Abstract

There is an increasing body of evidence demonstrating a critical role for the bioactive lipid S1P (sphingosine 1-phosphate) in cancer. S1P is synthesized and metabolized by a number of enzymes, including sphingosine kinase, S1P lyase and S1P phosphatases. S1P binds to cell-surface G-protein-coupled receptors (S1P1–S1P5) to elicit cell responses and can also regulate, by direct binding, a number of intracellular targets such as HDAC (histone deacetylase) 1/2 to induce epigenetic regulation. S1P is involved in cancer progression including cell transformation/oncogenesis, cell survival/apoptosis, cell migration/metastasis and tumour microenvironment neovascularization. In the present paper, we describe our research findings regarding the correlation of sphingosine kinase 1 and S1P receptor expression in tumours with clinical outcome and we define some of the molecular mechanisms underlying the involvement of sphingosine kinase 1 and S1P receptors in the formation of a cancer cell migratory phenotype. The role of sphingosine kinase 1 in the acquisition of chemotherapeutic resistance and the interaction of S1P receptors with oncogenes such as HER2 is also reviewed. We also discuss novel aspects of the use of small-molecule inhibitors of sphingosine kinase 1 in terms of allosteryism, ubiquitin–proteosomal degradation of sphingosine kinase 1 and anticaner activity. Finally, we describe how S1P receptor-modulating agents abrogate S1P receptor–receptor tyrosine kinase interactions, with potential to inhibit growth-factor-dependent cancer progression.

Introduction

S1P (sphingosine 1-phosphate) is a bioactive lipid that binds to a family of specific GPCRs (G-protein-coupled receptors) (S1P1–S1P5) to induce cellular responses such as growth, survival and migration of mammalian cells [1]. S1P is formed by conversion of sphingosine into S1P, catalysed by SK (sphingosine kinase) (two isoforms called SK1 and SK2) and is removed by hydrolysis catalysed by S1P lyase or by dephosphorylation catalysed by S1PP (S1P phosphatase) (two isoforms called S1PP1 and S1PP2). Several intracellular targets of S1P have recently been identified, whereas receptor-mediated actions of S1P, subsequent to its release from a variety of cell types, are well established.

SK1 and cancer

There is compelling evidence to support a role for SK1 in human cancers. For instance, cancer cell lines have increased SK1 expression and are reliant on SK1 for survival and growth. Therefore cancer cells have been defined as having a ‘non-oncogene addiction’ for SK1 [2]. For instance, there is increased expression of SK1 mRNA transcript and/or SK1 protein in cancers of the stomach, lung, brain, colon, kidney and breast and non-Hodgkin’s lymphoma (see [3] for a review). Indeed, we demonstrated recently that high tumour expression of SK1 correlates with poor survival rates and induction of tamoxifen resistance in ER+ (oestrogen receptor) breast cancer patients (n = 304) [4,5]. The average survival time of these patients is reduced from 18 years to 7.5 years [4,5]. The time to recurrence of the disease in patients receiving tamoxifen is shortened by 8 years in the high SK1 tumour expression group. Therefore there is a need to identify new inhibitors of SK1 to provide opportunities for drug intervention and to open up new approaches involving combined therapies, where tamoxifen resistance might be ablated by inhibition of SK1.

We have used a breast cancer cell line [MCF-7 cells which express ERα, S1P2 and S1P3, but lack S1P1] that recapitulates, in part, the phenotype of the clinical ER+ patient cohort to define the mechanism by which SK1 reduces disease specific breast cancer patient survival and induces resistance to tamoxifen [4]. We demonstrated that S1P binding to S1P3 stimulates the accumulation of phosphorylated ERK1/2 (extracellular-signal-regulated kinase 1/2) into membrane ruffles/lamellipodia and the nucleus to promote migration of MCF-7 cells (Figure 1). Evidence for the involvement of S1P3 is based on results showing that siRNA (small interfering RNA) knockdown of S1P3 or pharmacological intervention with an S1P3 antagonist, CAY10444, abolished the S1P-stimulated activation of ERK1/2. The accumulation of phosphorylated ERK1/2 in membrane ruffles/lamellipodia and the nucleus of MCF-7 cells is prognostic of the formation of a ‘migratory phenotype’. The close proximity requirement

Key words: cancer, FTY720, G-protein-coupled receptor, metastasis, sphingosine kinase, sphingosine 1-phosphate.

Abbreviations used: BODIPY® 4,4-difluoro-4-bora-3a,4a-diaz-o-indacene; EFA5A, eukaryotic elongation factor 1A; EGF, epidermal growth factor; EGF receptor; ER, oestrogen receptor; ERK1/2, extracellular-signal-regulated kinase 1/2; GPCR, G-protein-coupled receptor; PAK1, p21-activated protein kinase 1; PARP, poly(ADP-ribose) polymerase; PDGF, platelet-derived growth factor; PDGFRβ, PDGF receptor β; PPI-a, prostate tumour inducer-1; RTK, receptor tyrosine kinase; S1P, sphingosine 1-phosphate; SIP, S1P phosphatase; siRNA, small interfering RNA; SK, sphingosine kinase; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

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of SK1 with the migratory apparatus in membrane ruffles/lamellipodia is evident from results showing that exogenous S1P promotes the relocalization of SK1 from the cytoplasm to membrane ruffles/lamellipodia of MCF-7 cells [4]. Moreover, this can be blocked by pre-treating the cells with CAY10444, thereby demonstrating functional regulation of SK1 by S1P/S1P3.

SK1 also regulates the redistribution of actin from focal adhesions to membrane ruffles/lamellipodia, required for migration [4]. MCF-7 cells exhibit a phenotype in which actin is clustered into adhesion foci that are concentrated at the cell periphery. S1P stimulation of MCF-7 cells redistributes actin into membrane ruffles and promotes migration, whereas siRNA knockdown of SK1 expression restores actin-containing adhesion foci contacts and SK1 inhibitor prevents migration. Thus SK1 functions in MCF-7 breast cancer cells to regulate the transition from adherence to migration. Interestingly, siRNA knockdown of SK1 also reduces S1P3 expression in these cells and this is associated with decreased stimulation of the ERK1/2 pathway by S1P, thereby preventing formation of the migratory phenotype. Thus SK1 might control the responsiveness of MCF-7 cells to S1P-induced formation of a migratory phenotype, by regulating expression of the S1P3 gene (SIPR3). These SK1-regulated processes in breast cancer cells provide a possible explanation for the decrease in survival of patients with high expression of SK1 in their tumours. In addition, tamoxifen resistance in ER+ breast cancer cells is induced by PKA1 (p21-activated protein kinase 1) and ERK1/2, which catalyse phosphorylation of Ser305 and Ser118 respectively in the ERα [6]. This results in ligand-independent genomic activity of ERα. Therefore S1P3/SK1 might induce tamoxifen resistance by activating PKA1 and ERK1/2, and, indeed, S1P stimulates the Thr423 phosphorylation of p65 PAK1 in MCF-7 cells [4].

**Role of SK1 in regulating ER+/HER2+-dependent breast cancer**

HER2 is a 185 kDa orphan RTK (receptor tyrosine kinase) and forms heterodimers with EGFR [EGF (epidermal growth factor) receptor] (HER1) and HER3. Amplification of the HER2/neu/c-erbB-2 gene is present in 30% of primary breast cancers and is correlated with poor prognosis [7,8]. We have demonstrated that HER2 increases SK1 mRNA (SPHK1) and protein expression and activity in ER+ MCF-7 HER2 cells [4] (which stably express recombinant HER2) when compared with MCF-7 cells that stably express the Neo vector alone. Elevated SK1 levels are associated with suppressed expression of HER2 and ablated migration of MCF-7 HER2 cells in response to S1P, due to an SK1-induced deactivation/degradation of p65 PKA1 (PKA1 is required for motility). Exogenous S1P (which binds to S1P3) also fails to promote accumulation of phosphorylated ERK1/2 into membrane ruffles/lamellipodia and the nucleus in MCF-7 HER2 cells (Figure 1). Therefore the high expression of SK1 induces tolerance to HER2 [4]. Indeed, when the 304 ER+ patients were stratified according to their
HER1–HER3 status, high cytoplasmic SK1 expression in the tumours was associated with increased patient survival and reduced resistance to tamoxifen [4], thereby demonstrating a protective role for SK1 in this tumour phenotype.

**SK1 inhibitors**

FTY720 (or fingolimod) is an immunosuppressant, which has been licensed by the U.S. Food and Drug Administration and the European Medicines Agency (Gilenya®) for treatment of relapsing multiple sclerosis. FTY720 is a sphingosine analogue that is taken up by cells and is converted into FTY720 phosphate by SK2. The FTY720 phosphate is subsequently released and binds to and activates S1P1, S1P3, S1P4 and S1P5, and for S1P1, this results in its proteolytic down-regulation to induce lymphopenia, thereby abating inflammation by immunosuppression [9]. However, we have discovered an entirely novel action of FTY720 as an inhibitor of SK1 activity [10]. In collaboration with R. Bittman (City University of New York, New York, NY, U.S.A.), we have synthesized analogues of FTY720 [10] that inhibit either SK1 and SK2 or neither: e.g. (S)-FTY720 vinylphosphonate for SK1 and SK2; (R)-FTY720 phosphate and (S)-FTY720 phosphate for neither. Others have also demonstrated that it is possible to develop inhibitors that are specific for SK1, such as SK1-1 [11]. We have also confirmed that established SK inhibitors, such as the thiazole SKi [2-((p-hydroxyanilino)-4-(p-chlorophenyl)thiazole] [10,12,13] inhibits SK1 and SK2 activity. Our inhibitor characterization studies demonstrate that FTY720 is a competitive inhibitor (with sphingosine) of SK1 with a \( K_i \) of 2 \( \mu M \). In contrast, (S)-FTY720 vinylphosphonate is an uncompetitive inhibitor (with sphingosine) of SK1, with a \( K_{in} \) of 14.5 \( \mu M \), whereas SKi is a mixed inhibitor of SK1 with a \( K_{in} \) of 17 \( \mu M \) and a \( K_{in} \) of 48.3 \( \mu M \) [13]. In addition, BODIPY® (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)–sphingosine and (S)-FTY720 regiosomer, which are weak substrates, are able to stimulate SK1 activity [13]. Thus (S)-FTY720 vinylphosphonate, BODIPY®–sphingosine and (S)-FTY720 regiosomer appear to bind to a putative allosteric site in SK1. In this regard, it is interesting to note that SK1 is an oligomeric protein (minimally a dimer) containing non-co-operative catalytic sites [13]. This molecular arrangement would be conducive to allosterism. Indeed, we have proposed that the allosteric site(s) exert an autoinhibition of the catalytic site in SK1 [13], and that (S)-FTY720 vinylphosphonate might stabilize this site to increase the lifetime of the inhibited enzyme (the ‘on’ state) [13]. In contrast, activators of SK1 might stabilize the ‘off’ state, thereby relieving inhibition of SK1 activity and resulting in activation of the enzyme [13]. FTY720 and (S)-FTY720 vinylphosphonate prevent the S1P-stimulated rearrangement of actin required for migration of MCF-7 cells (i.e. they recapitulate the effects of siRNA knockdown of SK1 [4]) [13], indicating that these molecules are effective SK1 inhibitors that display potential anticancer activity.

Interestingly, (S)-FTY720 vinylphosphonate (unlike FTY720 phosphate) failed to activate any S1P GPCR (measured by intracellular \( \text{Ca}^{2+} \) mobilization in HTC4 cells, separately overexpressing S1P1–S1P5) and was a full antagonist of S1P1, S1P3, and S1P4 (\( K_i \) values of 208, 15 and 1190 \( \text{nM} \) respectively) and a partial antagonist of S1P2 and S1P4 [14]. Thus (S)-FTY720 vinylphosphonate not only inhibits SK1 activity, but also exhibits S1P receptor antagonism, and this dual functionality might increase its therapeutic efficacy in terms of inducing apoptosis of cancer cells (Figure 2). FTY720 also modulates ceramide kinase [15], 14-3-3 protein [16], acid sphingomyelinase [17] and phospholipase A2 [18], and some of these actions may contribute to its anticancer activity. Indeed, the treatment of human cancer cells with FTY720 inhibits growth, whereas FTY720 phosphate induces growth of these cells [19].

We have also reported that the chronic treatment of MCF-7 breast cancer cells and LNCaP prostate cancer cells with SKi [12], FTY720 or (S)-FTY720 vinylphosphonate [10] induces the ubiquitin–proteasome degradation of SK1. Indeed, polyubiquitinated SK1 accumulates in cells treated with the proteasomal inhibitor MG132, whereas SKi partially reverses inhibition of the proteasome by MG132 [12]. The treatment of LNCaP cells with the lysosomal cathepsin B1 inhibitor CA074Me had no effect on the SKi-induced degradation of SK1a or SK1b [12]. There are at least two possible models that describe the interaction between SK1 inhibitors and SK1 resulting in proteasomal degradation.
of the enzyme. In model 1, SK1 inhibitors might bind to SK1, resulting in trapping of the inhibitor (due to a low dissociation rate). This might disable the enzyme and induce a conformational change (protein unfolding) that is a signal for the proteasomal degradation of SK1. In this regard, there might be participation of effectors involved in the UPR (unfolded protein response) [e.g. IRE-1α (inositol-requiring enzyme 1α), XBP1 (X-box-binding protein 1) and ATF6 (activating transcription factor 6)]. In model 2, the binding of inhibitor to SK1 induces reduced intracellular S1P formation and an increased formation of ceramide. Indeed, we have shown that treatment of cells with SKi induces an increase in intracellular C22:0-ceramide and a reduction in S1P levels in LNCaP prostate cancer cells [12]. Moreover, C2-ceramide activates the proteasome, and we have therefore proposed that the SKi-induced increase in intracellular ceramide might function to accelerate the rate at which polyubiquitinated SK1 is degraded by the proteasome. Indeed, inhibition of ceramide synthase with fumonisin B1, to reduce ceramide accumulation, partially reversed the effect of SKi on the proteasomal degradation of SK1 in LNCaP cells [12]. Models 1 and 2 are not mutually exclusive and, indeed, might operate together.

**SK1 and chemotherapeutic resistance**

SK1 appears to have a critical role in the acquisition of chemotherapeutic resistance and androgen independence in prostate cancer. For example, S1P signalling promotes proliferation of androgen-independent PC-3 prostate cancer cells [20], and overexpression of SK1 in prostate cancer cells produces resistance [21]. In addition, SK1-transfected LNCaP cells escape growth inhibition after androgen deprivation [22]. In contrast, SK1-inhibitor-treated radiation-resistant LNCaP cells undergo irradiation-induced apoptosis [23], and the loss of cell viability induced by chemotherapeutic agents (e.g. doxorubicin) in vitro and in vivo is correlated with reduced SK1 activity [24]. In this regard, we have studied SK1 in androgen-sensitive LNCaP cells and androgen-independent LNCaP-AI cells. Both cell types express SK1a and SK1b [12]. SK1a (GenBank® accession number NM_001142601) is a 42.5 kDa protein, whereas SK1b (GenBank® accession number NM_182965) is a 51 kDa protein identical with SK1a, but with an 86-amino-acid N-terminal extension. Kihara et al. [25] have previously identified a smaller highly unstable version of SK1 called ‘SK1b’ (a 34 kDa species), which is distinct from the 51 kDa SK1b described here.

We have reported that SKi induces PARP [poly(ADP-ribose) polymerase] cleavage in LNCaP cells, indicating that these cells undergo the onset of apoptosis when SK1a and SK1b are degraded by the proteasome [12]. In contrast, SKi-treated androgen-independent LNCaP-AI cells do not undergo apoptosis (there is no PARP cleavage) and SKi failed to induce proteasomal degradation of SK1b, whereas down-regulation of SK1a and its partial restoration by MG132 was observed [12]. Therefore ‘androgen escape’ of LNCaP-AI cells is associated with an up-regulation of SK1a and SK1b expression and altered regulation of SK1b that renders these cells refractory to SKi-induced apoptosis. We concluded that chemotherapeutic agents such as SKi might induce the onset of apoptosis in androgen-sensitive LNCaP cells because they reduce SK1a and SK1b expression levels below a threshold required for cell survival. Thus SKi might not induce apoptosis of LNCaP-AI cells because the total SK1 level is not reduced below this threshold [12]. Indeed, a combined treatment of LNCaP-AI cells with SK1 siRNA and SKi eliminates expression of SK1a and SK1b, and these cells are then forced to undergo apoptosis [12]. Therefore chemotherapeutic resistance might be influenced by the expression level of SK1.

**S1P receptors and cancer**

S1P receptors have a significant role in cancer progression. For instance, S1P inhibits motility through a mechanism involving S1P2 in GNS-334 glioblastoma cells [26]. In contrast, S1P stimulates migration of fibrosarcoma cells through S1P1 [27] and of gastric cancer cells through S1P3 [28]. A key question is whether aberrant S1P receptor function is associated with cancer progression. Towards this end, we analysed the cohort of 304 ER+ breast cancer patients (all of whom received tamoxifen therapy) in order to investigate the prognostic significance of S1P1, S1P2 and S1P3 and ERK1/2 expression [5]. High membrane S1P1 expression in the tumours was associated with shorter time to recurrence on tamoxifen in ER+ tumours, e.g. increased resistance to tamoxifen. Moreover, high cytoplasmic S1P1 and S1P3 expression (i.e. S1P receptors that might have undergone endocytosis) in the tumours was also associated with reduced disease-specific survival. High cytoplasmic S1P1/S1P3 and ERK1/2 expression in the tumours were also associated with an earlier development of tamoxifen resistance [5]. Therefore interaction between S1P1 and/or S1P3 and ERK1/2 might promote ER+ breast cancer progression and resistance. No correlation between S1P2 and survival or recurrence was established [5]. There are also emerging reports of mutation of S1P receptor genes in cancer [29], although the importance in terms of oncogenesis is not yet clear.

**S1P receptors and RTKs in cancer**

We have demonstrated that RTKs and GPCRs form unique signalling networks where components specific for each receptor class are combined to regulate a common signalling pathway e.g. PDGFRβ [PDGF (platelet-derived growth factor) receptor β]-S1P1 complexes [30,31]. This mechanism does not involve tyrosine phosphorylation of the RTK [30] or growth-factor-induced release of GPCR ligands that can act on close-proximity S1P receptors. The PDGFRβ tyrosine kinase activity, Gαβγ and β-arrestin function as multipliers of signal output from the PDGFRβ-S1P1 complex in response to PDGF [31–33]. Antisense oligonucleotide against S1P1 also reduces the PDGF- or S1P-induced stimulation of the ERK1/2 pathway [33].
Interestingly, there is an S1P1-modifying agent, SB649146, that can interrupt signalling from the PDGFRβ–S1P complex. Thus treatment of cells with SB649146 inhibits the PDGF-induced stimulation of ERK1/2 and migration [34]. The action of SB649146 can be explained by a model in which PDGFRβ is associated with a high-efficacy Gα–βγ–arrestin- and β-arrestin-coupling S1P1 conformation [31,34]. This S1P1 conformation is in equilibrium with a second lower-efficacy Gα-coupling S1P1 conformation that is not associated with PDGFRβ. SB649146 binds exclusively to and stabilizes this lower-efficacy Gα-coupling S1P1 conformation, but still stimulates binding of β-arrestin and thereby reduces the amount of Gαβγ that can be used by the PDGFRβ kinase by mass action. A similar RTK–S1P1 signalling complex is present in follicular thyroid carcinoma ML-1 cells, where VEGF-R2 (VEGF (vascular endothelial growth factor) receptor 2) forms a complex with S1P1. VEGF-stimulated ERK1/2 activation and haptotaxis is reduced by pertussis toxin, SB649146 and siRNA knockdown of S1P1, whereas S1P-stimulated activation of ERK1/2 is reduced by inhibition of the VEGFR2 tyrosine kinase [35]. An important question is whether these functional RTK–S1P1 complexes are operative in cancer and play a role, for instance in neovascularization of tumours. If this is the case, then it might be possible to use S1P receptor-modifying agents to inhibit growth-factor-dependent progression of cancers.

### S1P and oncopgenes

There are also partnerships between oncopgenes and components of the S1P signalling system. For instance, SK1 activation and foci formation are increased in NIH 3T3 fibroblasts overexpressing a mutant H-RAS (V12-Ras), which can be inhibited by overexpression of the G82D dominant-negative SK1 mutant [36]. Thus SK1 and H-Ras are connected in a sequential signalling pathway, e.g. possibly via Ras-dependent activation of ERK1/2 and subsequent ERK1/2-catalysed phosphorylation (Ser225 in SK1) and activation of SK1. In addition, SK1 is activated by the GDP-bound form of eEF1A (eukaryotic elongation factor 1A) [37]. The levels of eEF1A–GDP are regulated by a guanine-nucleotide-dissociation inhibitor termed TCTP (translationally controlled tumour protein), which, when overexpressed in cells, also activates SK1. More importantly, a truncated form of eEF1A1, termed PTI-1 (prostate tumour inducer-1), which is an oncogene and lacks the GDP/GTP-binding domain of eEF1A1, activates SK1. This regulatory mechanism governing SK1 activity appears to be essential for neoplastic transformation induced by PTI-1 [37]. HER2/ErbB2 also functionally interacts with S1P1 in ER− MDA-MB-453 breast cancer cells. Thus S1P binding to S1P1 in these cells stimulates the activation of ERK1/2 and this is contingent on HER2 and is independent of EGF release [38]. S1P stimulates the tyrosine phosphorylation of HER2, which might produce a S1P1–HER2 signalling platform that enables stronger activation of the ERK1/2 pathway in response to S1P [38]. Shida et al. [39] have also demonstrated that S1P stimulates the tyrosine phosphorylation of HER2 in MKN28 and MKN74 gastric cancer cells, but this is dependent upon metalloproteinase-dependent release of EGFR ligand.

### S1P lyase and S1PP

S1P lyase mRNA (SGPL1) expression is reduced in intestinal cancer [40] and in metastatic tumours [41]. However, SGPL1 mRNA levels are increased in ovarian cancer [42] and in chemotherapeutic resistant ovarian tumours [43]. Therefore the precise role of this enzyme in cancer has not been established. Nevertheless, the sensitivity of lung cancer cells to cisplatin and doxorubicin is increased by overexpression of S1P lyase [44], whereas S1P lyase is down-regulated in adenoma lesions of the Apcm1−/− mouse, thereby improving survival of these non-malignant cells [40]. S1PP1 knockdown also confers resistance to chemotherapy and increases both intracellular and extracellular levels of S1P [45].

### Conclusions

S1P has a critical role in promoting cancer progression. Indeed, we have reported a correlation of SK1 and S1P receptor expression in tumours with patient survival and tamoxifen resistance in ER+ breast cancer, and have elucidated some of the possible molecular mechanisms underlying these clinical associations. In addition, the use of small-molecule catalytic/allosteric inhibitors of SK1 activity and/or enhancers of the proteasomal degradation of SK1 or S1P receptor-modifying agents that abrogate S1P receptor–RTK partnerships offers unique opportunities for novel therapeutic strategies for the treatment of cancer.

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