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1	Bone regenerative potential of the selective sphingosine 1-phosphate receptor
2	modulator siponimod: in vitro characterisation using osteoblast and endothelial cells
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18 Abstract

19	The repair of critical bone defects remains a significant therapeutic challenge. While the
20	implantation of drug-eluting scaffolds is an option, a drug with the optimal pharmacological
21	properties has not yet been identified. Agents acting at sphingosine 1-phosphate (S1P)
22	receptors have been considered, but those investigated so far do not discriminate between the
23	five known S1P receptors. This work was undertaken to investigate the potential of the
24	specific $S1P_{1/5}$ modulator siponimod as a bone regenerative agent, by testing <i>in vitro</i> its
25	effect on cell types critical to the bone regeneration process.
26	hFOB osteoblasts and HUVEC endothelial cells were treated with siponimod and other S1P
27	receptor modulators and investigated for changes in intracellular cyclic AMP content,
28	viability, proliferation, differentiation, attachment and cellular motility.
29	Siponimod showed no effect on the viability and proliferation of osteoblasts and endothelial
30	cells, but increased osteoblast differentiation (as shown by increased alkaline phosphatase
31	activity). Furthermore, siponimod significantly increased endothelial cell motility in scratch
32	and transwell migration assays.
33	These effects on osteoblast differentiation and endothelial cell migration suggest that
34	siponimod may be a potential agent for the stimulation of localised differentiation of
35	osteoblasts in critical bone defects.
36	Keywords: Siponimod, Sphingosine 1-phosphate, S1P1 Receptors, Osteoblasts, HUVEC

37

38 1. Introduction

The restoration of tissue function after damage, involves complex interactions between
various cell types, local tissue matrix, and chemical mediators, in various combinations.
The creation of new vasculature via angiogenesis is essential for the regeneration of any

42 tissue, and in the case of bones, regeneration also involves the recruitment of osteoblast and 43 osteoclast precursors to the defect area, their differentiation into their mature phenotypes as 44 well as interaction between the two cell types, with bone-forming osteoblasts stimulating the 45 maturation of bone resorbing osteoclasts, which in turn stimulate osteoblast recruitment and 46 maturation.

47 Sphingosine 1-phosphate (S1P) is a lipid mediator that modulates many biological processes, 48 including calcium signalling, cell growth, differentiation, survival, motility and cytoskeleton 49 organization (Spiegel and Milstien, 2000). It acts via 5 known G Protein-Coupled Receptors 50 (S1P₁₋₅), which are widely expressed throughout the body (Hla, 2004). The role of S1P in 51 promoting angiogenesis is well-established (Waeber, 2013). This, taken together with the pleiotropic effects of S1P on bone cells (Sartawi et al., 2017), suggests that modulating S1P 52 53 signalling may promote bone repair. However, systemic administration of S1P agents did not 54 improve fracture healing in a murine femoral defect (Heilmann et al., 2013), indicating that 55 more localised approaches of delivering S1P and related analogues may be needed (Das et al., 2014b). 56

57 The pharmacological characterization of the various S1P-mediated responses, in bone and 58 other tissues, has been hampered by the lack of well-characterized specific agents (Salomone 59 and Waeber, 2011). S1P itself (with or without receptor antagonists), as well as the S1P 60 receptor modulator fingolimod (aka FTY720 or Gilenya®) have been investigated in *in vitro* 61 and *in vivo* models of bone repair (Sartawi et al., 2017), but these agents do not discriminate 62 between the 5 different receptor subtypes. Fingolimod, used clinically for the management of 63 relapsing remitting multiple sclerosis, is a potent agonist at all S1P receptor subtypes except 64 $S1P_2$ (Brinkmann et al., 2010). Its mechanism of action relies, at least in part, on the 65 redistribution of lymphocytes to secondary lymphoid tissues following fingolimod-induced S1P₁ receptor internalization, resulting in their depletion from the peripheral blood and 66 67 immunosuppression. Although fingolimod is relatively safe, activation of S1P₃ receptors by 68 this agent may be associated with adverse effects (Cugati et al., 2014; DiMarco et al., 2014). 69 Although the role of S1P₃ receptors in cardiac side effects may be unique to rodents (Gergely 70 et al., 2012), these off-target effects led to the discovery and development of the S1P₁/S1P₅ 71 selective agonist siponimod (aka BAF312, or Mayzent®) (Behrangi et al., 2019). In addition 72 to its improved selectivity profile, siponimod is not a pro-drug (fingolimod must first be 73 phosphorylated by sphingosine kinase 2) and has a shorter half-life that still allows once-74 daily oral dosing but enables rapid recovery of lymphocyte counts upon treatment cessation. 75 The effects of S1P and of fingolimod on cells relevant to bone repair have been extensively 76 investigated (Sartawi et al., 2017), but far less is known on the effect of siponimod on these 77 cells. The goal of these studies was therefore to test the effect of siponimod on the viability, 78 proliferation, differentiation, and chemotactic behaviour of osteoblast and endothelial cells, 79 with the aim of better understanding the potential of selective modulation of $S1P_1$ (or $S1P_5$) 80 receptors via localised delivery to repair critical bone defects.

81 2. Material and methods

82 Siponimod and fingolimod were kindly gifted from Novartis. D-erythro-Sphingosine 1-

83 phosphate was acquired from Enzo Life Sciences. Dulbecco's Modified Eagle's

84 Medium/Nutrient Mixture F-12 Ham, foetal bovine serum (FBS), L-Glutamine, penicillin-

85 streptomycin, Thiazolyl Blue Tetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO),

86 neutral buffered formalin (NBF), Fast Blue BB and Naphthol AS-MX phosphate, Roche 87 Bromodeoxyuridine cell proliferation kit were acquired from Sigma-Aldrich. Endothelial cells (HUVEC) and endothelial cell growth medium (ECGM) with associated supplements 88 89 were acquired from PromoCell. Pierce[™] PNPP Substrate Kit was acquired from Thermo Fisher Scientific. cAMP-Glo[™] Max Assay was acquired from Promega. Cell culture 90 91 plasticware was acquired from Sarstedt Ltd. Human foetal osteoblasts (hFOB 1.19 (ATCC® 92 CRL-11372TM)) cell line was acquired from ATCC. 93 2.1. Cell culture hFOB were maintained in DMEM/F12 supplemented with FBS (10%), L-glutamine (1%), 94 95 and penicillin-streptomycin (1%). Incubation was at 34 °C and 5% CO₂. HUVEC were maintained in supplemented ECGM as per supplier's instruction at 37 °C and in 5% CO₂. 96 97 Although this medium contains only 2% serum, some HUVEC experiments were performed under reduced serum conditions (1/10th standard cell culture supplement) to rule out an effect 98 99 of endogenous S1P (Hanel et al., 2007). 2.2. Siponimod solution 100 101 Siponimod was dissolved in DMSO and then diluted in PBS as required. DMSO concentration were limited to <0.5% v/v in cell culture experiments. The potential toxicity of 102 103 exceeding this concentration of DMSO was explored using hFOB and HUVEC cells (2.5*10⁴ 104 cell/well in 24 well plates) incubated with increasing concentrations of DMSO (0.32 - 3.2%

105 in cell culture medium) for two and three days respectively. Thereafter, resazurin 60 µl of a

106 560 μ M stock solution was added to wells for 3.5 hrs before acquiring fluorescence at

107 excitation 488 nm/emission 595 nm.

108 2.3. Viability and proliferation

109 To estimate the effect of siponimod on cellular viability, cells were seeded at a density of 110 $2.5*10^4$ cells per well in 24-well plates, the following day test drugs (100 nM siponimod or 111 PBS vehicle) were added to cell culture medium. Following two- and three-days incubation 112 (HUVEC and hFOB, respectively), 60 µl MTT solution (5 mg/ml stock) was added directly 113 to wells and incubated for 2 h away from light. Wells were then rinsed with PBS. Formazan 114 crystals were dissolved with DMSO and absorbance acquired at 570 nm using a Wallac 115 Victor 2 plate reader (Perkin Elmer).

To determine the effect of siponimod on cellular proliferation, cells were seeded in 24-well
plates at a density of 2.5*10⁴ cells per well. Siponimod (100 nM) or PBS vehicle were added
to the cell culture medium for two- and three-days (HUVEC and hFOB, respectively).
Thereafter, cells were detached using trypsin-EDTA 0.25%, diluted with cell culture medium,
and individual well cell numbers manually counted using a haemocytometer. Additional cell
counting experiments were conducted using hFOB cells maintained over 7 days (see section

122 2.4 for details)

As an additional measure of proliferation, cells were seeded in 96-well plates at densities of 1×10⁴ per well. Increased concentrations of siponimod, fingolimod, and S1P (all 1000 nM) were added the following day with fresh medium, and incubation continued for a further two days. Following treatment, BrdU was diluted in fresh medium and added to cells for 24 h. Thereafter cells were fixed for 30 min, then incubated for 90 min in a BrdU antibody solution, rinsed thoroughly with PBS followed by incubation with anti-BrdU substrate until sufficient colour development for plate reading at 405 nm.

130 2.4. Osteogenic differentiation

131 2.4.1. Para-nitrophenylphosphate

132 Alkaline phosphatase (ALP) is an early marker of osteoblast differentiation. *In vitro*

133 osteoblast differentiation was estimated using para-nitrophenylphosphate (pNPP) as a

134 substrate of alkaline phosphatase that is dephosphorylated into a yellow product (p-

135 nitrophenol), detectable by absorbance at 405 nm.

hFOB cells $(5*10^4)$ were seeded in 24-well plates and treated on day 0, 2, 4, and 6 with 1000

137 nM of either siponimod, fingolimod or S1P, with PBS vehicle as a control. Test agents were

138 added directly to standard hFOB medium. As a positive differentiation control, cells were

139 treated with osteogenic medium containing 50 μ g/ml ascorbic acid and 7.5 mM β -

140 Glycerophosphate. After 7 days, cells were detached with 100 µl trypsin-EDTA 0.25%,

141 diluted with 200 µl fresh medium and counted using a haemocytometer. The cells were then

142 transferred to 1.5 ml tubes and centrifuged at 3000 g for 5 min. Medium was aspirated from

143 each tube and replaced with 100 µl pNPP substrate solution, allowing 30 min for yellow

144 colour development. Absorbance was recorded at 405 nm using a Wallac Victor 2 plate

145 reader (Perkin Elmer). The absorbance value of each sample was divided by its respective

146 cell count, to normalize for differences due to cell numbers. Data are presented relative to the

147 positive osteogenic medium control in each independent replicate.

148 2.4.2 Fast blue staining

149 Because the pNPP-based assay above does not allow the determination of the fraction of

150 differentiating cells, as a complimentary measure of ALP, staining was performed using Fast

151 Blue BB and Naphthol AS-MX phosphate. hFOB cells (5*10⁴) were seeded in 24-well plates

and treated every other day with siponimod (10-1000 nM), PBS, or osteogenic medium for 7

153 days. Thereafter, cells were equilibrated in an alkaline buffer followed by incubation with

154 fast blue dye for 60 min. Using a BX51 microscope (OLYMPUS), three images were

acquired per well (with a 4x objective). Using ImageJ analysis software, the number of

stained cells and total cells was manually counted and expressed as a percentage of the total

157 cell number.

158 2.5. Migration

159 Cell migration assays were conducted for HUVEC and hFOB cells using the same techniques160 but using cell type specific media.

161 2.5.1. Wound healing (scratch) assay

162 Cells were seeded at $1*10^5$ cells/well in 24-well plates and grown overnight to produce a

163 nearly confluent monolayer. A linear scratch was created by hand using a 1 ml pipette tip,

164 wells were rinsed with PBS to remove debris and the cell culture medium was replaced as

165 follows: for hFOBs, DMEM/F12 supplemented with 0.1% FBS was used to reduce serum

166 bioactive lipid effects. Likewise, for HUVEC cells ECGM was supplemented with 1/10th the

167 usual supplement. Siponimod and S1P (delivered in 20 µl PBS) were then added directly to

168 cell culture medium. Brightfield images (4x objective) were acquired immediately and after 8

169 h using a BX51 microscope (OLYMPUS). Using the associated software, Stream

170 (OLYMPUS), the distance between the edges of the scratch wound was measured and the

171 change over time attributed to cellular migration into the empty space.

172 2.5.2 Transwell migration

173 Transwell migration was conducted to assess chemotactic activity of S1P agents. Cells were 174 seeded at $5*10^4$ cells in 100 µl of medium in the upper chamber of 8 µm pore poly ethylene 175 terephthalate transwell inserts. Siponimod, S1P, or fingolimod (delivered in 20 µl PBS) were 176 then added to the bottom chamber of the transwell system, which contained 600 µl of 177 medium. After 4, 8, or 24 h of incubation, culture medium was aspirated from the upper 178 chamber, inserts were fixed with 10% NBF for 15 min at room temperature, then stained with 179 0.5% crystal violet for 30 min at room temperature. Thereafter inserts were rinsed with water 180 to remove excess dye and the top side of the membrane was wiped with a cotton bud to 181 remove non-migrated cells. Finally inserts were dried on the bench, before the membrane was 182 visualized by light microscopy (BX43 microscope (OLYMPUS)). Five brightfield images per 183 insert were acquired (10x objective), stained cells were manually counted using imageJ 184 analysis software.

185 2.6. Cell attachment

The influence of siponimod on HUVEC attachment was investigated by seeding $5*10^4$ cells in 24 well plates using ECGM that was supplemented with $1/10^{\text{th}}$ standard supplement to reduce serum lipid effects. Siponimod and S1P (delivered in 20 µl PBS) were then added immediately to wells. After 4 h incubation, non-attached cells were removed by washing with PBS. Remaining cells were fixed with 10% NBF for 15 min, followed by staining with crystal violet 0.5% w/v for 30 min. Three brightfield images per well were acquired (10x objective), with stained cells manually counted using imageJ analysis software.

193 2.7. Cyclic AMP assay

The effect of siponimod on intracellular cAMP levels was determined using the cAMP-GloTM Max Assay (Promega). hFOB cells were seeded at $2*10^4$ cells per well in 96 well plates and cultured overnight. Cells were washed with PBS, then treated with forskolin, siponimod, forskolin & siponimod combined, and a control containing the DMSO vehicle (concentration 0.32 %) for 1 h. All conditions included 500 µM IBMX to inhibit phosphodiesterases. Luminescence was measured and cAMP concentrations calculated using a standard curve as per the manufacturer's protocol.

201 2.8. Statistical analysis

- 202 Results were expressed as mean \pm standard deviation (S.D.), unless otherwise stated.
- 203 Determining statistical significance was performed using one-way analysis of variance
- 204 (ANOVA) followed by Bonferroni post-test for multiple comparisons. Differences were
- 205 considered significant at P < 0.05. We used extra-sum-of-squares F tests as previously
- 206 described (Waeber and Moskowitz, 1995) to determine whether drug responses were
- 207 concentration-dependent (with the null hypothesis that data points were best fitted with a
- 208 horizontal line, i.e. showed no concentration dependence).

209 3. Results

210 3.1. Siponimod solution

211 Siponimod was applied to cells in *in vitro* experiments as an aqueous solution of PBS 212 containing a small concentration of DMSO as a cosolvent (0.32 % v/v). In order to determine 213 whether the use of DMSO as a cosolvent would negatively impact hFOB and HUVEC cells 214 used throughout this work, increasing concentrations of DMSO were applied to cells and 215 their viability determined by resazurin assay (Fig 1). For hFOB cells there was no statistically 216 significant change in cell viability when using 0.32 % DMSO compared to PBS control (91.5 217 \pm 24.2 % compared to 100 \pm 26.7 %). With increasing concentration of DMSO, cell viability 218 trended downwards albeit without achieving statistical significance until a DMSO 219 concentration of 3.22 % produced a significant fall in hFOB viability compared to PBS 220 control and the 0.32 % condition (4.8 ± 2.7 % compared to 100 ± 26.7 % and 91.5 ± 24.2 %, 221 P < 0.05). For HUVEC cells incubated with a concentration of 0.32 % DMSO there was no 222 statistically significant change in cell viability over the experimental duration compared PBS 223 control (99.4 \pm 9.3 % compared 100 \pm 4.2 %). As before DMSO showed a statistically 224 significant reduction in cell viability at increased concentrations of 3.22 % compared to PBS 225 and the 0.32 % condition (25.0 \pm 12.1 % compared to 100 \pm 4.2 % and 99.4 \pm 9.3 %, P < 226 0.05)

227 Fig 1.

3.2. Viability and proliferation

229 Siponimod effect on cell viability and proliferation was investigated using hFOB and

HUVEC cells. MTT assays were used to determine cell viability, with results presented as

absorbance at 570 nm expressed as a percentage of positive control (for hFOBs, fully

supplemented DMEM/F12, for HUVEC fully supplemented ECGM). Manual cell counting

233 was used to determine cell proliferation, with data presented as average cell numbers 234 expressed as a percentage of positive control. Viability of hFOB cells treated with 100 nM 235 siponimod were not significantly different to those treated with PBS vehicle ($42.1 \pm 7.6 \%$ 236 compared to 36.9 ± 8.6 %, ns), this lack of effect was also seen in manual cell counting 237 experiments comparing 100 nM siponimod and PBS vehicle (49.1 ± 4.1 % compared to 44.9238 \pm 0.8 %, ns) (Fig 2 A & B). For HUVEC 100 nM siponimod produced no significant 239 difference in viability compared to PBS vehicle (80.9 ± 8.8 % compared to 74.9 ± 8.2 %, ns). 240 There was similarly no statistically significant change in manual cell count results between 241 100 nM siponimod and PBS vehicle (68.1 \pm 12.7 % compared to 59.1 \pm 12.7 %, ns) (Fig 2 C 242 & D). Additionally, BrdU assay confirmed the absence of a proliferative effect for 1000 nM 243 siponimod on both the hFOB cells and HUVEC (Fig 2 E & F). The BrdU assays also showed 244 that there was no discernible effect on proliferation for either S1P or fingolimod (1000 nM). Over a 7-day experimental duration (Fig 2 G), none of siponimod, fingolimod or S1P (all 245 1000 nM) led to any statistically significant change in hFOB cell count compared to PBS 246 247 control (87.9 ± 16.5 % for siponimod, 88.7 ± 10.3 % for fingolimod, and 72.5 ± 18.7 % for 248 S1P compared to 100 ± 37.1 % for PBS control). Likewise, increasing concentrations of 249 siponimod (10-1000 nM (Fig 2 H)) did not show any statistically significant changes in cell 250 number compared to PBS control (91.2 \pm 5.0 % for 10 nM, 95.8 \pm 15.5 % for 100 nM, and 251 82.2 ± 24.2 % for 1000 nM compared to 100 ± 14.7 % for PBS control).

252 Fig 2.

253 3.3. Osteogenic differentiation

254 3.3.1. Para-nitrophenylphosphate

hFOB cells were incubated in medium containing equal concentrations (1000 nM) of either
siponimod, fingolimod, or S1P (Fig 3). Absorbance values for the ALP product p-nitrophenol

257	were normalised according to manual cell counts. Data is expressed as a percentage of the
258	osteogenic medium positive control across each replicate. Fig 3 A shows the results of the
259	comparison between the three investigated drugs (siponimod, fingolimod, and S1P). Whereas
260	1000 nM fingolimod showed no significant difference compared to PBS vehicle (44.8 \pm 2.7
261	% compared to 39.8 \pm 9.5 %), siponimod (1000 nM) increased absorbance/count compared to
262	PBS vehicle (68.4 \pm 9.7 % compared to 39.8 \pm 9.5 %, P < 0.05). This increase was not
263	significantly different from that induced by 1000nM S1P (78.1 \pm 10.3 % compared to 39.8 \pm
264	9.5 % for PBS vehicle, P < 0.05) and the response to siponimod ranging from 10-1000 nM
265	was concentration dependent (F statistic = 11.46 ; P = 0.0069) (Fig 3 B).
266	<i>Fig 3.</i>
267	3.3.2. Fast blue staining
268	Alkaline phosphatase staining (Fig 4) was performed to complement the pNPP-based
269	assessment above. Results represent the number of stained cells divided by the total number
270	of cells, expressed as a percentage. hFOB cells were incubated with three concentrations of
271	siponimod (10, 100, and 1000 nM). The concentrations 100 nM and 1000 nM resulted in an
272	increased fraction of stained cells (100 nM siponimod 3.7 \pm 0.6 (P < 0.05), and 1000 nM
273	siponimod 4.2 \pm 0.8 % (P < 0.05) compared to PBS vehicle 1.9 \pm 0.6 %) and the response
274	was concentration dependent (F statistic = 6.53 ; P = 0.038).
275	Fig 4.
276	3.4. Migration
277	3.4.1. Wound healing (scratch) assay
278	Scratch assays were performed to investigate whether the migratory response of hFOB and
279	HUVEC cells was increased by siponimod. While hFOB cells did not respond to 100 nM

280 siponimod after 8 h (Fig 5 A), HUVEC cells scratch wound closure was doubled in the

281	presence of 100 nM siponimod compared to PBS vehicle (45.8 ± 4.0 % compared to $22.5 \pm$
282	6.0 %, $P < 0.05$) (Fig 5 B). The concentration responsiveness of the effect was examined in a
283	separate series of experiments; while all siponimod concentrations (1, 10, and 100 nM)
284	produced a statistically significant increase in scratch wound closure compared to PBS
285	vehicle (40.9 \pm 5.0 %, 42.3 \pm 3.5 %, and 45.9 \pm 2.9 % compared to 22.7 \pm 7.2 %, P < 0.05)
286	(Fig 5 C), this response was found to not be concentration dependent (F statistic = 2.82 ; P =
287	0.14). The migratory response of HUVEC to 100 nM S1P was qualitatively similar to the
288	effect of siponimod (Fig 5 D); 40.6 \pm 3.6 % for S1P compared to 18.1 \pm 3.5 % for PBS, P $<$
289	0.05. However, these experiments were conducted independently, precluding a direct
290	comparison.
291	<i>Fig 5.</i>

292 3.4.2. Transwell migration

Following the data obtained from scratch wound assays, transwell migration assays were conducted to test the hypothesis that siponimod-enhanced migration of HUVEC was due to a chemotactic effect. Due to lack of scratch assay effect, hFOB cells were not investigated.

296 In transwell migration assays conducted under standard endothelial growth medium

297 conditions (2 % v/v serum), 100 nM siponimod added to the bottom chamber of the transwell

system resulted in a reduction in the number of migrated cells detected on the bottom side of

the membrane compared to PBS vehicle (26.9 \pm 7.4 % compared to 71.6 \pm 10.3 %, P < 0.05)

300 (Fig 6 A).

301 The concentration of S1P in serum is in the submicromolar range, i.e. sufficient to activate

302 S1P receptors (Thuy et al., 2014). In contrast, much lower S1P concentrations are detected in

303 tissues; this S1P gradient controls the trafficking of immune and hematopoietic stem

304 progenitor cells (Liu et al., 2011). To test the hypothesis that the "repulsive" effect of

305 siponimod added to the bottom chamber under high serum concentrations was due to 306 siponimod-induced receptor internalization, thereby blocking serum-induced cell migration, 307 we tested the effects of S1P and siponimod added to the bottom chamber under reduced 308 serum conditions (0.2% v/v serum). Under these conditions, 100 nM siponimod produced no 309 statistically significant change in the number of migrated cells compared to PBS vehicle after 310 4 h (40.3 \pm 11.0 % compared to 33.8 \pm 10.1 %, ns) (Fig 6 B). S1P (100 nM) alone resulted in 311 an increased number of migrated cells compared to PBS vehicle after 4 h (111.5 \pm 19.5 % 312 compared to 36.2 ± 16.7 %, P < 0.05) (Fig 6 C). When S1P was administered in combination 313 with 100 nM siponimod, the number of migrated cells was significantly reduced (20.4 ± 22.8 314 % compared to 111.5 ± 19.5 %, P < 0.05) (Fig 6 C). We then performed transwell migration assays over 8 h to more closely match scratch assay 315 316 conditions. Here 100 nM siponimod produced a statistically significant increase in the

number of migrated cells compared to PBS vehicle (34.7 ± 7.9 % compared to 10.8 ± 3.3 %,

318 P < 0.05) (Fig 6 D). As in the 4 h experiment, 100 nM S1P increased the number of migrated

cells compared PBS vehicle (106.3 \pm 11.9 % compared to 10.8 \pm 3.3 %, P < 0.05), an effect

320 that was antagonised by 100 nM siponimod (106.3 \pm 8.0 % compared to 16.8 \pm 7.3 %, P <

321 0.05) (Fig 6 D).

Migration over 24 h was investigated (Fig 6 E) and showed a substantial fall in the overall
number of migrating cells compared to experiments conducted at 4 and 8 h.

324 Fig 6.

325 3.5. Cell attachment

To further rule out the possibility that the siponimod-induced reduction of HUVEC migration (Fig 6 A) was caused by an effect on cell attachment, we examined the effect of various test agents on this parameter. Alone, 100 nM siponimod resulted in no statistically significant 329 change in cell attachment compared to PBS vehicle (67.0 ± 0.6 % compared to 58.6 ± 5.0 %,

- ns). S1P (100 nM) resulted in a statistically significant increase in cell attachment compared
- to PBS vehicle (88.4 \pm 6.4 % compared to 58.6 \pm 5.0 %, P < 0.05). This effect was
- antagonised when 100 nM siponimod was added with 100 nM S1P (88.4 ± 6.4 % attachment
- for S1P compared to 51.3 ± 2.7 % for siponimod/S1P, P < 0.05). Attachment in the presence
- 334 of siponimod and S1P was not statistically different from attachment in the PBS vehicle
- 335 condition (51.3 \pm 2.7 % compared to 58.6 \pm 5.0 %, ns).
- 336 *Fig 7*.
- 337 3.6. Cyclic AMP assay

Intracellular cAMP was quantified in an attempt to confirm the identity of the S1P receptor involved and to examine potential signalling mechanisms involved in the response to siponimod. Results showed that after 1 h, siponimod significantly inhibited forskolinstimulated increases in intracellular cAMP ($30.0\% \pm 22.9\%$ for 1 µM forskolin + 100 nM siponimod compared to 100 % ± 11.5 % for 1 µM forskolin, P < 0.05) (Fig 8). Siponimod alone did not lead to any significant change in baseline cAMP ($12.6 \pm 8.6\%$ for 100 nM siponimod compared to 10.2 ± 10.1 % for untreated control).

345 Fig 8.

346 4. Discussion

The overall aim of these studies was to assess the suitability of siponimod as a potential bone regenerative agent, to be eluted by a localised delivery device to stimulate repair in critical bone defects. With this goal in mind, we investigated the effect of siponimod on osteoblast and endothelial cells proliferation, differentiation and migration. Primarily, it was necessary to show that solutions of siponimod, prepared using DMSO as a co-solvent, did not 352 negatively impact cell viability. Therefore, experiments were conducted showing that the 353 concentration of DMSO used (0.32 %) was non-toxic in both hFOB cells and HUVEC. This 354 concurs with the literature, that a concentration less than 0.5 % should not impact cell 355 viability (Shah et al., 2019). Shifting focus to the viability assays proper, S1P is well 356 established in promoting endothelial cell proliferation, viability and survival, likely via the 357 S1P₁ or S1P₃ receptors (Kwon et al., 2001; Lee et al., 2000; Lee et al., 1999; Rikitake et al., 358 2002; Wang et al., 1999). Therefore, the lack of proliferative effect herein, as well as 359 siponimod's selectivity for receptors 1 and 5 may indicate that the S1P₃ receptor plays the 360 more important role. Another possibility is that siponimod is behaving like fingolimod, which 361 at concentrations below 250 nM has no effect on HUVEC viability but exhibits toxicity 362 above 250 nM (Schmid et al., 2007). Siponimod also had no effect on osteoblast 363 proliferation, perhaps explained by a possible role for siponimod in osteoblast differentiation, 364 pushing the cells towards a post-mitotic phase precluding extensive proliferation (Long, 2011). 365

ALP is commonly used as a marker of osteoblast differentiation. Here we show that exposure 366 367 to siponimod (but not to fingolimod) increased ALP activity, an effect equivalent to that seen 368 with the same concentration of S1P. Complementary ALP staining showed a corresponding 369 siponimod-induced increase in the number of stained cells. S1P and fingolimod have 370 previously been shown to increase markers of osteoblast differentiation as well as stimulating 371 the osteogenic differentiation pathway of osteoblasts (Brizuela et al., 2014; Lotinun et al., 372 2013; Matsuzaki et al., 2013; Sato et al., 2012) and mesenchymal stems cells (Hashimoto et 373 al., 2016; Hashimoto et al., 2015; Marycz et al., 2016; Pederson et al., 2008), but it is unclear whether S1P₁, S1P₃ or both receptor subtypes mediate these effects. Studies of S1P on 374 375 osteoblast differentiation with receptor antagonists have shown an exclusive role for S1P₃ in 376 osteoblast maturation (Brizuela et al., 2014), whereas S1P₁ receptors were shown to mediate

377 the effect of S1P and fingolimod on osteoblast differentiation, when used in conjunction with 378 bone morphogenetic protein 2 (Sato et al., 2012). There is conflicting evidence regarding the 379 effect of fingolimod, which has recently been shown to reduce markers of bone formation 380 (including ALP) in osteoblasts and chondrocytes (El Jamal et al., 2019). This study, which is 381 more in line with our findings with fingolimod, taken together with the effects presented 382 herein for siponimod, an S1P_{1/5} selective modulator, and the lack of detectable S1P₅ receptor 383 mRNA expression in pre-osteoblasts and osteoblasts (Roelofsen et al., 2008), suggest that 384 S1P₁ receptor stimulation is sufficient to induce osteoblast differentiation.

385 S1P is known to stimulate the migration of osteoblast precursors, osteoclasts, and endothelial 386 cells (Lee et al., 2000; Lee et al., 1999; Ohmori et al., 2001; Pederson et al., 2008; Roelofsen 387 et al., 2008; Ryu et al., 2002). Here we found that siponimod had no effect on the migration 388 of hFOB osteoblasts. This lack of effect suggests that previously reported effects of S1P on 389 osteoblast migration were mediated by a receptor other than S1P₁ or S1P₅, or that this effect 390 was dependent on the differentiation stage. While both S1P₁ and S1P₂ receptors have been 391 shown to regulate the migration of cells of the osteoblast lineage, they only did so in MC3T3-392 E1 pre-osteoblasts (Roelofsen et al., 2008). Upon reaching cell confluence, cultures of hFOB 393 cells express high levels of phenotypic markers associated with osteoblast differentiation 394 (Harris et al., 1995). It is therefore possible that the cells used in our studies were more 395 differentiated than the pre-osteoblasts known to migrate in response to S1P, although the 396 relatively small fraction of cells expressing ALP in our studies, even after one-week exposure 397 to osteogenic medium, would seem to argue against this explanation, leaving open the 398 possibility that species differences may account for the discrepant migration response in 399 hFOB (of human origin) and in murine MC3T3-E1 cells. Our studies did however show a 400 significant effect on endothelial cell migration, doubling HUVEC cell motility in scratch 401 assays. The effect of siponimod was found to be similar to that of S1P (Lee et al., 2000; Lee

402 et al., 1999; Ohmori et al., 2001; Ryu et al., 2002). However, the extent of this response did 403 not seem to depend on siponimod's concentration (1-100 nM). It is possible that a 404 concentration response relationship may have emerged with further independent experiments. 405 Alternatively, the effect may already have been maximal at 1 nM siponimod, reaching a 406 plateau thereafter. Indeed, siponimod is a potent and efficacious S1P₁ agonist, with subnanomolar EC₅₀ in [³⁵S]GTPyS binding assays (Gergely et al., 2012; Lukas et al., 2014). 407 408 However, while siponimod is often tested at 100 nM in published functional cell culture 409 experiments (Gentile et al., 2016; Lupino et al., 2019), we found only two reports showing a 410 concentration response curve in such preparations, in which siponimod mediated a response 411 with an EC50 of 15.8 nM (Gergely et al., 2012) and only showed a non-significant trend at 1 412 nM (O'Sullivan et al., 2016).

413 In transwell assays designed to test whether the effect of siponimod in the scratch assay was 414 due to increased chemokinesis or to chemotaxis, siponimod decreased the migration of 415 endothelial cells under standard growth medium conditions (2% v/v serum). We hypothesized 416 that siponimod may have internalized S1P receptors, blocking the effect of S1P present in the 417 growth medium. Indeed, when transwell assays were conducted under low serum conditions (0.2% v/v serum), siponimod had no significant effect on cell migration, and S1P stimulated 418 419 endothelial cell migration in a siponimod-sensitive manner and as effectively as serum. 420 To understand how siponimod interfered with S1P-mediated transwell migration, cell

421 attachment studies performed under similar conditions to the transwell assays showed that
422 siponimod interfered with S1P-mediated increases in cell attachment over 4 h. It is therefore
423 possible that decreased cell attachment may have contributed to the reduced transwell
424 migration observed after 4 h. Given that the effect of siponimod in scratch assays was
425 determined over 8 hr, additional 8 hr transwell assays were conducted and showed that while
426 siponimod still antagonised S1P mediated migration, it also induced a statistically significant

427 increase in cell migration when added on its own, albeit with an effect 3-fold weaker than the 428 effect of S1P. This may be due to persistent signalling after internalisation of the S1P₁ 429 receptor (Mullershausen et al., 2009), which may only lead to migration after long exposure 430 (8 h) to siponimod, but not after 4 h, and may also indicate that siponimod behaves similarly 431 to fingolimod, which has been shown to induce cellular motility in scratch assays 432 (Mullershausen et al., 2009) but impede HUVEC migration across a membrane (Ho et al., 433 2005; LaMontagne et al., 2006; Tanaka et al., 2013). The same experiments were conducted 434 over 24 h, however the substantial drop in the number of cells migrating indicated that the 435 extended experimental duration under low serum conditions may have impacted HUVEC viability. 436

437 The role of intracellular cAMP in bone remodelling and bone cell differentiation has long 438 been known (Rodan et al., 1975), but the relationship is complex. While some early studies 439 have shown that parathyroid hormone stimulate the in vitro differentiation of osteoblasts via 440 intracellular cAMP production (Nakatani et al., 1984), other reports show that the influence 441 of cAMP on ALP expression changes depending on the stage of osteoblast differentiation, 442 and parathyroid hormone may preferentially inhibit the differentiation of more mature 443 osteoblasts (Isogai et al., 1996). Expanding on the complexity of the relation between cAMP 444 and osteoblast differentiation markers, increasing levels of cAMP have been shown to result 445 in decreased ALP but increased osteocalcin expression (Romanello et al., 2001). More 446 recently, increasing cAMP levels have been shown to suppress osteoblast mineralisation 447 (Nishihara et al., 2018). Forskolin-induced cAMP is known to be inhibited by both 448 sphingosine 1-phosphate and fingolimod after 1 h incubation (Mullershausen et al., 2009), 449 with results similar to those shown for siponimod. This effect is most likely associated with 450 the inhibitory Gi protein coupled to the S1P₁ receptor, which is the mechanism through which S1P and fingolimod produce the same inhibitory effect. Based on the somewhat 451

452 contradictory existing literature, it is not straightforward to provide a mechanism linking the
453 adenylate cyclase inhibiting effect of siponimod on our hFOB osteoblasts and the effects of
454 this agent on hFOB cell differentiation. However, we hypothesize that siponimod may
455 maintain a chronically low level of intracellular cAMP through its interaction with S1P₁
456 receptors, resulting in increased ALP activity, indicative of increased osteoblast
457 differentiation.

Fingolimod, and now siponimod, both antagonise the chemotactic effect of S1P on
endothelial cells. Fingolimod has shown proangiogenic effects *in vivo*, ultimately improving
recovery of bone defects (Huang et al., 2012; Li et al., 2019). Therefore, given siponimod's
effect on osteoblast differentiation, it may be useful to incorporate siponimod into a localised
delivery device to investigate the effects of siponimod *in vivo* towards the same end.

463 One of the limitations of the current study is that it does not consider osteoclasts, or the 464 coupling between osteoblasts and osteoclasts (Pederson et al., 2008). This communication is 465 known to involve S1P receptors and would likely impact significantly on healing outcomes in any potential in vivo studies. Also, our ALP staining studies showed that the total fraction of 466 467 stained cells was relatively small, being less than 10% of the total number of cells. This may 468 be due to the use of a relatively early time point for analysis (7 days). Conducting the experiment over a longer duration, more consistent with the effect of a drug eluting scaffold 469 470 (Das et al., 2014a; Das et al., 2014b) may have led to a more meaningful effect on 471 differentiation.

472 5. Conclusion

The aim of this work was to investigate the potential of siponimod in a bone regenerative
context, ultimately towards its use in conditions of critical bone defects, as part of a localised
delivery device, but improving on the specificity of the eluted drug (Das et al., 2014a; Das et

al., 2014b; Huang et al., 2012; Li et al., 2019). These studies add to the relatively small
amount of literature on the functional effects of siponimod in cell culture models. In the
context of bone repair, the differentiation effect of siponimod on osteoblasts, taken together
with its effects on endothelial cells suggest that this selective S1P₁ modulator may be useful,
particularly in conditions of critical defects that remain a significant therapeutic challenge.
However, more robust *in vivo* experiments would be the next step before making any
determinative conclusions.

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Fig 1. Viability assay for hFOB and HUVEC cells incubated with increasing concentrations of DMSO. (A) hFOB resazurin assay after 72 h, (B) HUVEC resazurin assay after 48 h. Data is expressed as a percentage of PBS control and is presented as mean \pm S.D. from 3 independently repeated experiments (with 3 technical replicates). For hFOB cells, increasing DMSO concentrations were added to cell culture medium containing 1/10th standard serum supplement. For HUVEC, DMSO concentrations were added to cell culture medium containing 1/3rd standard supplements. Statistical analysis was performed by one-way ANOVA. There was no statistically significant difference between any group except for 3.22% DMSO, which was statistically different from all other groups (***: P < 0.001).

Fig 2. Viability and proliferation assays for hFOB and HUVEC cells. (A & B) hFOB MTT assay and cell count after 72 h, n=3 (4 technical replicates) (C & D) HUVEC MTT assay and cell count after 48 h, n=3 (4 technical replicates) (E & F) hFOB and HUVEC BrdU assay, n=3 (4 technical replicates) (G & H) hFOB cell count after 7 days incubation, n=4 (3 technical replicates). For A-F, data is expressed as a percentage (positive control set to 100%), for G & H data is expressed as a percentage of PBS control. For hFOB cells (in A-F), factors were added to cell culture medium containing 1/10th standard serum supplement, with standard growth medium (DMEM/F12) acting as positive control. For HUVEC (in A-F), factors were added to cell culture medium containing 1/3rd standard supplements, with standard growth medium (ECGM, containing 2% serum) acting as positive control. In G & H growth medium supplement was not altered. "n=" represents the number of independently repeated experiments. Data is presented as mean ± S.D., statistical analysis by one-way ANOVA. NS: No statistical significance. *: P < 0.05. **: P < 0.01. ***: P < 0.001.

Fig 3. Alkaline phosphatase activity as an early marker of differentiation in hFOB cells. (A) Effect of siponimod, fingolimod, and S1P (1000 nM) after 7 days, n=4 (3 technical replicates) (B) Siponimod concentration response over 10-1000 nM after 7 days, n=4 (3

technical replicates). For A & B, data represents pNPP absorbance at 405 nm divided by cell count, relative to the positive control (osteogenic medium containing 50 μ g/ml ascorbic acid & 7.5 mM β -glycerophosphate) in each independent replicate. ''n='' represents the number of independently repeated experiments. Data is presented as mean ± S.D., statistical analysis by one-way ANOVA. NS: No statistical significance. *: P < 0.05. **: P < 0.01. ***: P < 0.001.

Fig 4. Alkaline phosphatase (ALP) staining as an early marker of differentiation in hFOB cells. (A) Response to 10-1000 nM siponimod after 7 days, n=4 (3 technical replicates). Data represents the average number of manually counted Fast blue-stained cells divided by the total cell number. Osteogenic medium (containing 50 µg/ml ascorbic acid & 7.5 mM β-glycerophosphate) was used as a positive control. "n=" represents the number of independently repeated experiments. Data is presented as mean ± S.D. Statistical analysis was done using one-way ANOVA. NS: No statistical significance. *: P < 0.05. **: P < 0.01. ***: P < 0.001. The percentage of ALP-stained cells in the presence of osteogenic medium was significantly different from the percentage of stained cells in all other conditions (P < 0.001). B, C, and D are representative brightfield photomicrographs of hFOB cells exposed to PBS (B), 100 nM siponimod (C), and osteogenic medium (D); blue cells are cells with higher alkaline phosphatase activity, and hence a higher level of differentiation. Scale bar is 500 µm.

Fig 5. Wound healing (scratch) assay for hFOB and HUVEC cells. (A) Effect of 100 nM siponimod on hFOB cells, n=4 (3 technical replicates), (B) Effect of 100 nM siponimod on HUVEC cells, n=7 (3 technical replicates), (C) Siponimod concentration response over 1-100 nM on HUVEC cells, n=3 (3 technical replicates); (D) Effect of 100 nM S1P on HUVEC, n=2 (3 technical replicates). Data represents the percentage closure of the scratch wound after 8 h. "n=" represents the number of independently repeated experiments. Data is presented as

mean \pm S.D., statistical analysis by one-way ANOVA. *: P < 0.05. **: P < 0.01. ***: P < 0.001. E-J: Representative photomicrographs for the experiment shown in (B) are shown at 0 and 8 h: (E,H) vehicle (PBS) control, (F, I) positive control (FBS), and (G, J) 100 nM siponimod. The white arrowheads at the top and bottom of each photomicrograph show the edge of the manually created scratch wound. Images were acquired using a 4x objective, scale bars are 500 µm.

Fig 6. Transwell migration assay for HUVEC cells. (A) Effect of 100 nM siponimod under standard growth medium conditions (2 % serum) over 4 h, n=4 (3 technical replicates) (B) Effect of 100 nM siponimod under reduced serum conditions (1/10th standard cell culture supplement containing 0.2 % serum) over 4 h, n=5 (3 technical replicates) (C) Effect of 100 nM S1P alone and combined with 100 nM siponimod under reduced serum conditions for 4 h, n=3 (3 technical replicates) (D) Effect of 100 nM siponimod under reduced serum conditions over 8 h, n=5 (2 technical replicates) (E) migration over 24 h, n=3 (2 technical replicates. Data, presented as mean \pm S.D., represents the number of cells counted on the bottom side of a transwell membrane, expressed as a percentage (positive control set to 100%). "n=" represents the number of independently repeated experiments. Statistical analysis by one-way ANOVA; ns: No statistical significance. **: P < 0.01. ***: P < 0.001. (F-I) Representative photomicrographs for experimental conditions shown in (D): FBS (F), PBS (G), 100 nM siponimod (H) and 100 nM siponimod + 100 nM S1P (I). Scale bar: 500 µm.

Fig 7. Cell attachment assay for HUVEC cells. Effect of siponimod, S1P, and siponimod + S1P (all 100 nM) on cell attachment after 4 h incubation under reduced serum conditions, n=3 (2 technical replicates. Data, presented as mean \pm S.D., represents the number of cells attached to the well, expressed as a percentage (positive control set to 100%). "n=" represents the number of independently repeated experiments. Statistical analysis by one-way

ANOVA. NS: No statistical significance. ***: P < 0.001). (B-E) Representative images for experimental conditions shown in cell attachment assay: FBS (B), PBS (C), 100 nM siponimod (D) and 100 nM siponimod + 100 nM S1P (E). Scale bar: 500 μ m.

Fig 8. Effects of siponimod on intracellular cAMP in hFOB cells. The effect of 100 nM siponimod alone and in combination with 1 μ M forskolin, n=4 (3 technical replicates). Cells were incubated in the presence of the indicated drugs for 1 h. Data, presented as mean \pm S.D., represents the concentration of intracellular cAMP, expressed relative to 1 μ M forskolin. "n=" represents the number of independently repeated experiments. Statistical analysis by one-way ANOVA. ***: P < 0.001.





















