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ROLE OF SPHINGOSINE 1-PHOSPHATE RECEPTORS, SPHINGOSINE KINASES AND SPHINGOSINE IN CANCER AND INFLAMMATION

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Abstract--Sphingosine kinase (there are two isoforms, SK1 and SK2) catalyses the formation of sphingosine 1-phosphate (S1P), a bioactive lipid that can be released from cells to activate a family of G protein-coupled receptors, termed $S1P_{1-5}$. In addition, S1P can bind to intracellular target proteins, such as HDAC1/2, to induce cell responses. There is increasing evidence of a role for S1P receptors (e.g. $S1P_4$) and SK1 in cancer, where high expression of these proteins in ER negative breast cancer patient tumours is linked with poor prognosis. Indeed, evidence will be presented here to demonstrate that $S1P_4$ is functionally linked with SK1 and the oncogene HER2 (ErbB2) to regulate mitogen-activated protein kinase pathways and growth of breast cancer cells. Although much emphasis is placed on SK1 in terms of involvement in oncogenesis, evidence will also be presented for a role of SK2 in both T-cell and B-cell acute lymphoblastic leukemia. In patient T-ALL lymphoblasts and T-ALL cell lines, we have demonstrated that SK2 inhibitors promote T-ALL cell death via autophagy and induce suppression of c-myc and PI3K/AKT pathways. We will also present evidence demonstrating that certain SK inhibitors promote oxidative stress and protein turnover via proteasomal degradative pathways linked with induction of p53- and p21-induced growth

arrest. In addition, the SK1 inhibitor, PF-543 exacerbates disease progression in an experimental autoimmune encephalomyelitis mouse model indicating that SK1 functions in an anti-inflammatory manner. Indeed, sphingosine, which accumulates upon inhibition of SK1 activity, and sphingosine-like compounds promote activation of the inflammasome, which is linked with multiple sclerosis, to stimulate formation of the pro-inflammatory mediator, IL-1β. Such sphingosine like compounds could be exploited to produce antagonists that diminish exaggerated inflammation in disease. The therapeutic potential of modifying the SK-S1P receptor pathway in cancer and inflammation will therefore, be reviewed.

Introduction--Formation of the bioactive lipid, sphingosine 1-phosphate (S1P) is catalysed by sphingosine kinase. There are two isoforms of sphingosine kinase (SK1 and SK2) which differ in their subcellular localisations, regulation and functions (Pyne et al., 2009). The S1P formed by these enzymes can either be exported from cells (through transporter proteins e.g. *Spns2*) and act as a ligand on a family of five S1P-specific G protein coupled receptors ($S1P_{1-5}$) (Blaho and Hla, 2014) or can bind to specific intracellular target proteins. For instance S1P formed by nuclear SK2 inhibits HDAC1/2 activity to induce *c-fos* and *p21* expression (Hait et al., 2009). Dephosphorylation of S1P is catalysed by S1P phosphatase and the sphingosine formed is then acylated to ceramide catalysed by ceramide synthase isoforms (Stiban et al., 2010). S1P can also be irreversibly cleaved by S1P lyase to produce (*E*)-2 hexadecenal and phosphoethanolamine (Degagné et al., 2014). The interconversion of ceramide to sphingosine and S1P has been termed the sphingolipid rheostat (Newton et al., 2015). In this model, shifting the balance toward ceramide induces apoptosis, while predominance of S1P formation promotes cell survival. For instance, ceramide activates protein phosphatase 2A (Dobrowsky et al., 1993), which dephosphorylates phosphorylated AKT (Zhou et al., 1998) and thereby alters BAD/Bcl2 regulation to induce apoptosis (Zundel and Giaccia, 1998). In contrast, S1P promotes cell survival, involving for instance, activation of the extracellular signal regulated kinase-1/2 (ERK-1/2) pathway (Pyne et al., 2009). However, the sphingolipid rheostat exhibits greater complexity, as certain ceramide species regulate processes other than apoptosis, such as autophagy and proliferation. This suggests temporal and spatial regulation, where the functionality of the sphingolipid rheostat is governed by compartmentalised signalling involving, for instance, ceramide synthase isoforms that produce different ceramide species with specific stress-dependent signalling functions that govern a defined cellular outcome e.g. apoptosis *versus* proliferation. The conversion of S1P to (*E*)-2 hexadecenal and phosphoethanolamine is also considered an exit point in the

sphingolipid metabolic pathway, but (*E*)-2 hexadecenal has potential signalling functions (Kumar et al., 2011) and both (*E*)-2 hexadecenal and phosphoethanolamine can be further metabolised to produce phospholipids that have additional defined signalling functions in cells (Nakahara et al., 2012). Therefore, the regulation of the sphingolipid rheostat in different cellular compartments is likely to impact significantly on lipid signalling pathways that regulate cell context specific physiology and pathophysiology.

S1P receptors, sphingosine kinase and cancer--S1P has been implicated in regulating cellular processes, some of which underlie the hallmarks of cancer. First, over-expression of SK1 promotes the Ras dependent transformation of fibroblasts into fibrosarcoma (Xia et al., 2000). Second, S1P promotes neovascularisation of tumours (LaMontagne et al., 2006). Third, S1P promotes inflammatory responses involved in cancer progression. Thus, S1P enhances colitis associated cancer via an amplification loop involving SK1, $S1P_1$, NFκB, STAT3 and IL-6 (Liang et al., 2013; Pyne and Pyne, 2013). Fourth, SK1 maintains the survival of cancer cells (a process termed 'non-oncogenic' addiction (Vadas et al., 2008)), promotes acquisition of replicative immortality and drives hormone-independent growth in prostate and breast cancer (for review see Pyne and Pyne, 2010). Fifth, S1P binding to $S1P_1$/$S1P_3$ promotes growth and enhances migration of cancer cells (for review see Pyne and Pyne, 2010) and SK1 regulates microenvironmental interaction between cancer cells and tumour associated myofibroblasts to promote metastasis (Albinet et al., 2014; Pyne and Pyne, 2014). Sixth, inhibition/down-regulation of SK1 blocks the Warburg effect to which cancer cells are addicted for ATP production and anabolic metabolism (Watson et al., 2013).

From a clinical perspective, we have established that high tumour expression of SK1 is associated with reduced survival and increased disease recurrance rates in estrogen receptor

(ER)-positive breast cancer patients (Watson et al., 2010; Long et al., 2010a; Ohotski et al., 2013). In addition, larger, more vascularised treatment resistant tumours are formed when cancer cells over-expressing SK1 are injected or implanted into mice (for review see Pyne and Pyne, 2010). There is also a substantial body of evidence to demonstrate a role for S1P receptors in cancer cell migration (for review see Pyne and Pyne, 2010). Moreover, we established that high $S1P_1$ and $S1P_3$ expression in tumours of ER positive breast cancer patients are associated with poor prognosis (Watson et al., 2010). We have also used a breast cancer cell line (MCF-7 cells) to demonstrate that S1P binding to $S1P_3$ stimulates the accumulation of phosphorylated ERK-1/2 into membrane ruffles/lamellipodia and the nucleus to promote migration of MCF-7 breast cancer cells (Long et al., 2010a). Moreover, S1P binding to $S1P_3$ promotes the re-localisation of SK1 from the cytoplasm to membrane ruffles/lamellipodia of MCF-7 cells (Long et al., 2010a), thereby demonstrating functional regulation of SK1 by S1P/$S1P_3$. In addition, siRNA knockdown of SK1 reduces $S1P_3$ expression and ERK-1/2 activation in response to S1P, indicating that SK1 and $S1P_3$ function in an amplification loop to promote ER positive breast cancer progression (Long et al., 2010a). Therefore, SK1 regulates the expression level of $S1P_3$, and this enables ER positive breast cancer cells to potentially match S1P released into the microenvironment with the required effector response.

We also reported that high tumour expression of $S1P_4$ and SK1 are associated with shortened disease-specific survival and disease recurrence times in patients with ER negative breast cancer (Ohotski et al., 2012). Stratification of the patient cohort to include only ER, PgR, HER2 negative tumours established a detrimental effect of tumour $S1P_4$ receptor expression on disease-specific survival (Pyne et al., 2012). Therefore, $S1P_4$ is an important biomarker for poor prognosis in ER negative breast cancer patients (Pyne et al., 2012). Furthermore,

SK1 and $S1P_4$ are functionally linked in ER negative MDA-MB-453 cells as S1P stimulation of the ERK-1/2 pathway mediated by $S1P_4$ is blocked by SK1 inhibitors (Ohotski et al., 2012). We have also demonstrated that oncogenic HER2/ErbB2 functionally interacts with $S1P_4$ in ER negative MDA-MB-453 breast cancer cells (Long et al., 2010b). Thus, S1P binding to $S1P_4$ in these cells stimulates activation of ERK-1/2 and this is contingent on HER2 and is independent of EGF receptor transactivation. Therefore, siRNA knockdown of HER2 or $S1P_4$, but not EGF receptor or $S1P_2$ receptor ablated S1P-stimulated ERK-1/2 activation and this was recapitulated using the ErbB2 inhibitor II, but not the EGF receptor tyrosine kinase inhibitor, AG1478 (Long et al., 2010b). Moreover, treatment of MDA-MB-453 breast cancer cells with S1P stimulated the tyrosine phosphorylation of HER2 and this was blocked by the $S1P_{2/4}$ antagonist, JTE-013 (Long et al., 2010b). Therefore, the $S1P_4$-HER2 signalling platform enhances signalling gain in response to S1P (**Figure 1**); this is significant as ERK-1/2 is implicated in promoting metastasis. Indeed, high $S1P_4$ expression in tumours of ER negative breast cancer patients is also correlated with node positive status, suggesting a role for $S1P_4$ in metastasis (Ohotoski et al., 2012; Pyne et al., 2012). The link between S1P and metastasis is further evidenced by studies from Ponnusamy et al. (2012), who demonstrated a reduction in systemic S1P inhibits TRAMP-induced prostate cancer growth in $TRAMP^{+/+}Sk1^{-/-}$ mice or lung metastasis of various cancer cells in $Sk1^{-/-}$ mice. SK1 loss promotes the expression of breast carcinoma metastasis suppressor 1 (Brms1). Furthermore, S1P binding to $S1P_2$ reduces Brms1 expression in these cancer cells. The finding that host S1P can increase dissemination of breast cancer cells suggests complex interplay between these cancer cells and the microenvironment. This is supported by the studies of Albinet et al. (2014), who demonstrated an important role of melanoma SK1 in promoting the differentiation of fibroblasts into myofibroblasts. The myofibroblast SK1 then

stimulates S1P-dependent metastasis of melanoma cells by providing S1P that is released and binds to the $S1P_3$ receptor on melanoma cancer cells (Albinet et al., 2014).

We have also identified a functional link between $S1P_4$ and $S1P_2$ receptors in ER negative breast cancer cells. Thus, pre-treatment of HA-$S1P_2$ over-expressing MDA-MB-231 cells with the $S1P_4$ antagonist CYM50367 or SK2 inhibitors or with $S1P_4$ or SK2 siRNA promotes the nuclear accumulation of HA-tagged $S1P_2$ and this is associated with growth arrest (Ohotski et al., 2014). These findings identify for the first time a signalling pathway in which $S1P_4$ functions to prevent nuclear translocation of $S1P_2$ to promote the growth of ER negative breast cancer cells. We proposed that heterodimerisation of $S1P_2$ and $S1P_4$ at the plasma membrane in response to S1P might provide a molecular trap that prevents translocation of $S1P_2$ to the nucleus. This suggests that plasma membrane and nuclear $S1P_2$ might elicit opposite effects on cancer progression. Thus, plasma membrane $S1P_2$ might function in concert with $S1P_4$ to promote cancer progression, while nuclear $S1P_2$ is associated with growth arrest. Tyrosine phosphorylated Src (Y416) also translocates to the nucleus of MDA-MB-231 cells treated with SK2 inhibitors or SK2 siRNA, but not $S1P_4$ siRNA (Ohotski et al., 2014).

SK2 and T-ALL--Acute lymphoblastic leukaemia is the most common type of paediatric cancer, affecting approximately one in every 2,000 children, mostly at between 2 and 5 years of age. Despite aggressive treatment approaches, including transplantation and new salvage regimens, most children with relapsed T-cell acute lymphoblastic leukaemia (T-ALL) will not be cured. In contrast, the clinical prognosis for adults with T-ALL, which is more common, is very poor indeed, with cure rates of, at best, 40%. T-ALL represents a malignant disorder arising from the neoplastic transformation of T-cell progenitors. Treatment options

include radiation therapy and prednisolone or dexamethasone, vincristine, asparaginase (paediatric patients) and daunorubicin (used in adult ALL). The development of chemo-resistance to these established therapeutic agents is a significant problem for effective treatment of this cancer. Current novel approaches for treatment of T-ALL include targeting phosphatidylinositol 3-kinase (PI3K)/mTOR and Notch1 signalling (gamma-secretase inhibitors). However, PI3K/mTOR inhibitors increase Notch-c-Myc activity and this can result in chemo-resistance in T-ALL (Shepherd *et al.* 2013). In addition, one major side effect of first-line Notch inhibitors is gastrointestinal toxicity and diarrhoea (Garber, 2007). Notch inhibitors might also promote certain cancers as Notch is a tumor suppressor (Nicolas *et al*. 2003). Therefore, there is a significant unmet medical need in the treatment of T-ALL and new and more effective therapeutic strategies displaying less toxicity and lack of chemo-resistance are required.

We have synthesised two structurally distinct, moderately potent, SK2-selective inhibitors ((*R*)-FTY720 methylether (ROMe), K_i =16 μM (Lim et al., 2011a) and F-02 ((2*S*, 3*R*) sphinganine derivative), K_i = 22 μM (Byun et al., 2013). ROMe induces the autophagic death of T-ALL cell lines and patient lymphoblasts (EC_{50} = 10 μM; (Evangelisti et al., 2014)) and no resistant sub-population of cells survive. Similarly, F-02 kills the entire population of T-ALL cells. Significantly, treatment of T-ALL cells with ROMe also reduces phosphorylated AKT (S473) and c-Myc levels, which are prognostic markers for T-ALL disease progression. Moreover, SK2 inhibitor genetic signatures are correlated to a publicly available gene expression dataset derived from paediatric T- and B-ALL patients (Wallington-Beddoe et al., 2014). In addition, SK2 has been implicated in maintaining cancer cell survival in various tumours. Thus, siRNA knockdown of SK2 expression has been shown to induce autophagic death of several different cancer cell lines (Gao & Smith, 2011) and S1P, formed by SK2, can

inhibit HDAC1/2 to induce epigenetic regulation of genes involved in B-ALL, such as c-Myc (Hait et al., 2009; Wallington-Beddoe et al., 2014). Significantly, the death of T-ALL cells induced by ROMe is reversed by inhibitors of autophagy but not apoptosis (Evangelisti et al., 2014). In addition, preliminary studies demonstrate that shRNA knockdown of SK2 in T-ALL cells induces autophagy and reduces colony formation (unpublished data, Evangelisti, Martelli, Pyne and Pyne). The key issue here is that the moderately potent SK2 inhibitor, ROMe kills the entire population of T-ALL cells (i.e. no sub-population survives). This contrasts with the effect of a moderately potent inhibitor of both SK1 and SK2 activity. For example, SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole)) kills only ~70% of the T-ALL cell population. A residual sub-population survives because of an endoplasmic reticulum stress response that is induced by SK1 inhibition. This effect appears to over-ride the concurrent inhibition of SK2 activity, thereby preventing autophagic death. Consistent with this, the highly selective inhibitor of SK1, PF-543, K_i = 14 nM (Byun et al., 2013) fails to kill T-ALL cells. These findings provide a strong rationale for the development of highly selective SK2 inhibitors for treatment of B-ALL and T-ALL.

To date, we have used X-ray crystallography and structural activity relationship/homology modelling to deduce the requirements for producing highly potent and selective SK1 and SK2 inhibitors. In this regard, we are the first group to successfully crystallize SK1 bound with PF-543 in the sphingosine binding site at 1.8 Å resolution (Wang et al., 2014). Using our existing homology model and the crystal structure of PF-543-bound SK1, we have identified the key structural requirements to promote inhibitor selectivity for SK2 over SK1. Our models suggest that the region that accommodates the head group of the SK2 selective inhibitor, ROMe, appears more restricted in SK1 than SK2 due to two key amino acid differences (V304/L517 in SK2 corresponding to I260/M358 in SK1b, which contains an 86

amino-acid N-terminal extension on SK1a). Compounds such as ROMe with 3D-steric demand in this region impart selectivity for SK2 over SK1.

Ubiquitin-proteasomal degradation of SK1--We have synthesised and characterised a number of structurally similar SK1 selective orthosteric inhibitors, including RB-005 (1-(4-octylphenethyl)piperidin-4-ol) (Baek et al., 2013) and 55-21 (1-deoxysphinganine analogue) (Byun et al., 2013) and allosteric SK1 inhibitors, such as (*S*)-FTY7200 vinylphosphonate (Lim et al., 2011b). A common feature of these inhibitors is that they induce the ubiquitin-proteasomal degradation of SK1 in solid cancer cell lines (Loveridge et al., 2010; Tonelli et al., 2010; Lim et al., 2011b) and proliferating vascular smooth muscle cells (Loveridge et al., 2010; Baek et al., 2013; Byun et al., 2013). This leads to inhibition of DNA synthesis (Byun et al., 2013) and is recapitulated by siRNA knockdown of SK1 expression.

The ubiquitin-proteasomal degradation of SK1 is correlated with either binding and/or inhibition of SK1 catalytic activity. Thus, PF-543, which exhibits a K_i of 14nM for SK1 induces proteasomal degradation of SK1 at nM concentrations in solid cancer cells (Byun et al., 2013). Therefore, the ubiquitin-proteasomal degradation of SK1 appears to be conformationally directed by inhibitor binding. Nevertheless, PF-543 lacks cytotoxic effects in 1483 head and neck cancer cells (Schnute et al., 2012). However, the complexity of the sphingolipid rheostat is not well understood, and PF-543 might inhibit other sphingolipid enzymes that neutralise the effect of inhibiting/down-regulating SK1 activity. For instance, the accumulation of ceramide is essential for induction of apoptosis, and PF-543 fails to modulate ceramide levels in 1483 head and neck cancer cells (Schnute et al., 2012). Indeed, we have shown that the SK1 selective inhibitor RB-005 also inhibits CerS5 activity, which would prevent ceramide formation (unpublished data, Pyne, Pyne and Futerman).

However, other very poor inhibitors of SK1 and SK2 such as SKi (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole)) also induce the proteasomal degradation of SK1 via a mechanism that is only partially dependent on direct binding to SK1 (Loveridge et al., 2010). We have demonstrated that SKi induces the proteasomal degradation of SK1a and SK1b (which has an 86 amino-acid N-terminal extension compared to SK1a) in androgen-sensitive LNCaP prostate cancer cells and this results in a reduction in S1P levels and an increase in sphingosine and C22:0 and C24:0 ceramide levels and the induction of apoptosis (Loveridge et al., 2010). SKi also induces proteasomal degradation of SK1a in androgen-independent LNCaP-AI cells, but fails to reduce SK1b levels (Loveridge et al., 2010), which is possibly a consequence of a compensatory increase in SK1b mRNA expression in these cells. This is associated with a failure to increase C22:0 and C24:0 ceramide levels and the absence of apoptosis (Loveridge et al., 2010; Lim et al., 2012). Sensitivity to apoptosis can be restored to these cells by either using the allosteric inhibitor, (*S*)-FTY720 vinylphosphonate (Tonelli et al., 2010) or by combined treatment with SK1 siRNA (to prevent mRNA translation of SK1a and significantly, SK1b) and SKi (Loveridge et al., 2010). Further, evidence supporting the concept that modulation of the sphingolipid rheostat can result in apoptosis, is provided by the finding that the siRNA knockdown of SK1 in MCF-7 cells induces accumulation of ceramide and promotes apoptosis of these cells (Taha et al., 2006) and this can be recapitulated with SK1 inhibitors, which induce proteasomal degradation of SK1 (Tonelli et al., 2010).

Treatment of androgen sensitive LNCaP prostate cancer cells with SKi also induces the proteasomal degradation of several proto-oncogenes with short stability half-lives (e.g. c-Myc, Cyclin D1) leading to catastrophic collapse of signalling networks that normally

function to inhibit apoptosis of these cells (Loveridge et al., 2010). This is associated with a substantial increase in the levels of several different molecular species of dihydroceramide (Loveridge et al., 2010) and we therefore, proposed that SKi might inhibit dihydroceramide desaturase. Dihydroceramide desaturase is the enzyme that catalyses the formation of ceramide from dihydroceramide. The inhibition of dihydroceramide desaturase by SKi (Cingolani et al., 2014) would therefore lead to the accumulation of dihydroceramide and this appears to induce activation of the proteasome. Consistent with this, the dihydroceramide desaturase inhibitor, fenretinide also promotes the degradation of SK1a in androgen-independent LNCaP-AI prostate cancer cells (unpublished data, McNaughton, Pyne and Pyne). Dihydroceramide levels also increase in response to SKi in androgen-independent LNCaP-AI prostate cancer cells.

ABC294640 is an SK2 specific inhibitor (K_i = 10 μM, (French *et al.* 2010)) with no inhibition of SK1 activity up to 100 μM. ABC294640 is currently in clinical trials for treatment of solid tumours and refractory/relapsed diffuse large B-cell lymphoma. Interestingly, ABC294640 induces the proteasomal degradation of c-Myc in multiple myeloma cancer cells (Venkata et al., 2014), an effect that is reminiscent of that induced by SKi in prostate cancer cells (Loveridge et al., 2010; Watson et al., 2013). Indeed, we have found that ABC294640 induces the proteasomal degradation of SK1a in both androgen-independent LNCaP-AI prostate cancer cells and HEK 293T cells (unpublished data, McNaughton, Pyne and Pyne). Therefore, ABC294640 cannot be defined as a SK2 specific inhibitor as it removes SK1a from cancer cells via an indirect mechanism.

The treatment of androgen-independent LNCaP-AI prostate cancer cells with SKi or ABC294640 or fenretinide induces the expression of p53 and p21 (unpublished data,

McNaughton, Pyne and Pyne; Watson et al., 2013). In contrast, SK1 selective (PF-543) or SK2 selective (ROMe) inhibitors or siRNA knockdown of SK1 or SK2 fail to induce p53/p21 expression (unpublished data, McNaughton, Pyne and Pyne). These findings suggest that the effect of SKi and ABC294640 on p52/p21 expression are likely mediated by dihydroceramide desaturase inhibition as this is recapitulated by fenretidine. The increase in dihydroceramide levels combined with the proteasomal degradation of SK1a and possibly inhibition of SK2 activity promotes growth arrest associated with an increase in the expression of markers of senescence in androgen-independent LNCaP-AI cells. Although, SKi increases dihydroceramide levels in androgen-sensitive LNCaP cells, p53 expression is not elevated in these cells suggesting the absence of a key regulatory step involved in controlling p53 in this cell line.

In summary, the SKi-induced proteasomal degradation of SK1a and SK1b is linked with apoptosis of androgen-sensitive LNCaP prostate cancer cells. SK2-selective inhibitors do not induce the apoptosis of androgen-sensitive LNCaP prostate cancer cells. In contrast, androgen-independent LNCaP-AI cells are resistant to apoptosis in response to SKi and instead undergo a p53/p21-dependent growth arrest that appears to be a consequence of an inhibitory action on dihydroceramide synthase, SK1a and possibly SK2. This pathway is summarised in **Figure 2**.

Sphingosine kinase 1, Sphingosine and the inflammasome--Multiple sclerosis is an autoimmune inflammatory demyelinating disease that involves destructive effects of reactive T-lymphocytes. A prognostic relationship exists between IL-1β levels, disease progression and mutation of the NOD-like receptor family, pyrin domain containing 3 (*Nlrp3*) gene, and which is associated with multiple sclerosis-like lesions (Compeyrot-Lacassagne et al., 2009;

Dodé, et al., 2002). This is supported by evidence showing that NLRP3 (*Nlrp3*$^{-/-}$) mice develop mild symptoms in an experimental autoimmune encephalomyelitis (EAE) model and this is associated with reduced numbers of IFNγ- and IL-17-expressing TH cells in peripheral lymphoid tissues and the spinal cord (Gris et al., 2010; Inoue et al., 2012). Microglial cells are activated by IL-1β and these cells stimulate T-lymphocytes with self-antigen during the development of EAE. Furthermore, IL-1 receptor deficient mice develop mild EAE (Sutton et al., 2006). There is also a link between the NLRP3 and the sphingosine mimetic, FTY720, which has been shown to activate PP2A and to stimulate IL-β release from macrophages (Luheshi et al., 2012). FTY720 modulates the immune response by preventing egress of T-lymphocytes from lymph nodes (Hla and Brinkmann, 2011). FTY720 (Gilenya^{TM}) is licenced for oral treatment of relapsing multiple sclerosis and is a prodrug, which is phosphorylated by SK2. FTY720 phosphate is a functional antagonist of $S1P_1$, causing its proteasomal degradation and removal from T-lymphocytes (Hla and Brinkmann, 2011). Since T-lymphocytes use an S1P gradient to egress from lymph nodes, FTY720 is able to prevent this by creating $S1P_1$ null T-lymphocytes that do not respond to the S1P gradient. This halts egress of T-lymphocytes from lymph nodes, thereby ablating their action on the CNS in multiple sclerosis.

We have demonstrated that administration of the SK1 inhibitor, PF-543 (0.4 mg/kg) to mice increases disease progression in the EAE model of muliple sclerosis and this was associated with a considerable increase in the infiltration of CD4^{+} T-cells, CD11b^{+} monocytes and F4/80^{+} macrophages in the spinal cord (**Figure 3**). EAE is also associated with increased SK1 expresssion in the spinal cord, possibly due to the influx of inflammatory cells expressing this enzyme (**Figure 3**). These findings indicate that SK1 functions in an anti-inflammatory manner in EAE. During sterile inflammation, danger-associated molecular

patterns (DAMPs) are liberated from dead cells to promote the innate immune responses. The NLRP inflammasome senses DAMPs leading to recruitment of the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Caspase 1, which cleaves pro-IL-1β to produce bioactive IL-1β, is then recruited to the inflammasome and activated (Brough and Rothwell, 2007). DAMPs might also be released from damaged lysosomal membranes, which is regulated by lysosomal proteases, such as cathepsin B (Hornung and Latz, 2010). Recently, Luheshi et al., (2012) demonstrated that sphingosine stimulates NLRP3-dependent release of IL-1β from LPS-stimulated macrophages. This involves the sphingosine-dependent activation of protein phosphatase 2A and/or protein phosphatase 1 (PP2A/PP1). We have assessed the effect of sphingosine on IL-1β release from macrophages, primarily as the removal of sphingosine by SK1 could account for its anti-inflammatory function in EAE. Treatment of differentiated U937 macrophage-like cells with sphingosine alone or in the presence of LPS, enhanced IL-1β release (Boomkamp et al., 2015). The increase in LPS-stimulated IL-1β release by sphingosine was reduced by pretreatment of cells with the caspase-1 inhibitor, Ac-YVAD-CHO, consistent with a model in which sphingosine stimulates the inflammasome (Boomkamp et al., 2015). However, this enhancement was also reduced by pretreatment of cells with the cathepsin B inhibitor, CA074Me, but not by the PP2A inhibitor, okadaic acid, indicating a predominant role for lysosomal destabilization in mediating the effect of sphingosine in differentiated U937 macrophage like cells (Boomkamp et al., 2015). Therefore, the anti-inflammatory function of SK1 in EAE might be partly related to its ability to reduce the bioavailability of sphingosine, thereby ablating its DAMP activity in modulating the innate immune response.

We have also assessed the effect of ether glycerol lipid, 77-6 ((2*S*, 3*R*)-4-(Tetradecyloxy)-2-amino-1,3-butanediol) which is chemically similar to sphingosine and is a substrate for

sphingosine kinases, on IL-1β release from macrophages. Treatment of differentiated U937 macrophage-like cells with 77-6 enhanced IL-1β release; either alone or in the presence of LPS. However, in contrast with sphingosine the effect of 77-6 on IL-1β release is not affected by cathepsin B inhibitor, but is diminished by the caspase-1 inhibitor, Ac-YVAD-CHO (Boomkamp et al., 2015). One could also argue that enhancing inflammasome activity with such sphingosine mimetics might represent a useful strategy for boosting innate immunity against invading pathogens. An additional strategy would be to identify allosteric activators of SK1 to increase S1P levels *in vivo* to promote an anti-inflammatory effect, which is independent of the S1P gradient. This is based on the finding that T-cell redistribution in lymphoid organs is unaffected in SK1 knockout mice (Allende et al., 2004). Alternative approaches to develop new therapeutics in the multiple sclerosis area focus on S1P receptor modulators and S1P lyase inhibitors. S1P lyase catalyses the degradation of S1P (Cosconati and Novellino, 2014) and inhibitors are therefore intended to raise lymphoid compartment S1P levels *in vivo* in order to eliminate the S1P gradient, thereby preventing T-cell egress.

Conclusion--The therapeutic potential of targeting the S1P signalling pathway in cancer and inflammation provides significant promise. This extends to targeting S1P receptor systems and the enzymes involved in promoting formation/degradation of S1P. Compounds can be designed to force 'mild' or 'severe' phenotypes based on whether they conformationally induce proteasomal degradation of SK1 or cause activation of the proteasome to instigate catastrophic collapse of cancer signalling networks. In terms of multiple sclerosis, the basis of intervention might be to produce activators of sphingosine kinase and/or sphingosine antagonists that prevent functioning of the inflammasome.

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ABBREVIATIONS

ASC, Apoptosis-associated speck-like protein containing a caspase recruitment domain; DAMPs, Danger associated molecular patterns; EAE, experimental autoimmune encephalomyelitis; HER2, Human epidermal growth factor related receptor 2; ERK, extracellular signal regulated kinase; mTOR, mammalian target of rapamycin; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family, pyrin domain containing 3; IFNγ, interferon gamma; IL-1β, interleukin-1beta; PI3K. Phosphoinositide 3-kinase; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; SK, sphingosine kinase; S1P, sphingosine 1-phosphate; $S1P_1$, sphingosine 1-phosphate receptor-1; TH, T helper cells.

Figure legends

Figure 1 The functional interaction between $S1P_4$ and HER2 in estrogen receptor negative breast cancer cells. S1P binding to the $S1P_4$ receptor induces the tyrosine phosphorylation of HER2 and the functional interaction between HER2 and $S1P_4$ increases signalling gain in terms of the S1P-induced stimulation of the ERK-1/2 pathway that might be linked to metastasis. In contrast with other studies which demonstrate that the EGF receptor is a preferred partner of HER2, there is no functional interaction between EGF receptor, HER2 and $S1P_4$ in estrogen receptor negative MDA-MB-231 breast cancer cells.

Figure 2 The mechanism of action of SKi and ABC294640 on growth arrest of androgen-independent LNCaP-AI cells. SK1 selective inhibitors induce the ubiquitin-proteasomal degradation of SK1. SKi is a weak inhibitor of SK1 and SK2. However, SKi and ABC294640 (SK2 selective inhibitor) also induce an activation of the proteasome that results in degradation of SK1 (possibly regulated via a dihydroceramide-dependent mechanism) and induce an increase in p53 and p21 expression. Therefore, androgen-independent LNCaP-AI prostate cancer cells undergo a p53/p21-induced growth arrest.

Figure 3 Effect of the SK1 inhibitor, PF-543 on EAE disease progression. C57BL/6 mice were immunized subcutaneously on the back with 100 μg of MOG_{35-55} peptide (Sigma Genosys) in 50 μl of PBS emulsified with an equal volume of CFA (total 125 μg of *Mycobacterium tuberculosis*, strain H37RA, Difco, Detroit MI). Each mouse also received intraperitoneally (i.p.) 100 ng/200 μl of PTX (Sigma, UK) 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. PF-543 (0.4mg/kg w/v) was administered i.p. daily from day 0 of

MOG immunization. The EAE control group received i.p. injection daily of the 20% cyclodextrin vehicle. The figure shows clinical disease prgression in the EAE group *versus* EAE plus PF-543. Also shown are images of SK1 expression and $CD4^+$ cell, F4/80 macrophage and $CD11^+$ cell infiltration in the spinal cord in the EAE plus PF-543 group *versus* EAE. Also shown is the inhibition of SK1 activity by PF-543 using a radiometirc assay described by Lim et al., 2011b. The results are shown as the % control SK1 activity (where control = 100% SK1 activity).

Figure 1

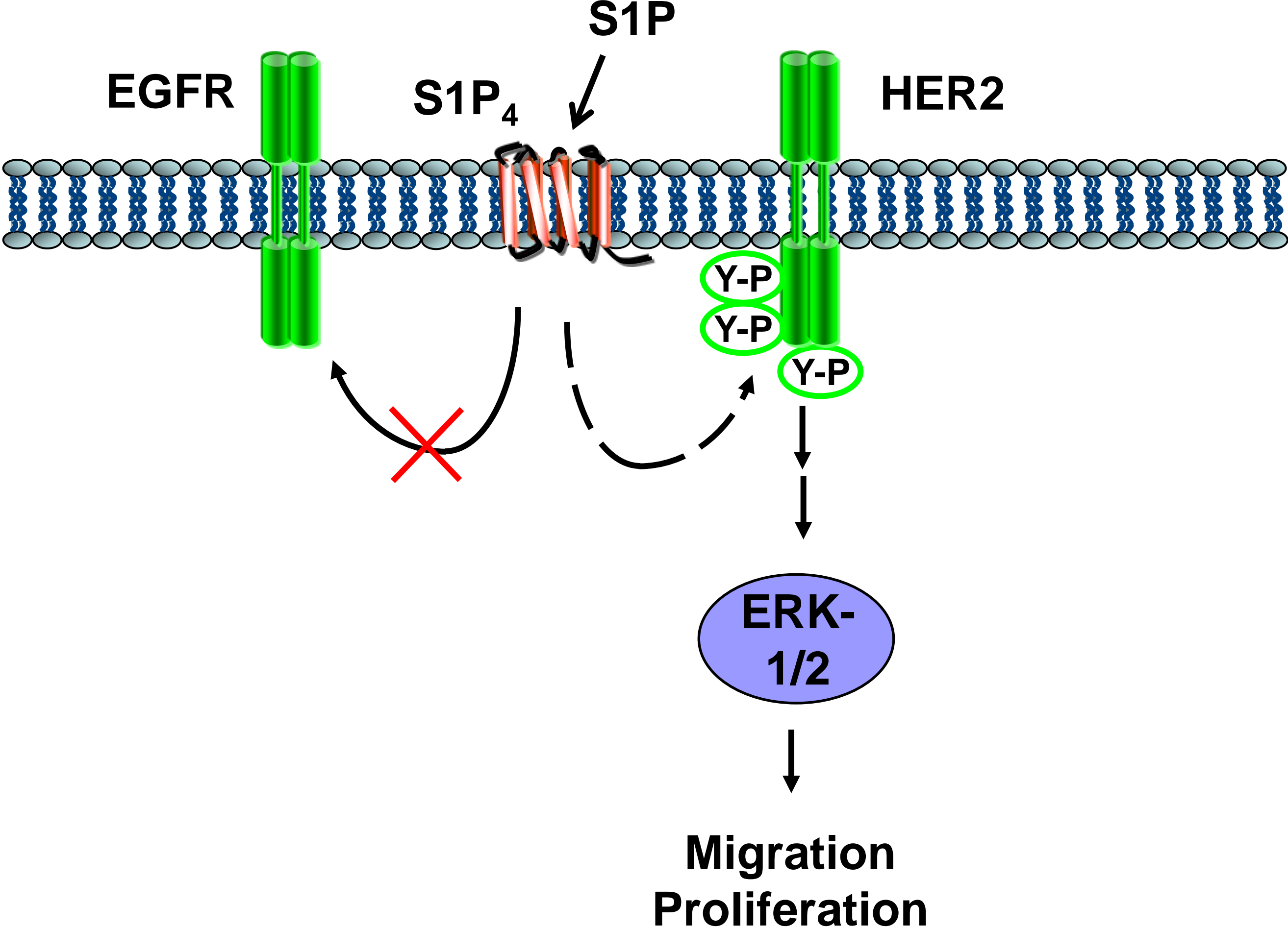

Figure 2

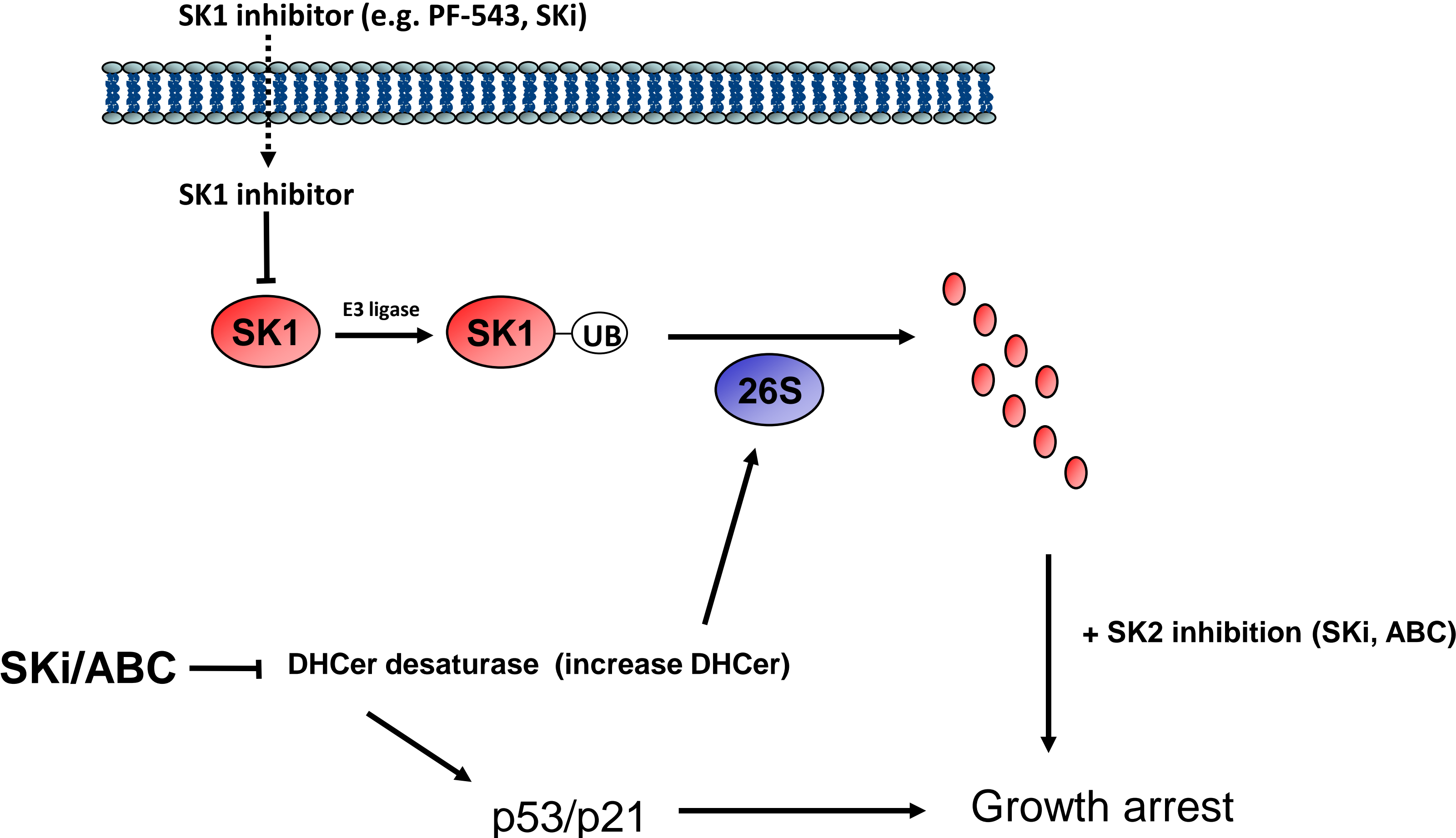

Figure 3