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Effect of sphingosine kinase modulators on interleukin-1β release, sphingosine 1-phosphate receptor 1 expression and experimental autoimmune encephalomyelitis

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FIGURES: 8, TABLES: 0Abstract

Background and Purpose: The sphingosine analogue, FTY720 (Gilenya^{R}) alleviates clinical disease progression in multiple sclerosis. Here we variously assessed the effects of an azide analogue of (*S*)-FTY720 vinylphosphonate (compound 5; a sphingosine kinase 1 activator), (*R*)-FTY720 methyl ether (ROMe, a sphingosine kinase 2 inhibitor) and RB-020 (a sphingosine kinase 1 inhibitor and sphingosine kinase 2 substrate) on IL-1β formation, sphingosine 1-phosphate levels and $S1P_1$ expression. We also assessed the effect of compound 5 and ROMe in an experimental autoimmune encephalomyelitis (EAE) model.

Experimental Approach: We measured IL-1β formation by macrophages, sphingosine 1-phosphate levels and $S1P_1$ expression levels *in vitro* and clinical score and inflammatory cell infiltration into the spinal cord *in vivo*.

Key Results: Treatment of differentiated U937 macrophages with compound 5, RB-020 or sphingosine (but not ROMe) enhanced IL-1β release. This data suggests these compounds might be pro-inflammatory *in vitro*. However, compound 5 or ROMe reduced disease progression and infiltration of inflammatory cells into the spinal cord in EAE and ROMe induced a reduction in $CD4^+$ and $CD8^+$ T-cell levels in the blood (lymphopenia). Indeed, ROMe induced a marked decrease in cell surface $S1P_1$ expression *in vitro*.

Conclusion and Implications: This is the first demonstration that an activator of SK1 (compound 5) and an inhibitor of SK2 (ROMe, which also reduces cell surface $S1P_1$ expression) have an anti-inflammatory action in EAE.

Introduction

Sphingosine 1-phosphate (S1P) is formed by the phosphorylation of sphingosine and this reaction is catalysed by two isoforms of sphingosine kinase (SK1 and SK2), which are encoded by different genes and exhibit distinct subcellular localisations, biochemical properties and functions (see Pyne & Pyne, 2011 for review). Once produced, S1P can either be exported from cells (through transporter proteins e.g. *Spns2* and certain ABC transporters) and act as a ligand on a family of five S1P-specific G protein coupled receptors ($S1P_{1-5}$) (Blaho & Hla, 2014) or, if retained within the cell, bind to and regulate specific intracellular target proteins. For instance, SK2 catalyses the formation of S1P in the nucleus and the subsequently formed S1P inhibits HDAC1/2 activity to induce *c-fos* and *p21* expression (Hait *et al*., 2009).

There is a strong link between S1P and multiple sclerosis (MS), which involves reactive T-lymphocytes in an autoimmune inflammatory demyelinating disease. Indeed, the sphingosine analogue FTY720 is licensed for oral treatment of relapsing MS under the trade name Gilenya^{TM}. FTY720 is a pro-drug, which is phosphorylated by SK2 and functionally antagonises sphingosine 1-phosphate receptor 1 ($S1P_1$), resulting in its proteasomal degradation and removal from T-lymphocytes (Hla and Brinkmann, 2011). This traps the T-lymphocytes in lymph nodes as they cannot egress due to their inability to sense an S1P gradient, which requires surface expression of $S1P_1$ on the T cell. The consequence of this is to prevent T-cell action on the CNS in MS.

Innate immunity to pathogens employs pathogen-associated molecular patterns (PAMPs) in inflammatory cells to stimulate IL-1β and IL-18 release (Schroder & Tschopp, 2010; Takeuchi & Akira, 2010). Danger-associated molecular patterns (DAMPs) that include sphingosine, released from dead cells, also induce innate immune responses (Chen & Nunez, 2010) by activating the NLRP3 ((NOD-like receptor family, pyrin domain containing 3) inflammasome**.** This involves assembly of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) with the inflammasome. Caspase-1 is then recruited to the complex where it cleaves pro-

IL-1β to produce IL-1β (Brough & Rothwell, 2007). There is also a link between *Nlrp3* and multiple sclerosis (MS) and between NLRP3 and sphingosine. Thus, IL-1β levels, *Nlrp3* gene mutation and MS-like lesions are correlated in the central nervous system (CNS) (Compeyrot-Lacassagne *et al*., 2009; Dodé, *et al*., 2002). Indeed, *Nlrp3*$^{-/-}$ mice exhibit milder symptoms in experimental autoimmune encephalomyelitis (EAE), an animal model for MS disease. In addition, sphingosine and the sphingosine analogue, FTY720 (2-amino-2-[2-(4-ocytlphenyl)ethyl]-1,3-propanediol) stimulate NLRP3-dependent release of IL-1β from LPS-stimulated macrophages (Luheshi *et al*. 2012). We also reported that sphingosine stimulates the caspase-1-dependent release of IL-1β from differentiated U937 macrophages via a mechanism that involves cathepsin B and lysosomal destabilisation (Boomkamp *et al*., 2016). The pro-inflammatory action of FTY720, in terms of promoting IL-1β release, and its anti-inflammatory action, in terms of inhibiting T-cell trafficking therefore appear opposed, although the latter is linked with the clinical efficacy of FTY720 in MS.

We have previously synthesised an azide analogue of (*S*)-FTY720 vinylphosphonate (a pan-S1P receptor antagonist (Valentine *et al*., 2011)): compound 5 ((*S,E*)-3-azido-3-(hydroxymethyl)-5-(4-octylphenyl)pent-1-enylphosphonic acid), which activates SK1 in lysates of HEK 293 cells over-expressing this enzyme and therefore is either a direct or indirect effect (Liu *et al*., 2013). Similarly, we have synthesised (*R*)-FTY720 methyl ether (ROMe; (*R*)-2-amino-2-(methoxymethyl)-4-(4-octylphenyl)butan-1-ol), which is a SK2 selective inhibitor (Lim *et al*., 2010) and RB-020 (1-(4-Octylphenethyl)piperidin-4-yl)methanol), which is a SK1 inhibitor and a SK2 substrate (Baek *et al*., 2013). Since these compounds are FTY720 analogues, we variously assessed their effect on IL-1β release, S1P levels and $S1P_1$ expression and compared the effect of compound 5 and ROMe in an experimental autoimmune encephalomyelitis (EAE) model of MS. In this regard, we have previously shown that PF-543 (which is an inhibitor of SK1 (Schnüte *et al*., 2012)) exacerbated EAE disease progression (Pyne *et al*., 2016). We have therefore tested the

hypothesis that compound 5 (SK1 activator) might abrogate EAE disease progression due to enhanced anti-inflammatory activity of SK1, particularly as this might offer novel approaches for therapeutic intervention in MS.

Methods

Cell Culture

All cells were maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. U937 monocytic cells (ATCC) were maintained in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% (v/v) foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (complete RPMI) and differentiated to macrophages using phorbol 12-myristate 13-acetate (PMA) as previously described by others (Boomkamp *et al*., 2016; Shepherd *et al*. 2004; Twomey *et al*. 1993). Briefly, cells were plated at 1×10^6 cells/ml in complete RPMI supplemented with 4 nM PMA. Medium was replenished after two days. After 4 days, the cells were cultured in complete RPMI in the absence of PMA for a further 24 hours. It is standard practice to differentiate U937 myeloid to macrophages to study inflammatory responses e.g. IL-1β release.

Human lung microvascular endothelial cells (HLMVECs) (Lonza, San Diego, CA, USA) were cultured in complete endothelial basal media (EBM-2) containing the growth factors and 10% (v/v) foetal bovine serum. Contact inhibited monolayers revealed typical cobblestone morphology and stained positive for acetylated LDL uptake.

CCL39 (Chinese hamster fibroblast) cells that were stably transfected with myc-tagged $S1P_1$ (Rutherford *et al*., 2013) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX™ supplemented with 10% (v/v) foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells grown to confluence on 12 well plates were quiesced for 24 hours prior to treatment with FTY720 (100 nM) or compound 5 (10 μM) or RB-020 (0.1-10 μM) or

ROMe (0.1-10 μM) for 24 hours. Cell lysates were analysed by SDS-PAGE and western blotted for proteins of interest (see below). CCL39 cells are used as standard for over-expression of recombinant proteins ($S1P_1$) in order to allow testing of compounds for target engagement.

LNCaP-AI (androgen-independent) prostate cancer cells were maintained in RPMI supplemented with 1% (v/v) L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) lipid-stripped foetal calf serum. LNCaP-AI cells are used as a model system since the effect of SK1 and SK2 inhibitors on SK1 expression in these cells is well established (Tonelli *et al*., 2010, McNaughton *et al*., 2016).

Interleukin-1β release

U937 cells were either untreated or treated with the indicated compounds alone for 1 hour or stimulated with 1 μg/ml LPS for 2 hours followed by the compounds, as indicated, for 1 hour. A series of experiments was performed with matched control and LPS treatments (where indicated) but in which not all compounds were necessarily assessed at the same time. Supernatants were collected and assayed for IL-1β protein expression by ELISA according to the manufacturer's instructions (Boomkamp *et al*., 2016).

Western Blotting

Following treatment, cells were lysed in sample buffer (62.5 mM Tris-HCl (pH 6.7), 0.5 M sodium pyrophosphate, 1.25 mM EDTA, 1.25% (w/v) sodium dodecyl sulphate, 0.06% (w/v) bromophenol blue, 12.5% (v/v) glycerol and 50 mM dithiothreitol). Proteins were separated on a 10% (v/v) acrylamide/bisacrylamide gel, and transferred to a nitrocellulose Hybond membrane. Membranes were blocked in 5% (w/v) bovine serum albumin (BSA) in TBST buffer (20mM Tris-HCl (pH 7.5), 48mM NaCl, 0.1% (v/v) Tween20) for 1 hour at room temperature prior to incubation with anti-myc tag primary antibody (diluted in blocking buffer) to detect myc-tagged

$S1P_1$ overnight at 4ºC. Following three washes in TBST, membranes were incubated with HRP-conjugated anti-IgG secondary antibody (diluted in blocking buffer) for 1 hour at room temperature. Immunoreactive protein bands were visualized using enhanced chemiluminescence. Blots were stripped and re-probed for ERK-2 or actin to ensure equal protein loading (Boomkamp *et al*., 2016).

FACS Analysis

Peripheral blood from naive, control or compound 5- or ROMe-treated mice was collected into EDTA to prevent clotting and red blood cells removed with RBC lysis buffer (eBioscience). Cells were added to FACS tubes ($0.5x10^6$ cells per tube) and incubated (30 minutes in the dark at 4°C) with FITC-conjugated anti-CD4 and PerCP-Cy5.5-conjugated anti-CD8 (1:500 dilution). Cells were washed and resuspended in 0.5 ml FACS buffer (1 % (w/v) BSA in phosphate buffered saline (PBS)) prior to quantification using a BD FACSCanto system and BD FACSDiva software (BD Biosciences). Similarly, CCL39 cells, grown in 12 well plates, were treated with vehicle (DMSO) or compounds, as described in the figure legends. These cells were added to FACS tubes ($0.2x10^5$ cells per tube) and incubated with allophycocyanin (APC)-conjugated $S1P_1$ antibody (1:50 dilution; 30 minutes in the dark at 4°C). Cells were washed twice with FACS buffer, resuspended in 0.25 ml FACS buffer with 12.5 µl propidium iodide (to allow exclusion of dead cells) and quantified.

S1P measurement

HLMVECs, cultured in 35 mm dishes to ~90% confluence, were labelled with 40 µCi/ml of [^{32}P] orthophosphate in phosphate free DMEM medium for 3 h. The radioactive medium was aspirated and cells incubated with vehicle (DMSO) or the compounds for 1 h in phosphate free DMEM medium containing 0.1% (w/v) fatty acid free BSA (1 ml final volume) for 1 h prior to addition of

exogenous sphingosine (2 μM) in 0.1% (w/v) fatty acid free BSA for an additional 1 h. Lipid were extracted by addition of 1 ml methanol containing 1% (v/v) 12 N HCl, scraped into tubes followed by an additional 1 ml of methanol containing 1% (v/v) 12 N HCl. To this methanolic extract, 2 ml of $CHCl_3$ and 1 ml of 0.1N NaOH were added, vortexed and the lower $CHCl_3$ layer collected, dried under N_2 and subjected to TLC for S1P separation and quantification (Tonelli *et al*., 2013).

EAE Mouse Model

C57BL/6 mice were purchased from Harlan (Oxford, UK) and maintained at the Biological Procedure Unit, University of Strathclyde. All experiments were performed under the guidelines of the UK Home Office Animals (Scientific Procedures) Act 1986 and were compliant with the ARRIVE guidelines for experiments involving animals (McGrath *et al*., 2010). Female mice at the age of 7–12 weeks were used in all experiments.

EAE Induction and Clinical Evaluation

EAE induction was performed as previously described (Jiang *et al*., 2009). Mice were immunized subcutaneously on the back with 100 μg of Myelin Oligodendrocyte Glycoprotein Peptide Fragment 35-55 (MOG_{35-55}) in 100 μl of PBS emulsified with an equal volume of Complete Freund's Adjuvent (CFA, total 350 μg of *Mycobacterium tuberculosis*, strain H37RA). Each mouse also received intraperitoneally (i.p.) 100 ng/100 μl of pertussis toxin (PTX) in PBS on days 0 and 2 post immunization. EAE was scored according to a 0 - 5 scale as follows: 0, no clinical sign; 1, complete loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb involvement; 5, moribund. In a previous study (in house), the maximum clinical score in EAE mice was 2.625 ± 0.7048 (mean ± SD, n=20). Assuming a change in clinical score of 1.4 to be of clinical relevance, the number of mice required to detect this at 80% statistical power (2 sided, 5% significance) was calculated to be 6 mice per group. 18 females from separate litters

born within 5 days of each other were used. These were then randomly divided them into 3 cages of 6 and each cage randomly designated to a specific treatment (Cage A = compound 5, Cage B = ROMe and Cage C = vehicle) in a non-blinded manner (due to available expertise).

To confirm that the infiltration of immune cells in the spinal cord tissue and subsequent inflammation and demyelination was EAE specific, control mice were immunised with a similar protocol including CFA and PTX, but with PBS in place of MOG_{35-55} peptide. Our data show that these mice displayed no signs of clinical disease, sickness or discomfort, and they gained weight throughout the course at a rate similar to naïve mice. The total number of mice employed in the study was 28.

Preparation of Compounds, Dosage and Route of Administration

All compounds were dissolved in a vehicle solution of 20% cyclodextrin in PBS. Compound 5 was prepared as a 1.55 mg/ml stock. The dosage given was 12.4 mg/kg (the approximate *in vivo* concentration is 30 μM assuming 100% bioavailability). ROMe was prepared as a 0.404 mg/ml stock. The dosage given was 3.234 mg/kg (the approximate *in vivo* concentration is 10 μM assuming 100% bioavailability). The dose of compound 5 and ROMe used in the EAE model was based on the EC_{50}/IC_{50} for SK activation/inhibition, respectively (Liu *et al*., 2013; Lim *et al*., 2010), and assumed 100% bioavailability. The compounds were all administered i.p. daily from day 0 of MOG_{33-55} immunization at the above doses. The EAE vehicle control group received i.p. injection daily of the 20% cyclodextrin vehicle.

Immunohistochemical Staining

Mice were euthanized in a CO_2 chamber and their spinal cords flushed out with PBS by hydrostatic pressure using a 19G syringe. Tissues were immediately frozen in OCT and 8μm thick sections were stained with specific primary antibodies for CD4, CD45 and F4/80 overnight.

Sections were then washed and incubated with biotinylated secondary antibody and streptavidin-HRP before being detected with ImmPACT AMEC red peroxidise substrate. Sections were then washed in distilled water and counterstained with haematoxylin. Isotypes with matching IgG were used as negative control and showed no staining in all tissues.

Statistical analysis

Statistical analysis employed unpaired t-test, one-way ANOVA with Bonferroni post-test, two-way ANOVA with Bonferroni post-test or repeated measures ANOVA (using GraphPad *Prism*™, V4.0, **(**GraphPad Software, San Diego)), as appropriate and as indicated in the figure legends. Data was considered to be significant when $p < 0.05$. Values of *n* for *in vitro* experiments are derived from independent experiments rather than within-experiment replicates. Densitometric values (represented as mean +/- SD) were normalized using the corresponding data for actin for the same samples and were obtained from 5 independent experiments. Statistical analysis was undertaken using one way ANOVA with Tukey's post hoc test.

Materials

Cell culture media (RPMI, DMEM with GlutaMAX™) and supplements (penicillin, streptomycin, L-glutamine) were obtained from Life Technologies (Paisley, UK). Foetal bovine serum was from Seralabs (Sussex, UK). Fine chemicals including phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), the caspase-1 inhibitor (Ac-YVAD-CHO), PP2A inhibitor (okadaic acid), pertussis toxin, cyclodextrin, HRP-conjugated anti-mouse IgG secondary antibody and haematoxylin were from Sigma (Poole, UK); propidium iodide was from eBioscience (Altrincham, UK). Antibodies employed included anti-myc (to detect myc-tagged $S1P_1$) (# sc-40, Santa Cruz, Wembley, UK), ERK-2 (# 610104, Transduction Laboratories, Oxford, UK), actin (# A2066, Sigma, Poole, UK), anti-$S1P_1$-APC (# FAB7089A, R & D Systems, UK), biotinylated

secondary antibody, anti-CD4, anti-CD45 and anti-F4/80 and streptavidin-HRP (eBioscience, Hatfield, UK). ImmPACT AMEC red peroxidise substrate was from Vector Laboratories, Peterborough, UK. Dithiothreitol was purchased from Enzo (Exeter, UK), nitrocellulose Hybond membrane from GE Healthcare (Little Chalfont, UK) and IL-1β protein ELISA kits from R&D Systems (Abingdon, UK), Myelin Oligodendrocyte Glycoprotein Peptide Fragment 35-55 (MOG_{35-55}) (Sigma Genosys, Haverhill, UK) and Complete Freund's Adjuvent from Difco (Detroit, MI). Compound 5, RB-020 and ROMe were synthesized as described previously (Liu *et al*., 2013; Lim *et al*., 2010; Baek *et al*., 2013). Compound 5 was gifted by Young Ah Kim (City University of New York).

Results

Effect of compound 5 and RB-020 on IL-1β release

We have previously shown that the azide analogue of (*S*)-FTY720 vinylphosphonate (compound 5) is an activator of SK1 and is not a substrate for either SK1 or SK2 (Liu *et al*., 2013). We have also shown that RB-020 is an SK1 inhibitor and SK2 substrate (Baek *et al*., 2013) and that (*R*)-FTY720 methylether (ROMe) has a K_i =16 μM for SK2 inhibition (Lim *et al*., 2011) with no inhibitory effect on SK1 at concentrations of 100 μM. See Fig. 1A for chemical structures. In addition, we have previously shown that SK1 inhibitors induce the proteasomal degradation of SK1 to create SK1 null cells (Tonelli *et al*., 2010; McNaughton *et al*, 2016). In androgen-sensitive prostate cancer cells, this can lead to apoptosis (Loveridge *et al*., 2010). Therefore, we tested the effect of compound 5, RB-020 and ROMe on SK1 expression levels in androgen-independent LNCaP-AI prostate cancer cells. We show here that the SK1 inhibitor, RB-020 induces a decrease in SK1 expression in LNCaP-AI cells but, as expected, the SK1 activator, compound 5 (10 μM) and the SK2 inhibitor, ROMe (0.1-10 μM) were without effect on SK1

expression (Fig. 1B). These findings are consistent with previous findings which demonstrate that the proteasomal degradation of SK1 is associated with SK1 inhibitors.

We have previously shown that ROMe modulates cellular sphingolipid levels with a reduction in S1P and an increase sphingosine levels (Watson *et al.,* 2013). Here we show that the SK1 inhibitor/SK2 substrate RB-020 and the SK1 selective nM potent inhibitor, PF-543 (Schnüte *et al*., 2012) reduce intracellular S1P, formed from exogenously added sphingosine in HLMVECs (Fig. 1C). In contrast, compound 5 increased S1P formation from exogenously added sphingosine in HLMVECs (Fig. 1C), consistent with this compound functioning as an activator of SK1 in these cells. Compound 5 activates recombinant SK1 by ~ 50% at 30 μM in lysates from HEK 293 cells over-expressing this enzyme (Liu *et al*., 2013) and this is consistent with the 28% increase in S1P formed from exogenously added sphingosine (Fig. 1C). Interestingly RB-020 is very heavily phosphorylated in HLMVECs (phosphorylated RB-020: 30527 ± 2304 dpm per 10^6 dpm in total phospholipids), consistent with our previous finding that RB-020 is a substrate for purified SK2 (Fig. 1C, Baek *et al*., 2013).

Given their chemical similarity with FTY720, we investigated the effect of compound 5 or sphingosine or RB-020 on IL-1β release from macrophages. We show here that treatment of differentiated U937 cells with compound 5 (10 μM) or FTY720 (10 μM) or sphingosine (20 μM) significantly increased IL-1β release and IL-1β release showed an upward trend in the presence of RB-020 (10 μM) ($p = 0.06$, Fig. 2A). There is evidence that PAMPs (e.g. LPS/TLR4) and DAMPs (e.g. sphingosine) can functionally interact to regulate NLRP inflammasome activity and IL-1β formation (Netea *et al*., 2009; Escamilla-Tilch *et al*., 2013; Luheshi *et al*. 2012). Therefore, we tested the effect of the compounds in the presence of LPS. LPS stimulated IL-1β release in differentiated U937 macrophages (Fig. 2B). Moreover, treatment of these cells with compound 5 (10 μM), FTY720 (10 μM) or RB-020 (10 μM) increased IL-1β release in the presence of LPS (Fig. 2B, upper panel). The effects of compound 5 was not additive with sphingosine (Fig. 3),

suggesting a common mechanism of action for this effect. To investigate whether sphingosine kinase inhibitors increase IL-1β release, we tested the effect of the SK2 selective inhibitor, ROMe and the SK1/2 inhibitor SKi, (2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole). We have previously demonstrated that ROMe (SK2 inhibitor) has no effect on LPS-induced IL-1β release (Boomkamp *et al*., 2016) and this is shown here (Fig. 2B, lower panel). In addition, SKi had no effect on IL-1β release in the presence of LPS (Fig. 2B, lower panel). The effect of compound 5 on IL-1β release in the presence of LPS was reduced by pretreatment of cells with the caspase-1 inhibitor, Ac-YVAD-CHO (Fig. 4A), thereby confirming a role for the inflammasome. Finally, treatment of cells with the PP2A inhibitor, okadaic acid (0.5 μM), had no effect on the ability of compound 5 to increaseIL-1β release in the presence of LPS (Fig. 4B).

Effect of compound 5 and ROMe on disease progression in EAE

We have previously shown that PF-543 (which is a nM potent inhibitor of SK1) exacerbates EAE disease progression (Pyne *et al*., 2016). Therefore, we tested the effect of the SK1 activator, compound 5 on EAE disease progression and compared it with the SK2 inhibitor, ROMe. Both compound 5 and ROMe markedly reduced EAE disease progression (clinical score) (Fig. 5A). The efficacy of these compounds is remarkable with 11 out of 12 mice exhibiting no symptoms whatsoever. EAE mice have a massive infiltration of immune cells in the spinal cord, which was not evident in PBS control tissues (Fig. 5B), confirming that the presence of immune cells in the CNS was EAE specific. Compound 5 or ROMe induced a considerable decrease in leukocyte infiltration including $CD45^+$ leukocytes, $CD4^+$ T-cells and $F4/80^+$ macrophages in the spinal cord of EAE mice (Fig. 5B). Immunohistochemical staining of spinal sections demonstrated that there are no infiltrating inflammatory cells in mice treated with compound 5 or ROMe (Fig. 5B). In addition, ROMe (but not compound 5) induced a reduction in $CD4^+$ and $CD8^+$ T-cell levels in the blood (lymphopenia) (Fig. 5C).

Effect of compound 5 and RB-020 on $S1P_1$ expression

Since FTY720, when converted by SK2 to FTY720-phosphate, is a functional antagonist of $S1P_1$ (by inducing proteasomal degradation of $S1P_1$) and this underlies part of its action in alleviating MS disease progression (Bigaud *et al*., 2014), we assessed the effect of compound 5, RB-020 or ROMe on $S1P_1$ expression levels in CCL39 cells that stably over-express myc-tagged $S1P_1$ (Boomkamp *et al*., 2016). This expression system is used for analyzing $S1P_1$ modifying agents as it is very well established that FTY720 (specifically FTY720 phosphate) induces proteasomal degradation of $S1P_1$ (Oo *et al*., 2007). In common with FTY720, which significantly reduced EAE severity in mice and $S1P_1$ levels in CCL39 cells (Boomkamp *et al*. 2016), compound 5, RB-020 or ROMe (0.1-10 μM) induced a reduction in $S1P_1$ levels (Supplementary Fig. 1). Indeed, proteolytic fragments of $S1P_1$ were detected in cells treated with compound 5 or RB-020 (Supplementary Fig. 1). We also assessed the cell surface expression of $S1P_1$ on CCL39 cells by FACS analysis. Treatment of CCL39 cells with RB-020, ROMe (0.1-10 μM) or FTY720 induced a 70-80% reduction cell surface expression of $S1P_1$ while compound 5 induced a ~ 40% reduction (Fig. 6). Therefore, the ROMe-induced reduction in $CD4^+$ and $CD8^+$ T-cell levels in the blood might be determined by a threshold cell surface $S1P_1$ expression level.

Discussion

We have presented *in vivo* data to show that ROMe and compound 5 exhibit remarkable efficacy in reducing disease progression in an EAE model. However, the mechanisms by which this occurs differ. We demonstrate that compound 5 (unlike ROMe) does not induce a reduction in $CD4^+$ and $CD8^+$ T-cells levels in the blood. Indeed, ROMe recapitulates the effect of FTY720 (in use for MS therapy (as Gilenya^R)) in substantially reducing cell surface $S1P_1$ expression (> 70%) in CCL39 cells. Therefore, it is possible that the effect of ROMe on EAE progression

involves modulation of $S1P_1$ and inhibition of T-lymphocyte trafficking from lymph nodes. However, the effects on EAE disease progression might also be related to the ability of ROMe to inhibit SK2 (Fig. 7), which is known to affect gene expression (Hait *et al*., 2009), as this compound induces a marked reduction in the infiltration of leukocytes and F4/80 macrophages into the spinal cord of EAE mice. Indeed, SK2 knockout mice are protected from EAE and this is associated with a partial lymphopenia (Imeri et al., 2016). In addition, S1P enhances the development of Th17 cells (Liao et al., 2007), which are implicated in the pathogenesis of MS and it is possible that SK2 regulates this compartment specific pool of S1P. Th17 cells were found primarily within central memory T cells and FTY720 reduced blood central memory T cells, including RORγt and IL-17-producing T cells, by >90% (Mehling et al., 2010). S1P also inhibits Tregs function, thereby preventing the suppressive effect of Tregs on Th17 formation (Liu et al., 2009). Therefore, inhibition of S1P formed by SK2 might block these processes to alleviate EAE disease progression. There is also close link between Th17 and Th1. Indeed, Th1 cells can be formed from Th17 (Harbour et al., 2015). In this regard, IL-12 is a Th1 cytokine and the IL12β1 receptor associates with SK2 and increases IL-12-induced STAT4-mediated transcriptional activity while dominant negative SK2 in Th1 cells reduced IL-12 stimulated IFNγ formation and WT SK2 enhanced it (Yoshimoto et al., 2003). Finally, the SK2 inhibitor, ABC294640 has been shown to be anti-inflammatory, consistent with the findings here with ROMe. ABC294640 reduced TLR4 expression, NF-κB activation, pro-inflammatory cytokine/chemokine production, adhesion molecule expression and the infiltration of monocytes/macrophages and neutrophils (Liu et al., 2012).

The lack of effect of the SK1 activator, compound 5 on $CD4^+$ and $CD8^+$ cell levels in the blood is consistent with its lesser effect on cell surface expression of $S1P_1$ in CCL39 cells, i.e. $S1P_1$ levels may not fall below a threshold required to prevent egress from lymph nodes *in vivo*. The mechanism by which compound 5 affects $S1P_1$ expression levels might involve direct binding

of this compound to the receptor and subsequent degradation of the receptor (Fig. 7). This is based on the finding that the parent compound, (*S*)-FTY720 vinylphosphonate is able to bind to and antagonise $S1P_1$ (Valentine *et al*., 2010). However, the effect of compound 5 *in vivo* involves a novel anti-inflammatory mechanism of action that is distinct from that of FTY720, whereby SK1 regulates a compartment specific pool of S1P which functions in an ant-inflammatory manner (Fig. 7). This is supported by the demonstration that SK1 is a negative regulator of Th1 and Th0 cells and siRNA knockdown of SK1 enhances IL-2, TNFα and IFNγ release in response to TCR stimulation (Yang et al., 2005). Moreover, over-expression of SK1 reduces these effects in response to TCR stimulation (Yang et al., 2005). In addition, the loss of SK1 potentiates induction of the pro-inflammatory chemokine RANTES and multiple chemokines and cytokines (Adada et al., 2013). Significantly our findings are the first to demonstrate that an activator of SK1 has this (or any) effect *in vivo*.

The effect of compound 5 on disease progression in the EAE model does not appear to be hindered by its stimulatory effects on IL-1β release, as detected in an *in vitro* cellular system (Fig. 7). Indeed, the major effect of RB-020 and compound 5 on IL-1β release more likely relates to their ability to mimic sphingosine and FTY720 and this is supported by the finding that both sphingosine and FTY720 enhance LPS-stimulated IL-1β release. However, SK inhibitors such as ROMe or SKi do not increase IL-1β. One possible explanation is that these SK inhibitors might not induce sufficient accumulation of sphingosine and/or promote sphingosine formation in the correct cellular compartment where IL-1β processing occurs. In addition, despite structural similarity to FTY720, the failure of ROMe to induce IL-1β release suggest that the 3-OH group in FTY720 and sphingosine is required for inflammasome-dependent stimulation of IL-1β release.

The effectiveness of ROMe and compound 5 in reducing EAE disease progression is consistent with a pharmacological mode of action on S1P signalling. We therefore conclude that combined modulation of SK1 (activation) and SK2 activity (inhibition) and $S1P_1$ (down-

regulation) might provide an effective anti-inflammatory action to ablate disease progression in multiple sclerosis. Our data is also consistent with the partial protection from EAE observed in inducible Sgpl1 (S1P lyase) knockout mice (Billich *et al*., 2013) and the anti-inflammatory effect of S1P lyase inhibitors (Bagdanoff *et al*., 2010).

Therefore, we have demonstrated that compound 5 exhibits a novel mechanism of action compared with FTY720 and this provides the rationale for future development of SK1 activators in the treatment of MS.

Author contributions

Conception and design – SP, NJP, HRJ; acquisition of data and analysis – SP, SDB, MB, NMacR, MMcN; Data interpretation – NJP, SP, HRJ; Drafting the work and critical revision – NJP, SP, HRJ, MB, SDB.

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Figure legends

Figure 1. Chemical structures and effects on SK1 expression and S1P formation in cells. (A) Chemical structures of FTY720, compound 5, RB-020 and ROMe. (B) Western blot showing the effect of compound 5 (10 μM), RB-020 (10 μM) and ROMe (10 μM) on the expression levels of SK1 in LNCaP-AI cells. Actin was used as a loading control. Results are representative of 5 independent experiments. Also shown is the densitometric quantification represented as a bar graph. * $p < 0.05$ *versus* control. (C) Bar graphs showing the effect of compound 5 (30 μM), RB-020 (10 μM) and PF-543 (100 nM) on S1P formation from exogenously added sphingosine in HLMVECs. Results are expressed as means +/- SD for three independent experiments * $p < 0.05$ (one-way ANOVA with Bonferroni post-test; basal [^{32}P]S1P = 718 + 169dpm; plus sphingosine [^{32}P]S1P = 6154 + 569 dpm).

Figure 2. Compound 5, RB-020 and FTY720, but not ROMe or SKi, enhance IL-1β release in the presence and absence of LPS in differentiated U937 cells. Differentiated U937 cells were treated with (A) sphingosine (20 μM) , compound 5, FTY720 or RB-020 (all at 10 μM) alone for 2 hours or (B) LPS (1 μg/ml) for 2 hours and compound 5, RB-020, FTY720, ROMe or SKi (all at 10 μM) for a further 1 hour and the supernatants assayed by ELISA for IL-1β released (pg/ml). (A) Results are expressed as means +/- SEM for independent experiments with matched controls (n=5 for Sph, FTY720, RB-020 and n=6 for compound 5); * $p < 0.05$ *versus* control using an unpaired t-test. (B) Results are expressed as means +/- SEM for independent experiments with matched controls and LPS (n=5, ROMe panel; n=6, compound 5, RB-020 and SKi panels; n=8; FTY720 panel); * $p < 0.05$ *versus* control using one-way ANOVA with Dunnett's post-test; ** $p < 0.05$ *versus* LPS response and † not significant *versus* LPS response using one-way ANOVA with Bonferroni post-test.

Figure 3. Sphingosine or compound 5 enhances IL-1β release in the presence of LPS in a non-additive manner. Differentiated U937 cells were treated with LPS (1 μg/ml) for 2 hours

and then with sphingosine (20 μM) or compound 5 (10 μM) or both sphingosine and compound 5 for 1 hour and the supernatants assayed for IL-1β by ELISA. Results are expressed as means (IL-1β (pg/ml) released) +/- SEM for 6 independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni post-test. * $p < 0.05$ *versus* LPS alone; † no significant difference *versus* LPS/Sph or LPS/compound 5.

Figure 4. The effect of the Caspase-1 inhibitor Ac-YVAD-CHO or the PP2A inhibitor, okadaic acid on the enhancement of IL-1β release by compound 5 in the presence of LPS. Differentiated U937 cells were treated with vehicle or Ac-YVAD-CHO (10 μM) or okadaic acid for 15 min (black bars), stimulated with LPS (1 μg/ml) for 2 hours prior to treatment with compound 5 (10 μM) for 1 hour and the supernatants assayed for IL-1β by ELISA. (A) The effect of AC-YVAD-CHO on the increase in IL-1β release by compound 5 in the presence of LPS; (B) The lack of effect of okadaic acid on the increase in IL-1β release induced by compound 5 in the presence of LPS. Results are expressed as means (IL-1β (pg/ml) released) +/- SEM for 4 (Ac-YVAD-CHO) or 3 (okadaic acid) independent experiments. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test. No significant difference between LPS/Compound 5 *versus* LPS/compound 5/okadaic acid. * $p < 0.05$ for LPS/Compound 5 *versus* LPS/compound 5/Ac-YVAD-CHO.

Figure 5. Compound 5 or ROMe attenuate disease progression in EAE. EAE-treated mice were treated with vehicle alone or dosed with compound 5 (12.4 mg/kg) or ROME (3.23 mg/kg). (A) Clinical scores are plotted against time and are represented as means ± SEM for n=6 mice/treatment. Repeated Measures ANOVA was carried out. * $p < 0.05$ for compound 5 or ROMe *versus* vehicle. (B) Immunohistochemical staining of representative spinal sections with specific anti-CD4, anti-CD45 or anti-F4/80 antibodies. Arrows denote infiltrating cells. Results are representative of n=6 mice/treatment. We have included immunohistochemical staining of spinal sections from control mice (representative of results from 10 naïve mice) to show that there

are no infiltrating inflammatory cells. (C) FACS analysis of $CD4^+$ and $CD8^+$ cells. FACS plots are representative of n=6 animals/treatment group for vehicle, compound 5 and ROMe and 3 for naïve animals. Graphs show combined data; bars represent mean ± SEM. One way ANOVA was carried out. * $p < 0.05$ *versus* vehicle.

Figure 6. Analysis of S1P receptor surface expression in CCL39 cells. FACS analysis of CCL39 cells pretreated with compound 5 (30 μM), RB-020 (10 μM), ROMe (100 nM or 10 μM), or FTY720 (1 μM), on cell surface $S1P_1$ expression. Graphs show combined data; bars represent mean ± SEM for 5 independent (or for 100 nM ROMe, three independent experiments) experiments. One way ANOVA was carried out. * $p < 0.05$ *versus* control.

Figure 7. Schematic summarising the observed effects of ROMe and compound 5 in inflammatory cells involved in EAE and multiple sclerosis disease progression

Supplementary Figure 1. The effect compound 5, ROMe and RB-020 on myc-tagged $S1P_1$ expression in CCL39 cells. CCL39 cells stably over-expressing myc-tagged $S1P_1$ were treated with FTY720 (100 nM), compound 5 (10 μM), ROMe (0.1-10 μM) or RB-020 (0.1-10 μM) for 24 h. Cell lysates were then western blotted with anti-myc antibody. ERK-2 was used as a protein loading control. Results are representative of 3 independent experiments.

TARGETS	
Other protein targets[a]	**Enzymes**[e]
PAMP	Sphingosine kinase 1
DAMP	Sphingosine kinase 2
NLRP3	Sphingosine 1-phosphate lyase
GPCRs[b]	HDAC1
$S1P_1$	HDAC2
	Caspase 1
	ERK-2
	Hormone sensitive lipase (HSL)
	PKA

LIGANDS	
FTY720	ROMe
FTY720 phosphate	Sphingosine 1-phosphate (S1P)
IL-1β	Lipopolysaccharide (LPS)
IL-18	IL-12
PF-543	PMA
Sphingosine kinase inhibitor (SKi)	Okadaic acid

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e]Alexander et al., 2015a,b,c,d,e).*

ABBREVIATIONS

Compound 5: (*S,E*)-3-azido-3-(hydroxymethyl)-5-(4-octylphenyl)pent-1-enylphosphonic acid; DAMPs, Danger associated molecular patterns; DMEM, Dulbeccos Modified Eagles Medium; EAE, experimental autoimmune encephalomyelitis; ERK, extracellular signal regulated kinase; FTY720, 2-amino-2-[2-(4-ocytlphenyl)ethyl]-1,3-propanediol; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family, pyrin domain containing 3; IFNγ, interferon gamma; IL-1β, interleukin-1beta; PAMPs, Pathogen-associated molecular patterns; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; RB-020, 1-(4-Octylphenethyl)piperidin-4-yl)methanol; ROMe, (*R*)-FTY720 methyl ether, (*R*)-2-amino-2-(methoxymethyl)-4-(4-octylphenyl)butan-1-ol; SK, sphingosine kinase; S1P, sphingosine 1-phosphate; $S1P_1$, sphingosine 1-phosphate receptor-1; TLR, Toll-like receptors.