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Identification and characterisation of small molecule ligands that maintain pluripotency of human embryonic stem cells

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human embryonic stem cells, pluripotency, phosphodiesterase, adenosine deaminase

Abbreviations

ADA, adenosine deaminase; bFGF, basic fibroblast growth factor; EHNA, *Erythro*-9-(2-hydroxy-3-nonyl)adenine; GMP, good manufacturing practise; hESC, human embryonic stem cells; PDE, Cyclic nucleotide phosphodiesterase;

Abstract

Human embryonic stem cells (hESC) offer great potential for pharmaceutical research and development and, potentially, for therapeutic use. However, improvements in cell culture are urgently required to allow the scaleable production of large numbers of cells that maintain pluripotency. Supplementing feeder-free conditions with either *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) or readily synthesised analogues of this compound maintains hESC pluripotency in the absence of exogenous cytokines. When the hESC lines SA121 or SA461 were maintained in feeder-free conditions with EHNA they displayed no reduction in stem cell-associated markers such as NANOG, OCT4 and SSEA 4 when compared to cells maintained in full feeder-free conditions that included exogenously added basic fibroblast growth factor (bFGF). Spontaneous differentiation was reversibly suppressed by the addition of EHNA, but EHNA did not limit efficient spontaneous or directed differentiation following its removal. We conclude that EHNA or related compounds offers a viable alternative to exogenous cytokine addition in maintaining hESC cultures in a pluripotent state and might be a particularly useful replacement for bFGF for large scale or good manufacturing practise-compliant processes.

hESCs originate from the inner cell mass of the developing blastocyst and can be maintained in an undifferentiated state whilst remaining capable of differentiating to all cell types in the body [1,2]. They have huge potential as both research tools and for cell therapies. Furthermore, they offer great potential to revolutionise aspects of the drug discovery process [3-5]. For toxicology and drug efficacy studies, as well as for aspects of high-throughput and high-content screens, the widespread availability of large numbers of reproducibly defined populations of human cells would accelerate progress greatly. However, with the exception of certain white cells populations there remain substantial barriers to obtaining nucleated human cells in a routine, timely and cost-effective manner. hESCs have the potential to overcome such barriers but a number of technical challenges need to be overcome before this might be achieved. These include means to sustain hESCs, preferably in feeder layer-free monolayer culture through many passages, as proliferating, undifferentiated cells that still remain pluripotent, as well as means to then induce their differentiation to cells displaying key proteomic and functional, as well as visual phenotypic, markers of freshly isolated human cell types. Furthermore, aspects of quality assurance and control must be inbuilt to such programmes to ensure reproducibility. Within such programmes, the identification of small molecule ligands that either maintain the undifferentiated state or promote coherent development either towards fully differentiated cells or to precursor populations committed to differentiate towards a specific germ layer may be of particular use as a means to replace complex and often ill-defined combinations of biological growth promoters and inhibitors derived either from supportive feeder cells or via exogenously provided admixtures.

In efforts to identify such small molecule ligands that maintain the pluripotency of hESC lines we adopted a pair of distinct strategies. The first of these,

which will not be discussed further herein, was a series of high content screens employing libraries of small molecules, many of which were already known to have biological activity within various, diverse cellular signaling cascades. The second employed a much smaller number of compounds known to target pathways already well established to modulate cellular growth and differentiation. To facilitate the high content screening programme and to link this to the more directed small molecule identification programme, initial key requirements were to ensure the viability of hESCs in feeder-free conditions and the ability to generate and maintain monolayer cultures of the cells (**Figure 1**). These were achieved for both the SA121 and SA461 hESC lines [6] , supplied by Cellartis AB (Dundee, UK), on fibronectin-coated dishes with the use of a fully supportive medium that consisted of a 1:1 mixture of defined media [7] and conditioned VitrohES (VitroLife, Gothenburg, Sweden). Under these conditions both lines could be successfully passaged 1 in 4 using trypsin with routine addition of bFGF at a final concentration of 10ng/ml. Importantly, hESCs grown in these feeder-free conditions expressed a number of undifferentiated stem cell markers, including the transcription factors OCT-4 and NANOG, as well as the cell surface proteins SSEA3, SSEA4, TRA-160 and TRA-180, but did not express a wide range of markers indicative of individual germ layer differentiation. Such a marker profile strongly indicated the maintenance of a pluripotent phenotype. Conversion to feeder-free, monolayer culture allowed scale up of cell production and the potential for automation in cell culture, both pre-requisites for the high content studies and large scale screens. Such monolayer-maintained cells are also far more suitable to assess the effectiveness of small molecule ligands in the induction of cellular differentiation circuits. By contrast, this modification failed to address the need for addition of bFGF, a biological that is extremely expensive to acquire at good

manufacturing practice (GMP) grade, a requirement for translation to any therapeutic use of hESC.

It is well established that growth and differentiation of many cell types is either modulated by or under the direct control of cyclic AMP levels [8-9]. Cyclic nucleotide phosphodiesterases (PDEs) are a large and complex family of intracellular proteins and are the key, and often the only, cellular mechanism to reduce intracellular cyclic AMP levels [8-10]. We thus sought to explore if either broad spectrum cyclic AMP PDE inhibitors or highly selective inhibitors of specific PDE subtypes might modulate hESC viability, growth or differentiation or either modulate or replace the requirement for bFGF in maintaining the pluripotency of these cells in feeder-free culture. Interestingly, *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), a previously described PDE2 inhibitor [11-13], was able to maintain expression of markers of pluripotency, in the absence of added bFGF, throughout extensive periods that involved multiple passaging steps. By contrast, in the absence of bFGF and without addition of EHNA, pluripotency markers such as OCT4 were greatly reduced and the cells displayed clearly observable signs of spontaneous differentiation. Importantly, maintenance of hESC pluripotency in the presence of EHNA did not lock cells into a state unable to differentiate upon removal of this supplement. When cells grown in the EHNA-containing medium for more than 20 passages were allowed to differentiate passively by removal of EHNA and replacing fibronectin with gelatin in monolayer cultures containing 20% foetal calf serum, within 4 weeks a number of differentiation markers including PAX6 (ectoderm), alpha-feta protein (endoderm) and smooth muscle actin (mesoderm) were readily detected in the cultures. This indicated that, at least *in vitro*, cells grown in EHNA for multiple passages retained functional pluripotency. . However, the effect of EHNA was sufficiently robust that

when hESC were exposed to conditions that promoted neural differentiation, the continued addition of EHNA suppressed the induction of the neural marker PAX6 and maintained expression of pluripotency-associated markers including NANOG and OCT4.

These effects of EHNA were concentration-dependent but did not appear, however, to reflect inhibition of PDE2 because neither a second selective PDE2 inhibitor, BAY-60-7550 [13-14] nor the pan-PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX) [14], were able to mimic the effects of EHNA when added to cultures undergoing passive differentiation. Although EHNA is indeed an inhibitor of PDE2, it is perhaps better known as an inhibitor of the enzyme adenosine deaminase (ADA) [15]. Inhibition of ADA is anticipated to result in the accumulation of adenosine and deoxyadenosine and these compounds are known to have wide ranging effects on cells produced by a variety of mechanisms [16-21]. We also explored, therefore, whether other, structurally unrelated ADA inhibitors, including the highly potent inhibitor pentostatin [22-24] could mimic the effect of EHNA. Pentostatin, however, had no EHNA-like effects. Despite this lack of effect we also explored the potential role of phosphorylated metabolites of adenosine that might accumulate in the presence of an ADA inhibitor. The adenosine kinase inhibitor, ABT-702 was thus used in conjunction with EHNA. After 2 weeks under passive differentiation conditions the effects of EHNA were not ablated by the co-presence of ABT-702. Additionally, stimulation of the adenosine monophosphate regulated kinase (AMPK) with 5-aminoimidazole-4-carboxamide riboside (AICAR), a previously widely studied regulator of this enzyme [25-26] was unable to mimic the effect of EHNA. We have, therefore, been unable to provide evidence to suggest an important role for the phosphorylation of adenosine to the maintenance of pluripotency markers by

EHNA. Adenosine and related nucleotides are effectively exported from cells. It was thus possible that the effects of EHNA were mediated via export of accumulated adenosine that resulted in autocrine or paracrine activation of adenosine receptors of the G protein-coupled receptor class, that are expressed on the surface of many cell types [27]. However, each of subtype selective and non-selective adenosine receptor agonists and antagonists were unable to mimic or antagonise, respectively, the effects of EHNA. Finally, because a number of kinases and kinase-based cascades are known to influence stem cell function and survival [28-30] we also made preliminary efforts to investigate the possibility that EHNA might act through a previously undefined protein kinase-inhibitory action. However, when EHNA at 30 μM, a concentration sufficient to produce all the features of the maintainance of pluripotency, was evaluated using the Protein Kinase Panel service of the MRC National Centre for Protein Kinase Screening (University of Dundee, UK) it was unable to inhibit significantly any of the 80 kinases within the panel. These included GSK-3 [28], a number of ERK/MAPK pathway components [29] and fibroblast growth factor receptor [30], which have all previously been shown to be important for hESC pluripotency. As such, despite wide-ranging efforts to define the mechanism of action of EHNA to maintain hESC pluripotency this remains elusive, as is often the case with intact cell- and phenotype-based assays.

As a means to begin to help to define the chemical requirements of EHNA-mediated suppression of hESC differentiation and maintenance of pluripotency we embarked on a limited medicinal chemistry programme to understand the minimum structural requirements for function and to identify potential EHNA mimetics appropriate for cost-effective, large scale production. Over a series of more than 30 compounds that were synthesised or purchased from commercial sources the EHNA-

like activity failed to track with ADA inhibitory activity. Indeed, a number of compounds failed to display EHNA-like activity but were potent ADA inhibitors confirming, along with the lack of action of pentostatin, that ADA is not the key molecular target. However, a number of EHNA analogues and variants displayed essentially full EHNA-like activity as assessed by the ability to maintain expression of NANOG and prevent the development of expression of PAX6 as a marker of ectoderm development. Other compounds were partial in effect in that they mimicked EHNA in only one of these two characteristics. Importantly for future development and practical use of compounds that maintain hESC pluripotency, these studies have identified simplified compounds that can be synthesised in bulk at low cost, making them very attractive for large scale production and current-good manufacturing practice-compliant requirements for the production of hESC.

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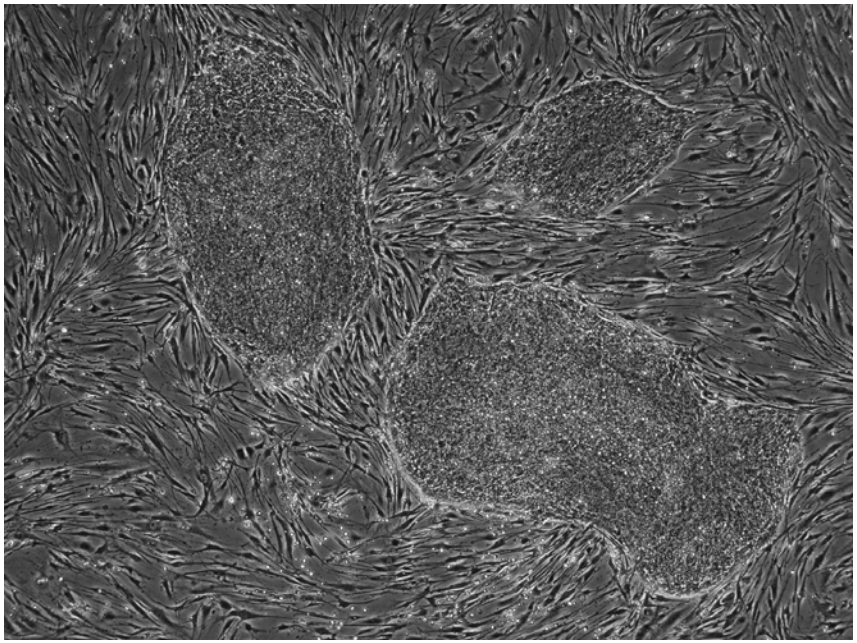
Figures and Figure Legends

Figure 1

Generation and maintainance of monolayer cultures of SA461 hESCs in feeder-free conditions

Colonies of SA461hESCs maintained on a mouse embryo fibroblast feeder layer (**A**) is compared with the same cells (**B**) after transfer to feeder free monolayer culture. See text for further details.

A



B

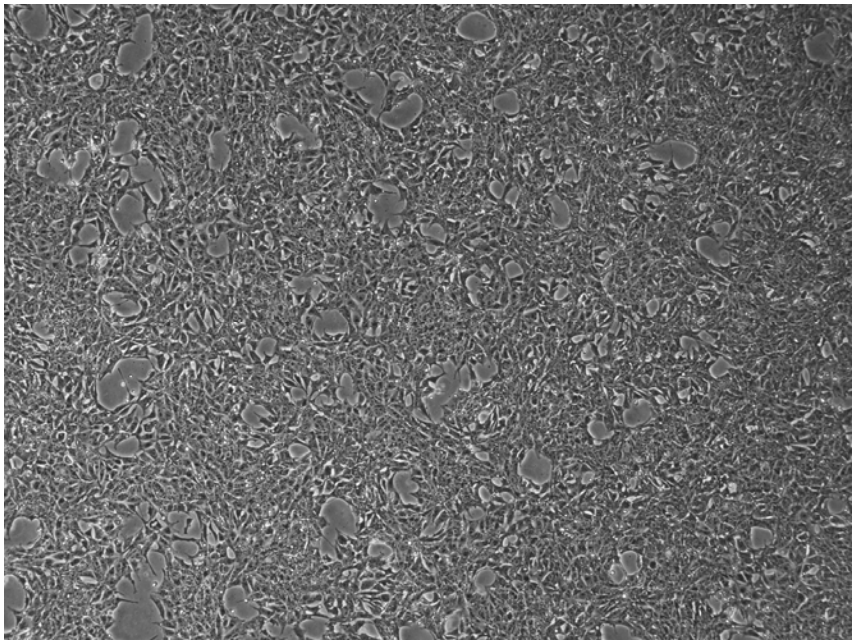


Figure 1