THE ROLE OF SPHINGOSINE 1-PHOSPHATE IN INFLAMMATION AND CANCER

Nigel J. Pyne[1], Jan Ohotski[1], Robert Bittman[2] and Susan Pyne[1]

[1]Cell Biology Research Group, Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK. [2]Department of Chemistry and Biochemistry, Queens College of the City University of New York, Flushing, New York 11367-1597, USA.

ABSTRACT--The enzymes that catalyse the formation of bioactive sphingolipid, sphingosine 1-phosphate, sphingosine kinase 1 and 2, are predictive markers in inflammatory diseases and cancer as evidenced by data from patients, knockout mice and the use of available molecular and chemical inhibitors. Thus, there is a compelling case for therapeutic targeting of sphingosine kinase. In addition, there are several examples of functional interaction between sphingosine 1-phosphate receptors and sphingosine kinase 1 that can drive malicious amplification loops that promote cancer cell growth. These novel aspects of sphingosine 1-phosphate pathobiology are reviewed herein.

The lipid sphingosine 1-phosphate (S1P) is a key regulator of cell growth, survival, invasion, lymphocyte trafficking, vascular integrity and cytokine production, and plays a central role in inflammatory disease and cancer. S1P is formed by phosphorylation of sphingosine, catalysed by sphingosine kinases 1 and 2 (SK1 and SK2) that differ in their biochemical properties, sub-cellular localization, and function (Pyne and Pyne, 2011). S1P is cleaved by S1P lyase to produce *trans*-2-hexadecenal and phosphoethanolamine. S1P can also be dephosphorylated by S1P phosphatases to recycle into sphingolipids (Pyne and Pyne, 2011). S1P is an agonist of S1P-specific G-protein coupled receptors, termed $S1P_{1-5}$, and also binds to intracellular protein targets, such as histone deacetylase 1 and 2 (HDAC1/2, which regulate gene expression) (for review see Pyne and Pyne, 2011).

S1P and inflammation

S1P has been linked to regulating the production of pro-inflammatory cytokines, Toll-like receptor signalling and inflammation. Inflammatory mediators might regulate SK1 via an ERK-catalysed phosphorylation of SK1 on S225; this promotes SK1 translocation to the plasma membrane, where it catalyzes the formation of S1P (Pitson et al., 2005). S1P is then transported out of the cell and/or partitions in the plasma-membrane, where it binds to a family of G-protein-coupled receptors ($S1P_{1-5}$) to induce a multitude of cell responses. This process has been termed ‘‘inside-out signalling’’ (Takabe et al., 2008). For instance, this mechanism leads to the expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) in response to tumour necrosis factor alpha (TNF-α) (Xia et al., 1998). SK1 and S1P are also required for TNF-α-induced cyclooxygenase 2 (COX2) and prostaglandin E2 (PGE2) production (Pettus et al., 2003). TNF-α also activates SK1, resulting in S1P accumulation in synoviocytes from rheumatoid arthritis patients, which promotes proliferation and cytokine production from these cells (Kitano et al., 2006). These studies, along with many others, have firmly established that TNF-α signalling leads to activation of the SK1/S1P pathway. Intracellular S1P also binds to and stimulates TRAF2 E3-ligase activity resulting in the lysine-63-linked polyubiquitination (signalling ubiquitination) of receptor interacting protein 1 (RIP1) (Alvarez et al., 2010). Polyubiquitinated RIP1 acts as a scaffold for the recruitment and phosphorylation of the IKK complex, followed by activation of NKκB, which is a key player in inflammation.

Lipopolysaaccharide (LPS) stimulation of Toll-Like Receptor-4 (TLR-4) in macrophages increases SK1 mRNA and enzyme activity, resulting in generation of S1P and induction of COX2 (Hammad et al., 2008). However, while siRNA knockdown of SK1 has no effect on LPS-stimulated inflammation, it does block TNFα-stimulated inflammation (Hammad et al., 2008) and protects against LPS-stimulated apoptosis of macrophages, demonstrating dual and distinct roles of SK1 in TNF and LPS inflammatory pathways. A

role for SK1 in LPS signaling was also observed by Wu et al., (2004) who demonstrated that SK inhibitors or siRNA knockdown of SK1 expression reduces LPS-stimulation of ERK-1/2 and p38 MAPK, and enhances LPS-stimulated JNK activation. In addition, over-expression of a dominant negative kinase inactive SK1 mutant blocks LPS-stimulated Elk-1 and NFκB transcriptional activity (Wu et al., 2004). SK1 mRNA and protein are also increased in LPS-activated microglia (Nayak et al., 2010), thereby implicating the SK1/S1P pathway in neuroinflammation. In addition, the transactivation of S1PR by IgE receptor, Fc3RI is necessary for mast cell degranulation and migration (Jolly et al., 2004), whereas cytokine production from mast cells requires SK2 (Oskeritzian et al., 2004).

There is also substantial evidence for a role of SK1 in animal models of inflammation. For instance, S1P levels are increased in wild-type mice with dextran sulphate-induced colitis, but not in *SK1*$^{-/-}$ mice, which have reduced local and systemic inflammation (Snider et al., 2009). In murine models of inflammatory disease, such as arthritis, chemical inhibition or siRNA knockdown of SK1 results in reduced inflammation (Lai et al., 2008). A role for SK1 is also evident in patients with rheumatoid arthritis, where there is an increase in S1P levels in the synovium (Alvarez et al., 2007). Other inflammatory signalling molecules, such as IL-1β, IFN-γ and IgE (Alvarez et al., 2007) also activate SK1 thereby providing additional compelling evidence for a role of SK1/S1P in the inflammatory response. Treatment of inflammatory disease using drugs that modulate S1P biology is already evident in the clinic, providing proof of concept that SK1 and S1P are *bone fide* targets for therapeutic intervention. The synthetic sphingosine analogue and pro-drug FTY720 (fingolimod, Gilenya^{TM}), is used as an oral drug for the treatment of multiple sclerosis (MS). FTY720 is taken up by cells, phosphorylated by SK2 and released as FTY720 phosphate. FTY720-phosphate binds to and induces functional antagonism of the S1P receptor, $S1P_1$, to induce lymphopenia, thereby inhibiting attack within the central nervous system by T-lymphocytes (Brinkmann et al., 2010). Egress of T-lymphocytes from the lymph nodes requires an S1P

gradient. In addition, FTY720 reduces pro-inflammatory cytokine release by astrocytes in multiple sclerosis (Choi et al., 2011). This is significant because FTY720 is also a SK1 inhibitor (Tonelli et al., 2010) and might suggest a role for SK1 in MS.

S1PR modulation with FTY720 has also been studied in mouse models of asthma. In this case, FTY720-treated mice previously sensitized to ovalbumin (OVA) exhibit reduced bronchial constriction and eosinophilia compared with vehicle-treated mice. FTY720 also prevents the migration of dendritic cells and inhibits T-lymphocyte activation (Idzko et al., 2006), and reduces hindpaw edema, joint destruction, and lymphocyte invasion in collagen-induced arthritis (CIA) and adjuvant-induced arthritis (AA) (Wang et al., 2007; Matsuura et al., 2000). Furthermore, disease progression in CIA is reduced in mice treated with SK1 siRNA, while SK2 siRNA increases disease incidence and severity. In addition, the SK2 selective inhibitor, ABC294640 (see below) reduces TLR4 expression, NFκB activation, TNF-α, IL-1β and CXCL-10 mRNA formation, ICAM1 expression and infiltration of monocytes/macrophages and neutrophils (Liu et al., 2012). ABC294640 also reduces CD4+ lymphocyte infiltration and IFN-γ production (Liu et al., 2012). In this regard, murine SK2 interacts with the IL-12 receptor β1, to promote IL-12 stimulated formation of IFN-γ (Yoshimoto et al., 2003). Additional evidence supporting a pro-inflammatory role for SK2 is the finding that adenoviral over-expression of wild-type SK2 enhances LPS-induced lung injury (Wadgaonkar et al., 2009).

These studies, along with many others, have firmly established a key role for SK1 and SK2 in inflammatory disease, and as such, both enzymes are targets for novel anti-inflammatory therapeutics. However, there is also evidence that SK2 may have an anti-inflammatory role, and therefore there is a need to provide clarity. For instance, SK2-deficient MCF-7 breast cancer cells exhibit increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory IL-10, which is associated with a

decrease in tumour growth (Samy et al., 2007). Moreover, when T-lymphocyte-deficient C.B-17 *scid* mice were injected with *SK2*$^{-/-}$ T-lymphocytes, pro-inflammatory cytokines levels increased and intestinal inflammation was more severe compared with mice receiving wild-type T-lymphocytes. This appears due to enhanced IL-2 responsiveness and increased expression of activated phosphorylated STAT5 (Samy et al., 2007).

Current advances in the identification of SK1 and SK2 inhibitors

Multiple options exist for the development of SK1 inhibitors. Enzyme kinetic studies show that many of the SK inhibitors that have been developed are competitive with sphingosine, but allosteric inhibitors are also an exciting option (see below). The Pyne lab recently showed that SK1 contains an allosteric site (Lim et al., 2011a) and that replacement of the amino group in (*S*)-FTY720-vinylphosphonate with an azido group, changes this compound from an allosteric inhibitor to an activator of SK1 (Liu et al., 2013). The first inhibitors of SK1 were sphingosine analogs, such as *D,L-threo*-dihydrosphingosine (DHS) which has a Ki of ~5 μM (Pyne and Pyne, 2011). N,N-Dimethylsphingosine (DMS), which was originally identified as a PKC inhibitor, also inhibits both SK isoforms. The first inhibitor that was highly selective for SK1 was the water-soluble sphingosine analog, SK1-I (Ki ~10 μM) (Paugh et al., 2008). Currently, the most potent nanomolar SK1-selective inhibitor is PF-543 (Schnute et al., 2012). Using a synthetic route from 4-octylphenethyl alcohol, we have produced a series of FTY720-like analogues that are SK1-selective inhibitors (e.g. RB-005) (Baek et al., 2013). ABC294640 (French et al., 2010) and (*R*)-FTY720 methylether (ROMe) (Lim et al., 2011b) are SK2-selective inhibitors with K_i values ~ 10 μM. With the very recent resolution of the crystal structure of SK1 (Wang et al., 2103), it may now be possible to understand the mechanisms of action of these compounds and their selectivity for SK1.

Allosteric inhibitors

Allosteric inhibitors and activators might bind to unique allosteric sites in SK1 or alternatively, might induce cooperative effects with bound substrate. For instance, (*S*)-FTY720 vinylphosphonate contains a hydroxyl group that can form a hydrogen bond with A339 via a water molecule. Therefore, (*S*)-FTY720 vinylphosphonate is expected to overlap the binding mode of sphingosine and should act as a competitive inhibitor with sphingosine. However, our kinetic study revealed that (*S*)-FTY720 vinylphosphonate is an uncompetitive inhibitor with sphingosine (Lim et al., 2011a), which requires that (*S*)-FTY720 vinylphosphonate binds to the SK1-sphingosine complex and not to the free enzyme. We and others have also reported that SK1 is a dimer (Kihara et al., 2006; Lim et al., 2011a); therefore, binding of sphingosine to the catalytic site in one of the monomers could promote binding of (*S*)-FTY720 vinylphosphonate to the catalytic site in the second monomer. In this manner, the binding of substrate and inhibitor molecules do not compete and inhibition is uncompetitive. Once (*S*)-FTY720 vinylphosphonate has bound to the second monomer it might reduce the phosphorylation of sphingosine by displacing the critical amino-acid residues away from sphingosine, thereby preventing deprotonation of the primary hydroxy group of sphingosine, which is required for catalysis.

Allosteric activators

The azido derivative of (*S*)-FTY720 vinylphosphonate is an activator of SK1 (Liu et al., 2013), as is a regioisomer of FTY720 (Lim et al., 2011a), suggesting that the amino group in (*S*)-FTY720 vinylphosphonate is essential for allosteric regulation and that these activators bind in a manner where there is overlap in the catalytic pocket**.** One possibility is that the binding of an activator in one of the monomers promotes binding of sphingosine to the catalytic site in the second monomer. In this manner, overall catalysis is more efficient compared with occupation of both sites with sphingosine. One would predict that as the activator concentration is increased, the activator should also bind in a manner that

overlaps the catalytic pocket of the second monomer. Under these conditions, both catalytic sites will be occupied with activator, thereby precluding binding of sphingosine. Under these conditions, the enzyme will effectively be inhibited. Indeed, we found that two azido analogues of (*S*)-FTY720 vinylphosphonate exhibit a bell-shaped concentration response curve, with activation of SK1 at low concentrations and inhibition at high concentrations (Liu et al., 2013). Allosteric inhibitors offer the prospect of conferring exquisite specificity for SK1. Furthermore, crystallisation studies and modelling might enable development of allosteric inhibitors with very high affinity, improving on current inhibitors that only have micromolar potency. Our studies indicate that SK1 contains an auto-inhibitory domain which is either stabilised in the 'on-state' by inhibitors or stabilised in the 'off-state' by activators (Lim et al., 2011a).

Use of inhibitors to interrogate TLR4- and TNF-α-dependent signaling

We propose that TNF-α-dependent activation of SK1 results in S1P which can be released from cells to act on S1P receptors and thereby promotes STAT3-mediated IL-6 formation (Fig. 1). Indeed, binding of S1P to $S1P_1$ receptor has been shown to promote NKκB and STAT3 activation and IL-6 formation in colitis-induced cancer, which is driven by aberrant macrophage pathology (Liang et al., 2013; Pyne and Pyne, 2013). The role of SK/S1PR 'inside-out' signalling can be tested by assessing the effect of S1PR antagonists and siRNA knockdown (compared with scrambled siRNA) of S1PR on TNF-α- and S1P-stimulated NFκB activation, STAT3 phosphorylation and IL-6 formation. Alternatively, S1P might stimulate TRAF2/receptor interacting protein 1 (RIP1) signalling and IKK activation. S1P derived from SK2 might enhance LPS signalling by binding to intracellular targets in the MyD88-dependent pathway to amplify signal transmission from TLR4, expression of which is regulated by SK2 (Liu et al., 2012).

S1P and cancer

Role of SK1 in inflammation-linked cancer

There is substantial evidence of a role for SK1 in numerous cancers (Pyne and Pyne, 2010; Pyne et al., 2012). There is increased expression of SK1 in stomach, lung, brain, colon, kidney and breast cancers and non-Hodgkins lymphoma (Pyne and Pyne, 2010). In addition, high expression of SK1 in patient tumours, including breast cancer and astrocytoma grade 4, is associated with poor clinical prognosis (Van Brocklyn et al., 2005; Watson et al., 2010; Ohotoski et al., 2012). There is also a link between inflammation and cancer in terms of the participation of SK1. Thus, high expression of SK1 is associated with metasatic colon cancer (Kawamori et al., 2009). Indeed, the colon carcinogen, azoxymethane increases SK1 expression and S1P levels in tumours (Kawamori et al., 2009). Moreover, SK1 knockout mice subjected to azoxymethane develop significantly less aberrant crypt foci formation and exhibit reduced colon cancer progression (Kawamori et al., 2009). Indeed, silencing of SK1 reduces COX2 and PGE2 production in HT-29 colon cancer cells (Kawamori et al., 2006). S1P also promotes a malicious amplification loop involving $S1P_1$, NFκB, IL-6 and STAT3 to promote colitis-induced colon cancer (Liang et al., 2013). Moreover, Liang et al. (2013) have recently demonstrated that FTY720 ablates colitis-induced colon cancer via inhibition/down-regulation of SK1 expression.

Role of SK1 in regulating cell survival

We have shown that the treatment of androgen-sensitive LNCaP cells with the SK inhibitor, SKi ((2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole) (French et al., 2003)), induces the proteasomal degradation of SK1 (Loveridge et al., 2010). SKi also promotes formation of pro-apoptotic diadenosine 5',5'''-P^1,P^3-triphosphate (Ap3A) (Watson et al., 2013). Tryptophanyl-tRNA synthetase uses ATP, ADP and tryptophan to produce Ap3A, which binds to the effector, FHIT (fragile histidine triad protein) tumour suppressor gene product (Vartanian et al., 1997; Fischer and McLennen, 2008) and is removed by FHIT

hydrolase activity. Growth suppression induced by FHIT involves up-regulation of the cell cycle regulator and cyclin-dependent kinase inhibitor, $p21^{waf1}$ (Sard et al., 1999). FHIT has also been shown to interact with ferridoxin reductase to produce reactive oxygen species (ROS) and to thereby induce apoptosis of cancer cells (Trapasso et al., 2008). Therefore, since Ap3A accumulates when SK1 is degraded by the proteasome in response to SK inhibitors, we propose that SK1 limits the tryptophanyl-tRNA synthetase-catalysed formation of Ap3A or promotes FHIT hydrolase activity. This might prevent FHIT-dependent activation of ferridoxin reductase and ROS formation and this leads to cancer cell survival. The finding that SK inhibitor increases Ap3A levels are significant as over-expression of FHIT induces apoptosis in cancer cells with FHIT gene abnormalities (Ji et al., 1999). Moreover, *Fhit* knockout mice are predisposed to tumour development, and *Fhit* gene therapy reduces tumour burden (Pichiorri et al., 2008). In addition, down regulation of FHIT protein is linked with extra-prostatic extension and Gleason score > 8 in prostate cancer and breast cancer. Evidence strongly suggests the involvement of germ-line variations of FHIT in prostate cancer risk. Restoration of wild-type FHIT in 3p14.2-deficient human lung cancer cells also inhibits cell growth and induces apoptosis (Deng et al., 2007). Thus, the SK1-FHIT axis may be one mechanism whereby SK1 regulates cell survival.

Role of SK1 in regulating amplification loops

We have reported that S1P promotes translocation of SK1 to the plasma-membrane of MCF-7 breast cancer cells via an $S1P_3$-dependent mechanism (Long et al., 2010). Moreover, silencing of SK1 reduces $S1P_3$ expression and results in a decrease in S1P/$S1P_3$-stimulated ERK-1/2 activation and migration of MCF-7 cells (Long et al., 2010). Therefore, SK1 regulates responsiveness of these cancer cells to S1P by increasing $S1P_3$ expression levels. This might produce a positive amplification loop of $S1P_3$-mediated invasive signalling in breast cancer cells (Fig. 2).

Role of SK1 in regulating the Warburg effect

We have also demonstrated that SK1 inhibitors, such as SKi, induce the ubiquitin-proteasomal degradation of SK1 by activating the proteasome (Loveridge et al., 2010). We have also performed the first metabolomic analysis in prostate cancer cells treated with SKi, under conditions where SK1 is degraded by the proteasome (Loveridge et al., 2010). The findings suggest that SK1 promotes the Warburg effect (Fig. 3), which is an essential survival pathway for cancer cells to obtain ATP for biosynthetic and catabolic metabolism (Watson et al., 2013). Treatment of androgen-sensitive prostate cancer cells with the SK1 inhibitor, SKi, increases glycolytic metabolites and *(R)-S*-lactoyl-glutathione levels (Watson et al., 2013); the latter is formed from methylglyoxal (a highly reactive glycolytic byproduct that is apoptotic in prostate cancer cells). Treatment of androgen-sensitive prostate cancer cells with SKi also increases glucose 6-phosphate utilisation by the pentose phosphate pathway in order to provide the anti-oxidant NADPH to counter oxidative stress responses (Watson et al., 2013). The glutathione system uses NADPH to recycle glutathione (GSH). In this case, the protection afforded by NADPH toward oxidative stress is inadequate and the prostate cancer cells appear to be overwhelmed by ROS; this might explain the subsequent apoptotic response. SKi also promotes the proteasomal degradation of c-Myc, a consequence of ceramide-dependent activation of the proteasome, which might account for the effect of this inhibitor on the Warburg effect (Watson et al., 2013). Indeed, c-Myc is a master transcriptional regulator of the glycolytic pathway (Osthus et al., 2000; Shim et al., 1997). A reduction in c-Myc level is also found in polyps of *ApcMin*$^{-}$ *Sk1*$^{-/-}$ mice (Kohno et al., 2006), thereby confirming a regulatory link between SK1 and c-Myc expression.

Nuclear $S1P_2$ receptor signalling and interaction with SK1/SK2 and SRC

High estrogen receptor positive (ER^+) tumour expression of nuclear SRC and nuclear $S1P_2$ in patients is associated with longer disease-specific survival time and is therefore protective against mortality (Ohotski

et al., 2013). In addition, tumours with high levels of nuclear $S1P_2$ receptor have significantly reduced levels of nuclear SK1 (which is linked with poor prognosis). This suggests the presence of an active translocation mechanism for SK1 that is regulated by $S1P_2$ and which might account for its protective action in cancer patients (Ohotski et al., 2013). We have demonstrated that treatment of triple negative MDA-MB-231 breast cancer cells with the SK1 inhibitor, SKi, promotes the accumulation of ectopically expressed wild type $S1P_2$ along with tyrosine phosphorylated (Y416) Src (non-receptor tyrosine kinase) in the nucleus of these cells (Fig. 4). Our proposed model is that S1P prevents the nuclear localisation of $S1P_2$ and Y416 Src. Therefore, inhibition of SK1 with pharmacological inhibitors reduces S1P levels, enabling the translocation of $S1P_2$-Y416 Src to the nucleus. Indeed, treatment of these cells with S1P reduces the SKi-induced nuclear translocation of $S1P_2$-Y416 Src. Therefore, SK1 might protect against apoptosis of breast cancer cells by preventing nuclear $S1P_2$-Y416 Src localization and induced expression of apoptotic genes.

Conclusion

Although the crystal structure of SK1 has been resolved, this has been achieved with SKi, a low-affinity SK1 inhibitor. The functional interaction with a high-affinity inhibitor is necessary to determine the complete structural architecture of the catalytic site. This may lead to the development of better SK1 inhibitors that can be used to induce apoptosis of cancer cells and to enhance anti-inflammatory activity. However, a recent study showed that a nanomolar inhibitor of SK1 (PF-543) failed to kill cancer cells *in vitro* (Schnute et al., 2012). This observation has led to serious questions about whether SK1 is a viable therapeutic target in cancer, despite a wealth of evidence for a critical role of SK1 in cancer cell survival, growth, transformation, metastasis and neovascularisation. The high binding affinity of PF-543 for SK1 might indicate the possibility that this compound might also bind to other enzymes that use sphingosine as a substrate e.g. ceramide synthases. Inhibition of ceramide synthase is likely to neutralize the effect of

inhibiting SK1 activity on cell growth and survival by preventing formation of ceramide from sphingosine that is accumulates as a result of inhibiting/down-regulating SK1. Thus, development of more potent SK inhibitors requires binding modalities that confer specificity toward SK over other sphingolipid metabolizing enzymes. This might be essential in order to potentiate killing of cancer cells by selective inhibition of SK1.

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Figure legends

Fig. 1. Schematic showing the possible role of TNF-α activated SK1 in $S1P_1$ receptor-mediated activation of NFκB, STAT3 and IL-6 formation and inflammation

Fig. 2. Schematic showing the SK1-$S1P_3$ amplification loop in ER^+ MCF-7 breast cancer cells that might drive invasiveness and metastasis.

Fig. 3. Schematic summarizing the role of SK1 in inhibiting proteasomal activity thereby maintaining c-Myc expression levels. c-Myc is a master transcriptional regulator of the glycolytic enzymes that drives

the Warburg effect and limits flux through the pentose phosphate pathway. SK1 also limits the formation of Ap3A and oxidative stress.

Fig. 4. Schematic summarizing how the SK1 inhibitor, SKi, promotes nuclear localization of $S1P_2$-Y416 Src in ER^- MDA-MB-231 cells that might contribute to the effect of these inhibitors on cancer cell growth, DNA synthesis and apoptosis.

Fig. 1

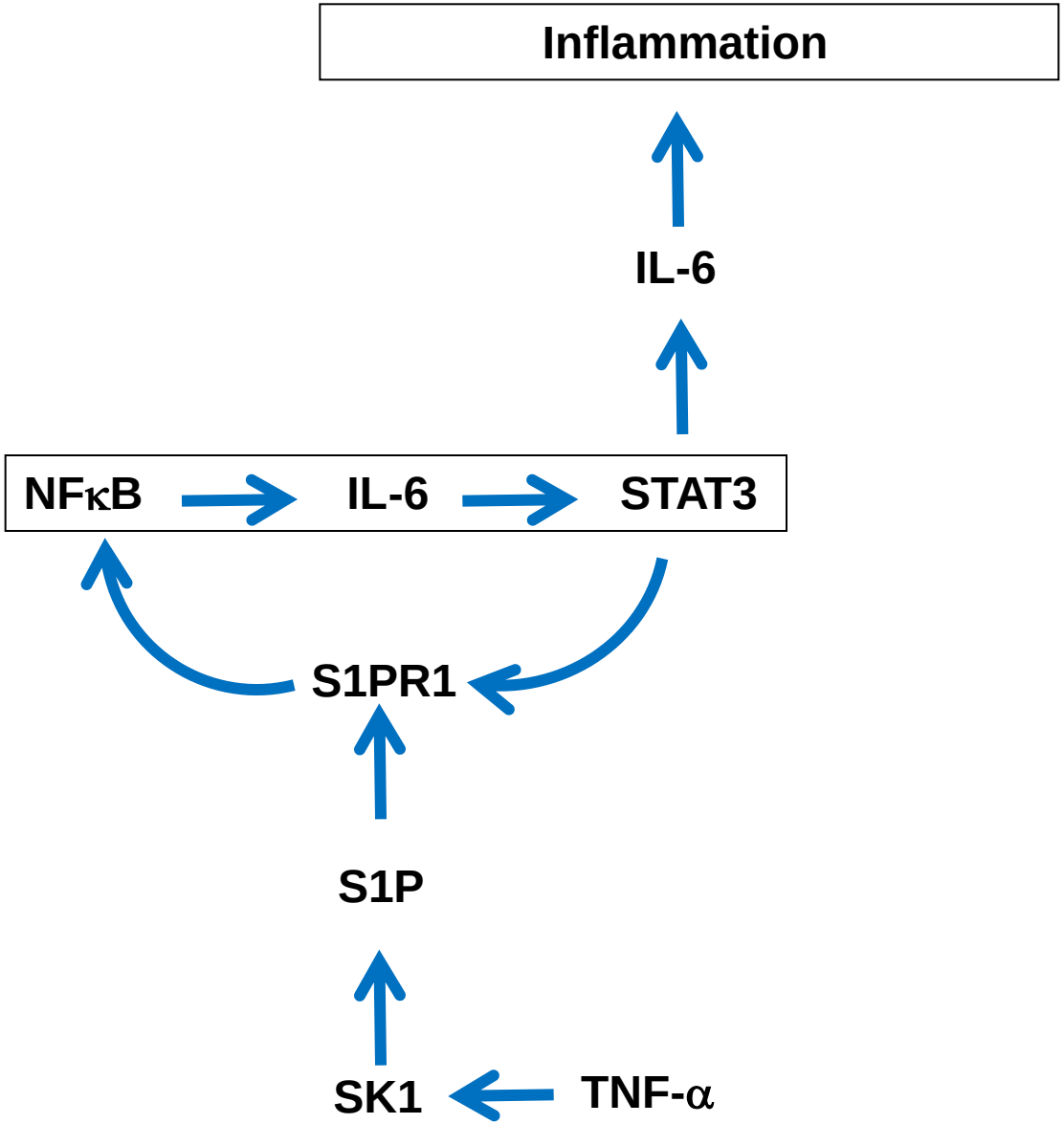


Fig. 2

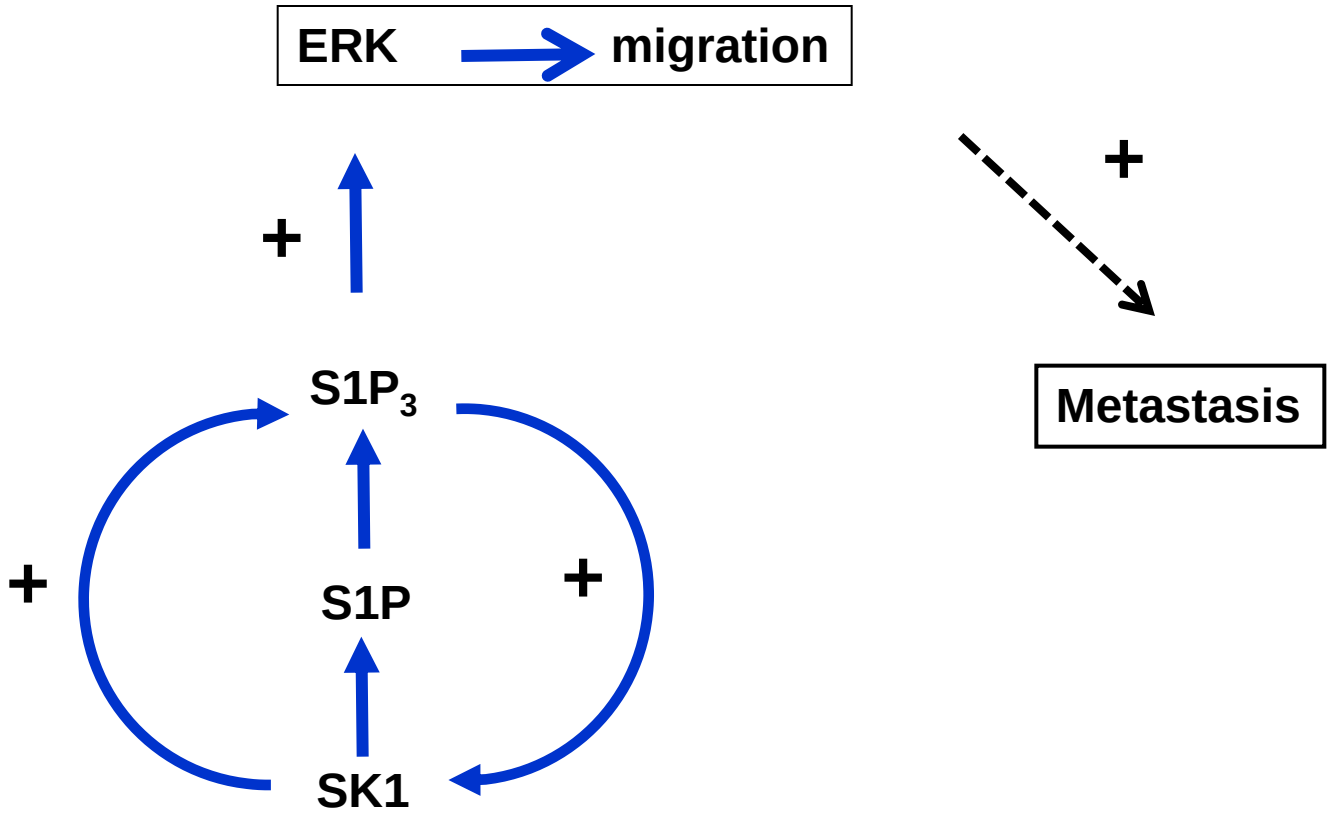


Fig. 3

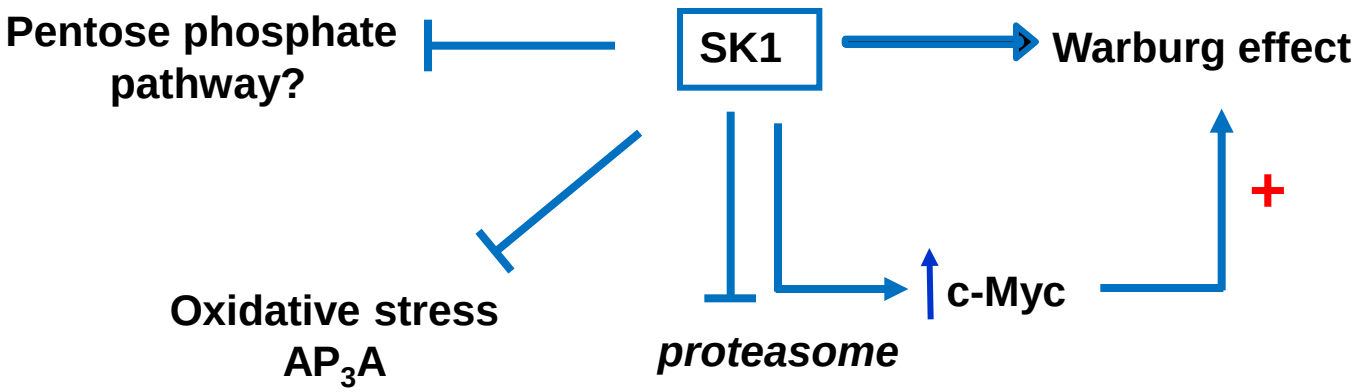
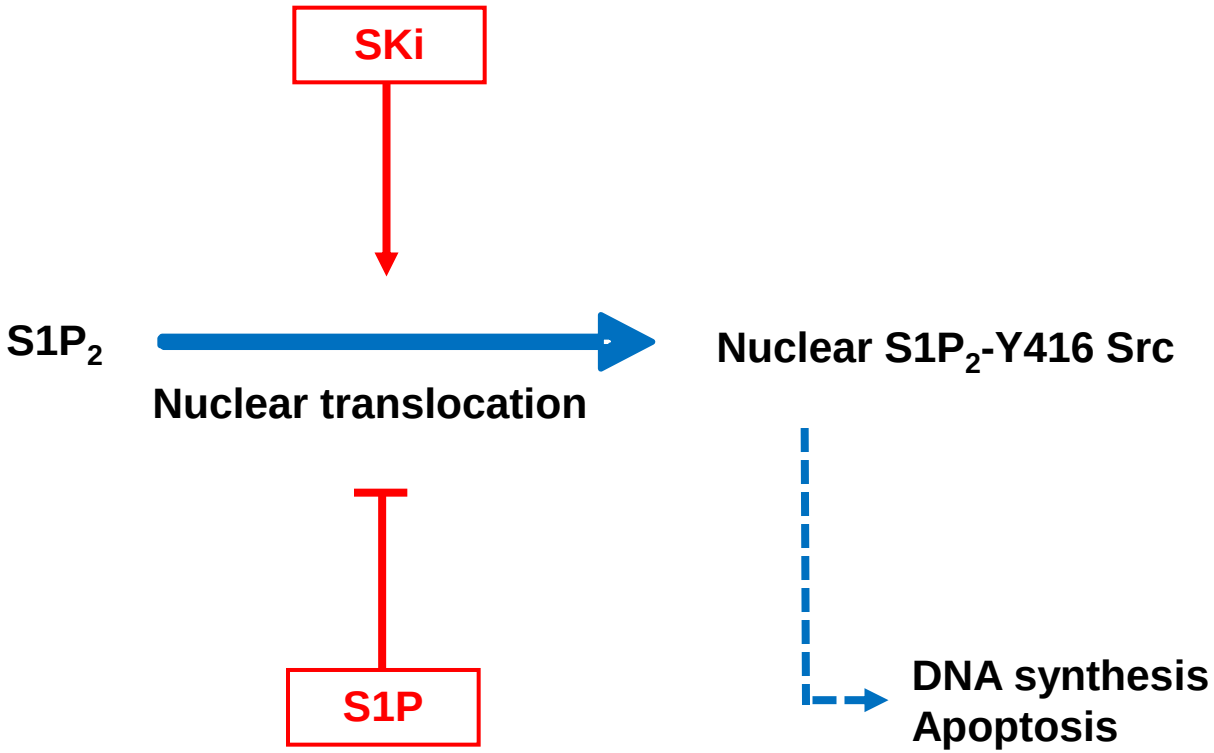

Pentose phosphate pathway?
SK1
Warburg effect
+
Oxidative stress
AP3A
proteasome
c-Myc

Fig. 4


SKi
S1P2
Nuclear translocation
Nuclear S1P2-Y416 Src
S1P
DNA synthesis
Apoptosis