining positive for NF-200. Cells treated with EC23 displayed a higher level of neuronal morphology with a 2.25-fold and 5-fold increase in β-III-tubulin- and NF-200-positive cells, respectively. The increase in expression of both neuronal markers in response to ATRA and EC23 reflects the formation of more mature neurons and is consistent with previous observations.[4b]

A likely explanation for the apparent greater potency of EC23 over ATRA may lie in its superior stability[4] and potential lack of metabolism. The result of this is a higher effective concentration of EC23 during incubation with cells under conditions where ATRA would be expected to both degrade and be metabolised. In addition, EC23 may provide stronger interactions with retinoid receptors than the natural ligands. Although EC23 is a potent inducer of neuronal differentiation, the mechanism through which this synthetic retinoid mediates its intracellular effects remains to be fully elucidated.

Conclusions

The experimental evidence demonstrates clearly that compound **3** is able to induce differentiation of the human TERA2.cl.SP12 embryonal carcinoma stem cells on a level comparable to that of the natural retinoid ATRA. Furthermore, at 0.1 μM, **3** demonstrates enhanced neural commitment and neurite outgrowth over ATRA with levels that are comparable to the potent stable synthetic retinoid EC23. Further characterisation is required to determine whether compound **3** demonstrates specificity for a particular retinoic acid receptor subtype, and if this may contribute toward the positive results observed.

The shorter, less bulky, thiazole containing compound **1** has been previously classified as a RARβ2 agonist. However, this molecule showed limited ability to modulate the differentiation of either the pluripotent TERA2.cl.SP12 or the neuroprogenitor ReNcell 197VM cell lines. Although RARβ2 signalling is understood to play a role in neurite outgrowth[9,10] it did not have a positive effect of neuritogenesis like ATRA or EC23 under the conditions tested. It remains possible that activation of RARβ2 is a necessary, but not sufficient, factor for neuronal differentiation by these types of human cells.

Overall these results further demonstrate how subtle modifications to the structure of small molecules, specifically the function of the bulky side chain, can have a significant effect on their biological activity. Such information about structure activity relationships will advance our ability to design new compounds developed for specific biological applications.

Acknowledgements

We are grateful to High Force Research Ltd. and the EPSRC for an Industrial CASE Award (to G.-L. Zhou).

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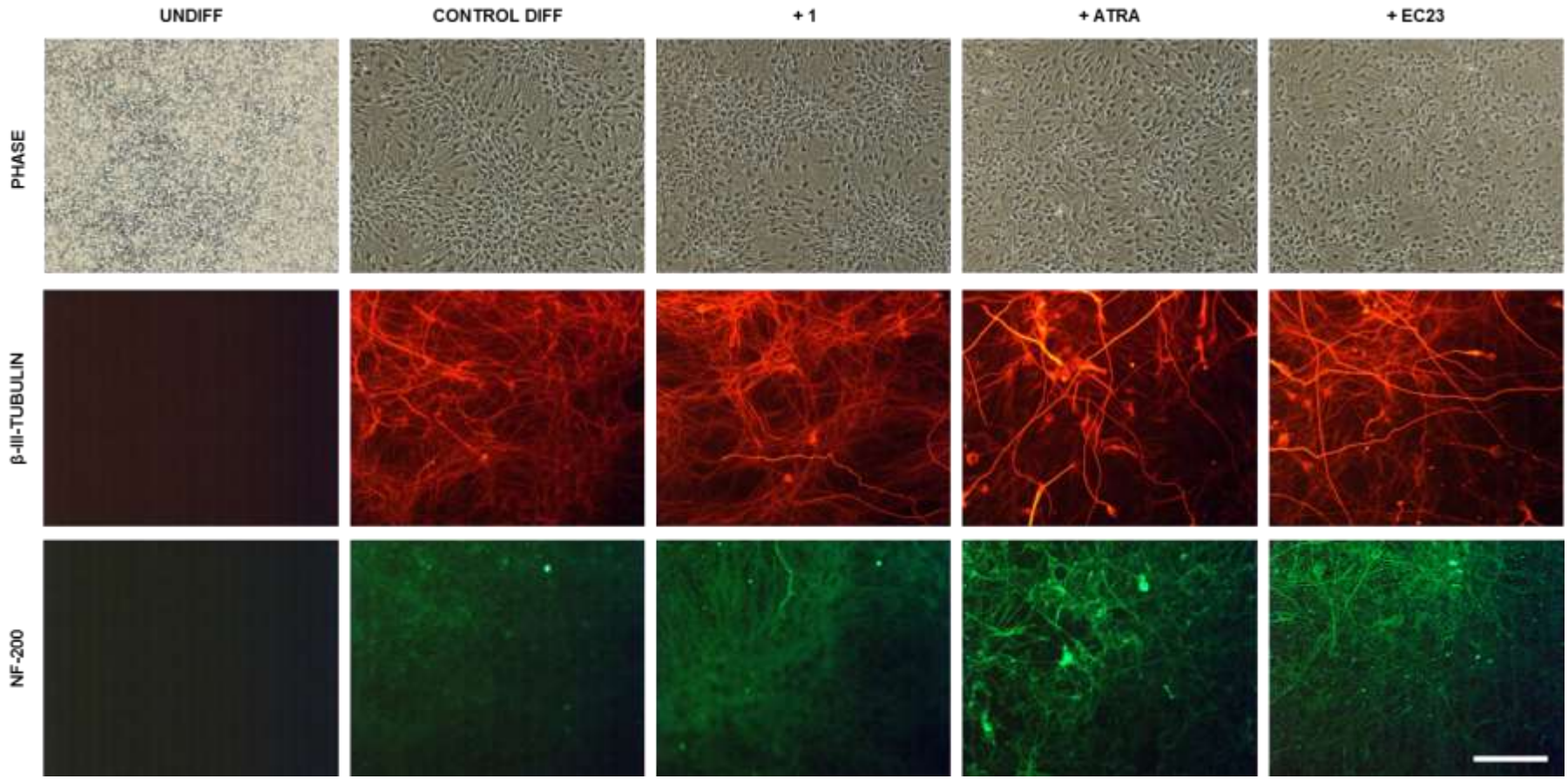

Fig. 5 Effect of supplementation of ReNcell 197VM culture media with 1 µM compound **1**, ATRA or EC23 on the induction of neural differentiation as observed by immunocytochemical staining for markers for β-III-tubulin (a general neuronal marker protein) and neurofilament protein NF-200 (a 200 KDa protein expressed in mature neurons) compared to undifferentiated (undiff) and differentiated (control diff) control cultures. Scale bar represents 100 µm.

Cite this: DOI: 10.1039/c0xx00000x

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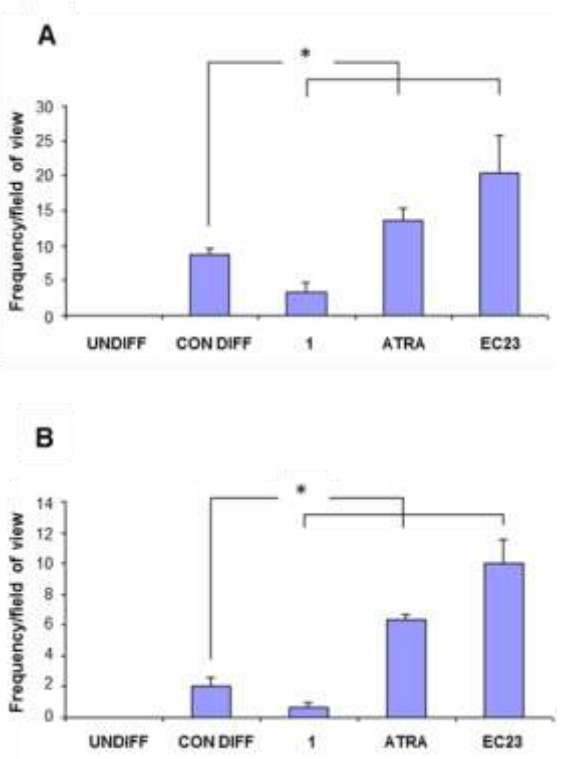

Fig. 6 Graphical representation of positive cell counts for the neuronal marker β-III-tubulin (A) and the mature neuronal marker NF-200 (B) in cultures exposed to 1 μM **1**, ATRA or EC23 compared to undifferentiated (undiff) and differentiated (con diff) control cultures. Triplicate analyses were performed for reproducibility with data representing mean ± SEM, $n = 3$, * < p=0.05.

Experimental

General experimental

Reagents were purchased from Sigma-Aldrich, Acros or Alfa-Aesar and used without further purification unless otherwise stated. Solvents were dried before use with appropriate drying agents. Where indicated, reagents were combined in an Innovative Technology Inc. nitrogen-filled (BOC) glovebox. All glassware was oven-dried (130 ˚C) prior to use. Microwave (MW)-assisted reactions were carried out in an Emrys™ Optimizer (Personal Chemistry) in septum-containing, crimp-capped, sealed vials with automatic wattage adjustment to maintain the desired temperature for a specified period of time. Reactions were monitored *in situ* by TLC, GC-MS or ^{1}H NMR spectroscopy to ensure consumption of starting materials before reaction workup. Thin layer chromatography (TLC) was performed on Polygram SIL G/UV254 plastic-backed silica gel plates with visualisation achieved using a UV lamp. Column chromatography was performed with Davisil Silica gel, 60 mesh. GC-MS was performed using an Agilent Technologies 6890 N gas chromatograph equipped with a 5973 inert mass selective detector and a 10 m fused silica capillary column (5% cross-linked phenylmethylsilicone) using the following operating conditions: injector temperature 250 ˚C, detector temperature 300 ˚C, oven temperature was ramped from 70 ˚C to 280 ˚C at 20 ˚C/min. UHP helium was used as the carrier gas. All NMR spectra were recorded on either Bruker Avance-400, Varian Mercury-400, Varian Inova-500 and Varian VNMRS 700 spectrometers at the following frequencies: ^{1}H: 200, 400, 500 and 700 MHz; ^{13}C: 176 MHz; ^{19}F: 658.4 MHz. NMR spectra were recorded in $CDCl_3$; tetramethylsilane (TMS) was used as the internal standard and spin multiplicities are indicated by the following symbols: s (singlet), d (doublet), t (triplet), m (multiplet). *J* coupling constants are given in Hz. ES-MS was performed by the Durham University departmental service using an Acquity TQD (Waters UK Ltd.) mass spectrometer and accurate mass measurements were obtained on a Thermo LTQ-FT spectrometer. Elemental analyses were carried out using an Exeter Analytical E440 machine. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer with an ATR attachment. Melting point values were measured on a Sanyo Gallenkamp apparatus and are uncorrected.

4-(4-Hydroxy-5-methyl-thiazol-2-yl)-benzoic acid ethyl ester (5)

Ethyl 4-cyanobenzoate (1.29 g, 7.4 mmol), 2-mercaptopropionic acid (0.64 mL, 7.4 mmol) and pyridine (0.59 mL, 7.4 mmol) were thoroughly mixed in a MW vial and heated at 150 °C for 75 minutes (5 x 15 min periods). The resulting yellow solid was dissolved in ethyl acetate (60 mL). Undissolved material was removed by filtration, and recrystallisation from ethyl acetate produced yellow crystals that were washed with acetonitrile to provide **5** as a yellow crystalline solid (1.50 g, 77%); mp 203-205 °C; ν_{max} (neat, cm^{-1}) 2980 (C-H), 1709 (C=O), 1580 (C=C), 1510 (C=C), 1470 (C=C), 1271 (C-O), 1100 (C-O); [**a:** δ_H (400 MHz, $CDCl_3$) 8.11 (2H, unsymmet. d, *J* 8.4 Hz, Ar), 7.88 (2H, unsymmet. d, *J* 8.4 Hz, Ar), 4.40 (2H, q, *J* 7.2 Hz, CH_2), 2.36 (3H, s, CH_3), 1.42 (3H, t, *J* 7.2 Hz, CH_3); δ_C (176 MHz, $CDCl_3$) 166.0, 159.1, 158.7, 136.5, 131.1, 130.3, 125.4, 105.6, 61.2, 14.3, 9.4 and **b:** δ_H (400 MHz, $CDCl_3$) 8.19-8.13 (4H, m, Ar), 4.41 (2H, q, *J* 7.2 Hz, CH_2), 4.29 (1H, q, *J* 7.6 Hz, CH), 1.76 (3H, d, *J* 7.6 Hz, CH_3), 1.43 (3H, t, *J* 7.2 Hz, CH_3); δ_C (176 MHz, $CDCl_3$) 193.8, 165.3, 159.1, 135.9, 135.5, 130.0, 128.6, 61.7, 49.4, 18.1, 14.2]; *m/z* (ESI) 264 (M + H); λ_{max} (EtOH) 244 nm (ε 4 950 M^{-1} cm^{-1}), 356 (5 000); Anal. calcd. for $C_{13}H_{13}NO_3S$: C, 59.30; H, 4.98; N, 5.32; found C, 58.99; H, 4.97; N, 5.34.

4-[4-(2-Ethoxy-ethoxy)-5-methyl-thiazol-2-yl]-benzoic acid ethyl ester (6)

Compound **5** (197 mg, 0.75 mmol), 2-bromoethyl ethyl ether (255 µL, 2.26 mmol), Cs_2CO_3 (268 mg, 0.83 mmol), KI (373 mg, 2.23 mmol) and CH_3CN (3.75 mL) were mixed in a MW vial and irradiated at 150 °C for 10 min. The filtrate was extracted with ethyl acetate (20 mL) and washed with brine (3 x 30 mL). The organic phase was dried with Na_2SO_4, filtered and concentrated onto Celite. Purification of the residue by flash chromatography produced **6** as a viscous yellow oil (193 mg, 77%); ν_{max} (neat, cm^{-1}) 2976 (C-H), 1715 (C=O), 1273 (C-O), 1106 (C-O); δ_H (400 MHz, $CDCl_3$) 8.05 (2H, unsymmet d, *J* 8.4 Hz, Ar), 7.89 (2H, unsymmet d, *J* 8.4 Hz, Ar), 4.54-4.50 (2H, m, CH_2), 4.40 (2H, q, *J* 7.2 Hz, CH_2), 3.81-3.77 (2H, m, CH_2), 3.61 (2H, q, *J* 6.8 Hz, CH_2), 2.33 (3H, s, CH_3), 1.41 (3H, t, *J* 7.2 Hz, CH_3), 1.24 (3H, t, *J* 6.8 Hz, CH_3); δ_C (176 MHz, $CDCl_3$) 166.1, 160.1, 157.7, 137.7, 130.7, 130.1, 125.0, 108.9, 69.8, 69.2, 66.6, 61.1, 15.2, 14.3, 9.4; *m/z* (ESI) 336 (M + H); λ_{max} (EtOH) 244 nm (ε 33 880 M^{-1} cm^{-1}), 352 (26 460); HRMS (ESI) calcd. for $C_{17}H_{22}NO_4S$ 335.1191 (M + H), found 335.1185.

4-[4-(2-Ethoxy-ethoxy)-5-methyl-thiazol-2-yl]-benzoic acid (1)

Compound **6** (183 mg, 0.55 mmol), $LiOH{\cdot}H_2O$ (68 mg, 1.62 mmol), H_2O (825 µL) and THF (2.75 mL) were mixed in a MW vial and irradiated at 150 °C for 8 minutes. The reaction mixture was acidified with 1M HCl, extracted with ethyl acetate (3 x 20 mL) and washed with brine (3 x 60 mL). After drying with $MgSO_4$ and removal of the solvent *in vacuo* the crude product was obtained and recrystallised from ethyl acetate to give **1** as a pale yellow crystalline solid (95 mg, 56%); mp 184-185 °C; ν_{max} (neat, cm^{-1}) 2858 (C-H), 2554 (O-H), 1673 (C=O), 1606 (C=C), 1432 (C=C) 1125 (C-O); δ_H (500 MHz, $CDCl_3$) 8.11 (2H, unsymmet d, *J* 8.5 Hz, Ar), 7.93 (2H, unsymmet d, *J* 8.5 Hz, Ar), 4.55-4.51 (2H, m, CH_2), 3.82-3.78 (2H, m, CH_2), 3.61 (2H, q, *J* 7 Hz, CH_2), 2.34 (3H, s, CH_3), 1.25 (3H, t, *J* 7 Hz, CH_3); δ_C (176 MHz, $CDCl_3$) 171.1, 160.5, 157.8, 138.9, 131.1, 129.7, 125.4, 109.7, 70.2, 69.6, 67.1, 15.6, 9.9; *m/z* (ESI) 308 (M + H); λ_{max} (EtOH) 240 nm (ε 2 760 M^{-1} cm^{-1}), 348 (2 940); Anal. calcd. for $C_{15}H_{17}NO_4S$: C, 58.61; H, 5.57; N, 4.56; found C, 58.29; H, 5.43; N, 4.19.

4-(5-Methyl-4-trifluoromethanesulfonyloxy-thiazol-2-yl)-benzoic acid ethyl ester (7)

DIPEA (2.65 mL, 15.2 mmol) was added to a stirred solution of **5** (2.0 g, 7.60 mmol) in CH_2Cl_2. The flask was cooled to -78 °C after which trifluoromethanesulfonic anhydride (4.4 g, 15.2 mmol) was carefully added. The reaction was allowed to warm slowly to RT and was stirred overnight under a nitrogen atmosphere. CH_2Cl_2 and water were added and the product extracted with three portions of CH_2Cl_2. The organic extracts were combined and washed three times with water. After drying with $MgSO_4$ the solvent was removed *in vacuo* and the brown residue purified by flash chromatography to give a yellow oil, R_f 0.32 (20% EtOAc/hexanes). Recrystallisation from hexane produced **7** as an off-white crystalline solid (2.1 g, 70%); mp 48-50 °C; ν_{max} (neat, cm^{-1}) 1712 (C=O), 1422 (C=C), 1274 (C=C), 1221 (C-O), 1134 (C-F), 1093 (C-F), 768 (C-H), 693 (C-H), 602 (C-H); δ_H (700 MHz, $CDCl_3$) 8.10 (2H, unsymmet. d, *J* 8 Hz, Ar), 7.91 (2H, unsymmet. d, *J* 8 Hz, Ar), 4.41 (2H, q, *J* 7 Hz, CH_2), 2.49 (3H, s, CH_3), 1.42 (3H, t, *J* 7 Hz, CH_3); δ_C (176 MHz, $CDCl_3$) 166.1, 161.4, 148.5, 136.4, 132.6, 130.6, 125.9, 122.6, 61.7, 14.7, 10.4; δ_F (658.4 MHz, $CDCl_3$) -72.5; *m/z* (ESI) 263 (M – CF_3SO_2), 396 (M + H), 418 (M + Na); λ_{max} (EtOH) 312 nm (ε23 100 M^{-1} cm^{-1}); Anal. calcd. for $C_{14}H_{12}F_3NO_5S_2$: C, 42.53; H, 3.06; N, 3.54; found C, 42.50; H, 3.07; N, 3.48.

4-(5-Methyl-4-phenyl-thiazol-2-yl)-benzoic acid ethyl ester (9)

Compound **7** (500 mg, 1.26 mmol), phenylboronic acid pinacol ester (284 mg, 1.39 mmol, 1.1 equiv), K_3PO_4 (537 mg, 2.53 mmol) and Pd(dppf)Cl_2 (28 mg, 0.0383 mmol, 3 mol%) were combined in DMF and H_2O (10:1) in a Young's tube under an inert nitrogen atmosphere and heated at 80 °C for 18 h. The mixture was dissolved in diethyl ether and washed twice with water. The organic layer was dried with $MgSO_4$ and the solvent removed *in vacuo* to give an orange/brown residue which was purified by column chromatography (3→5 % EtOAc/hexanes) to give a white solid, R_f 0.28 (10% EtOAc/hexanes). Recrystallisation from ethanol produced **9** as a white crystalline solid (246 mg, 60 %); mp 85-87 °C; ν_{max} (neat, cm^{-1}) 1706 (C=O), 1278 (C-O), 1100 (C-O), 860 (C-H), 768 (C-H), 699 (C-H); δ_H (700 MHz, $CDCl_3$) 8.10 (2H, unsymmet d, *J* 8.4 Hz, Ar), 8.03 (2H, unsymmet. d, *J* 8.4 Hz, Ar), 7.73 (2H, dd, *J* 7.7 and 1.4 Hz, Ar), 7.47 (2H, td, *J* 7.7 and 1.4 Hz, Ar), 7.38 (1H, tt, *J* 7.7 and 1.4 Hz, Ar), 4.40 (2H, q, *J* 7 Hz, CH_2), 2.64 (3H, s, CH_3), 1.42 (3H, t, *J* 7 Hz, CH_3); δ_C (176 MHz, $CDCl_3$) 166.5, 162.6, 153.0, 137.9, 135.2, 131.5, 130.5, 130.0, 129.0, 128.8, 128.1, 126.4, 61.5, 14.7, 13.4; *m/z* (ESI) 324 (M + H), 325 (M + 2H), 670 (2M + Na); λ_{max} (EtOH) 254 nm (ε22 400 M^{-1} cm^{-1}), 330 (18 100); Anal. calcd. for $C_{19}H_{17}NO_2S$: C, 70.56; H, 5.30; N, 4.33; found C, 70.62; H, 5.32; N, 4.44.

4-[5-Methyl-4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)-thiazol-2-yl]-benzoic acid ethyl ester (11)

Compound **7** (500 mg, 1.26 mmol), **10**[26] (477 mg, 1.52 mmol, 1.2 equiv), K_3PO_4 (537 mg, 2.53 mmol) and Pd(dppf)Cl_2 (28 mg, 0.0383 mmol, 3 mol%) were combined in DMF and H_2O (10:1) in a Young's tube under an inert nitrogen atmosphere and heated at 80 °C for 18 h. The mixture was dissolved in diethyl ether and washed twice with water. The organic layer was dried with $MgSO_4$ and the solvent removed *in vacuo* to give a white residue which was purified by flash chromatography, R_f 0.33 (8 % EtOAc/hexanes). Recrystallisation from ethanol afforded **11** as a fluffy white crystalline solid (410 mg, 75%); mp 161-162 °C; ν_{max} (neat, cm^{-1}) 1707 (C=O), 1270 (C-O), 1102 (C-O), 772 (C-H); δ_H (700 MHz, $CDCl_3$) 8.09 (2H, unsymm d, *J* 8.4 Hz, Ar), 8.03 (2H, unsymm d, *J* 8.4 Hz, Ar), 7.61 (1H, d, *J* 1.4 Hz, Ar), 7.49 (1H, dd, *J* = 8.4 and 1.4 Hz, Ar), 7.40 (1H, d, *J* 8.4 Hz, Ar), 4.40 (2H, q, *J* 7 Hz, CH_2), 2.63 (3H, s, CH_3), 1.75-1.70 (4H, m, 2 x CH_2), 1.42 (3H, t, *J* 7 Hz, CH_3), 1.34 (6H, s, 2 x CH_3), 1.32 (6H, s, 2 x CH_3) ; δ_C (176 MHz, $CDCl_3$) 166.5, 162.3, 153.5, 145.1, 144.9, 138.1, 132.3, 131.4, 130.4, 129.4, 127.2, 127.1, 126.4, 126.2, 61.5, 35.5, 35.4, 34.7, 34.6, 32.3, 32.2, 14.7, 13.3; *m/z* (ESI) 434 (M + H); λ_{max} (EtOH) 262 nm (ε27 400 M^{-1} cm^{-1}), 335 (17 600); Anal. calcd. for $C_{27}H_{31}NO_2S$: C, 74.79; H, 7.21; N, 3.23; found C, 74.67; H, 7.22; N, 3.17.

4-(5-Methyl-4-phenyl-thiazol-2-yl)-benzoic acid (2)

Compound **9** (100 mg, 0.31 mmol) and $LiOH \cdot H_2O$ (39 mg, 0.93 mmol) were mixed in THF/H_2O (2 mL, 3:1 ratio) in a MW vial and irradiated at 160 °C for 15 min after which the reaction mixture was acidified with 1 M HCl and ethyl acetate was added. The organic layer was washed twice with brine and dried with $MgSO_4$. After *in vacuo* solvent removal the residue was recrystallised from ethanol to give **2** as a white crystalline solid (81 mg, 88%); mp 266-269 °C; ν_{max} (neat, cm^{-1}) 2847 (O-H), 1674 (C=O), 1284 (C-O), 858 (C-H), 771 (C-H), 700 (C-H), 681 (C-H); δ_H (500 MHz, DMSO-d_6) 8.09 (4H, s, Ar), 7.80 (2H, dd, *J* 8 and 1.5 Hz, Ar), 7.55 (2H, td, *J* 8 and 1.5 Hz, Ar), 7.46 (1H, tt, *J* 8 and 1.5 Hz, Ar), 2.68 (3H, s, CH_3); δ_C (176 MHz, $CDCl_3$) 167.7, 162.3, 152.5, 137.5, 135.3, 131.2, 131.2, 129.4, 129.2, 128.8, 126.8, 13.6; *m/z* (ESI) 294 (M - H); λ_{max} (EtOH) 255 nm (ε23 000 M^{-1} cm^{-1}), 329 (19 000); Anal. calcd. for $C_{17}H_{13}NO_2S$: C, 69.13; H, 4.44; N, 4.74; found C, 68.79; H, 4.46; N, 4.74.

4-[5-Methyl-4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)-thiazol-2-yl]-benzoic acid (3)

Compound **11** (100 mg, 0.23 mmol) and $LiOH \cdot H_2O$ (29 mg, 0.69 mmol) were mixed in THF/H_2O (2 mL, 3:1 ratio) in a MW vial and irradiated at 160 °C for 15 min after which the reaction mixture was acidified with 1 M HCl and ethyl acetate was added. The organic layer was washed twice with brine and dried with $MgSO_4$. After *in vacuo* solvent removal the residue was washed with ethanol to give **3** as a white crystalline solid (80 mg, 85%); mp 276-278 °C; ν_{max} (neat, cm^{-1}) 2929 (O-H), 1681 (C=O), 1279 (C-O), 773 (C-H); δ_H (500 MHz, DMSO-d_6) 8.12-8.05 (4H, m, Ar), 7.66 (1H, d, *J* 1.5, Ar), 7.52 (1H, dd, *J* 8.5 and 1.5, Ar), 7.48 (1H, unsymmet. d, *J* 8.5, Ar), 2.66 (3H, s, CH_3), 1.75-1.72 (4H, m, 2 x CH_2), 1.35 (6H, s, 2 x CH_3), 1.33 (6H, s, 2 x CH_3); δ_C (176 MHz, DMSO-d_6) 167.8, 162.1, 153.0, 145.3, 145.0, 137.6, 132.5, 131.2, 130.6, 127.5, 127.2, 126.8, 126.7, 35.5, 35.5, 35.0, 34.8, 32.6, 32.5, 31.7, 13.6; *m/z* (ESI) 404 (M - H); λ_{max} (EtOH) 261 nm (ε27 000 M^{-1} cm^{-1}), 333 (17 000); Anal. calcd. for $C_{25}H_{27}NO_2S$: C, 74.04; H, 6.71; N, 3.45; found: C, 73.42; H, 6.75; N, 3.53.

Tissue culture

Stock solutions of ATRA (Sigma), EC23 (Reinnervate), compounds **1**, **2** and **3** were prepared in dimethyl sulfoxide (DMSO, Sigma) to concentrations of 10 mM. Aliquots of these stock solutions were stored at -80 °C in the dark and thoroughly defrosted in a water bath set at 37 °C prior to use. Unless otherwise stated all plastic-ware was purchased from Becton, Dickinson and Company. Phase contrast images of cultures were obtained using a light microscope (Nikon Diaphot 300) and photomicrographs were captured using digital photography (Nikon).

Human pluripotent TERA2.cl.SP12 embryonal carcinoma stem cells were cultured in DMEM (Sigma) supplemented with 10% FCS (Lonza), 2 mM L-glutamine (Lonza) and 100 active units each of penicillin and streptomycin (Lonza). Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C in a Sanyo CO_2 incubator and handled under sterile conditions in a Class 1 microbiological safety cabinet. Cultures were passaged using sterile acid-washed glass beads (VWR) or trypsinised using a solution of 0.25% (w/v) trypsin (Life Technologies) and 2 mM EDTA in PBS to obtain a single-cell suspension for counting. 96-well plates were used for cell viability studies and cultures intended for flow cytometric analyses were set up in T25 flasks.

Human neural progenitor ReNcell 197VM cells (Millipore) were maintained under the laboratory conditions described above. Before establishing cell cultures, a 20 µg/mL concentration of laminin solution was applied to all plastic-ware, incubated at 37 °C for 6 h and then rinsed once with culture medium. Cells were maintained in serum-free conditions with DMEM:F12 (1:1, Gibco) supplemented with B27 (Invitrogen), 2 mM L-glutamine, gentamycin (Gibco) and 50 mg/mL heparin solution (Sigma). For proliferation 10 ng/mL fibroblast growth factor (FGF) and 20 ng/mL epidermal growth factor (EGF) were added to the culture media before applying to cells. Cells were trypsinised as described above.

Neurite outgrowth assay

TERA2.cl.SP12 EC cells were maintained as described above. Aggregates of TERA2.cl.SP12 EC cells were produced by creating a single cell suspension and adding 1.5 x 10^6 cells per 20 ml of maintenance media to a 90 mm un-treated Petri dish. The cells were left to aggregate overnight and subsequently treated with the test compound. Aggregates were cultured for 21 days in the presence of the test compound to induce neural commitment and the production of neurospheres prior to analysis of neurite outgrowth. To induce neurite outgrowth a 48 well tissue culture plate, the well was coated with a solution of 10 µg/mL laminin (Sigma) and poly-D-lysine (Sigma) overnight. The tissue culture plastic was washed 3 times with sterile PBS prior to the addition of the neurospheres. Neurospheres were added to the permissive substrate in the presence of the mitotic inhibitors; 10 µM 5-fluoro-2-deoxyuridine; 10 µM Uridine and 1 µM cytosine-arabinoside in TERA2.cl.SP12 maintenance media described previously and incubated at 37 °C 5% CO_2 for 10days. Neurospheres where then fixed in 4% PFA and visualised by immunocytochemical staining of TUJ-1 antibody. Neurites were imaged using the Nikon Diaphot 300 and counted for quantification using Image J.

Cell viability assay

Cells were assessed for viability by combining a single cell suspension (achieved by the addition of 1 mL 0.25% trypsin/EDTA solution) of live cells with detached dead cells contained in the culture medium from each flask. Cell numbers were determined using a haemocytometer. Cells were required to be diluted 1:20 in a staining solution for the Viacount assay and added to a 96-well plate. The number of viable cells, percentage viabilities and total cell numbers were recorded in triplicate for each experimental condition.

Flow cytometric analysis of pluripotent TERA2.cl.SP12 EC cells

At each time point (except for the negative control flasks, which were processed for analysis after 3 d), cultures were washed with PBS and treated with trypsin to obtain a single-cell suspension as described above. Cells were washed three times with PBS to

ensure complete removal of trypsin. After centrifugation of the cell mixture and removal of the supernatant, the cell pellet was re-suspended in 0.1% bovine serum albumin (BSA) solution and counted using a haemocytometer. 200 000 cells/well were added to a 96-well plate according to the experimental plan. After centrifugation of the plate at 1000 rpm for 3 min and removal of the supernatant, cells were re-suspended in 50 µl of the appropriate primary antibody. Each triplicate set of culture conditions (ATRA, compounds **1**, **2** and **3**, as well as the DMSO-supplemented flasks [on day 3 only]) was analysed at each time point for expression of the following three antigens: SSEA-3 (antibody diluted 1:5 in 0.1% BSA), TRA-1-60 (diluted 1:10) and A2B5 (diluted 1:40). These primary monoclonal antibodies were used as they recognise specific cell surface antigens associated with globoseries glycolipids, glycoproteins and ganglioseries, displaying highly regulated expression profiles in response to differentiation of human EC cells.[27] One well containing undifferentiated control cells was incubated with the mouse myeloma marker anti-P3X as a negative control. The plate was incubated on ice for 1 h after which excess unbound antibody was removed by addition of 100 µl of ice-cold 0.1% BSA, centrifugation as above and removal of the supernatant. Two further washings were carried out with 180 µl of 0.1% BSA, after which cells were re-suspended with the fluorescent secondary antibody FITC (fluorescein isothiocyanate-conjugated) goat anti-mouse IgM (ICN Pharmaceuticals, Inc.; Aurora, OH; http://www.icnpharm.com) diluted 1:100 in 0.1% BSA. After incubating the plate in the dark for 1 h on ice, cells were washed three times with 0.1% BSA as described above, and re-suspended in 200 µl of 0.1% BSA for analysis.

Cell surface antigen expression on TERA2.cl.SP12 stem cells and their compound-induced derivatives was observed by indirect immunofluorescence and quantified using a flow cytometer (Guava Easycyte). A fluorescence threshold was set such that cells fluorescing with a greater intensity than approximately 95% of the cells incubated with the negative control antibody P3X were counted as antigen positive.

Immunocytochemistry with neuroprogenitor ReN197 VM cells and human pluripotent TERA2.cl.SP12 EC cells

Cells were fixed in 4% paraformaldehyde (PFA) in PBS (Sigma) for 30 min at room temperature (RT) and rinsed with PBS. Cell membranes were permeabilised by treatment with 1% Triton-X-100 (Sigma) in PBS for 10 min at RT. Nonspecific labelling was blocked by incubation on a bench-top shaker (Fischer Scientific) for 1 h at RT with a solution of 1% goat serum (Sigma) containing 0.2% Tween-20 (Sigma) in PBS. Primary antibodies were diluted in blocking solution and incubated with cells for 1 h at RT [β-III tubulin antibody (TUJ1, Covance, diluted 1:600); NF-200 antibody (AbCam, diluted 1:200)]. After washing three times for 15 min with PBS cells were incubated for 1 h in the dark with FITC-conjugated (anti-mouse Alexafluor 488, Invitrogen, diluted 1:600), Cy3-conjugated (anti-rabbit Cy3, JacksonLabs, diluted 1:600) fluorescently-labelled secondary antibodies and Hoechst 33342 nuclear staining dye (Molecular Probes, diluted 1:1000) in blocking solution. Cells were washed twice more with PBS and left in the final wash for immediate imaging. Fluorescence micrographs, including Hoecsht 3342, were acquired using the appropriate filter sets and an adapted digital camera (Nikon).

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