

Sphingosine Kinase Inhibitors and Cancer: Seeking the Golden Sword of Hercules

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Abstract

There is considerable evidence that sphingosine kinases play a key role in cancer progression, which might involve positive selection of cancer cells that have been provided with a survival and growth advantage as a consequence of overexpression of the enzyme. Therefore, inhibitors of sphingosine kinase represent a novel class of compounds that have potential as anticancer agents. Poor inhibitor potency is a major issue that has precluded successful translation of these compounds into the clinic. However, recent discoveries have shown that sphingosine kinase 1 is an allosteric enzyme and that some inhibitors offer improved effectiveness by inducing proteasomal degradation of the enzyme or having nanomolar potency. Herein, we provide a perspective about these recent developments and highlight the importance of translating basic pharmacologic and biochemical findings on sphingosine kinase into new drug discovery programs for treatment of cancer. *Cancer Res*; 71(21); 6576–82. ©2011 AACR.

The Role of Sphingosine Kinase in Cancer

The levels of the bioactive sphingolipid sphingosine-1-phosphate (S1P) are controlled by its synthesis (conversion of sphingosine to S1P, catalyzed by the 2 isoforms of sphingosine kinase SK1 and SK2) and removal (by cleavage of S1P catalyzed by S1P lyase or dephosphorylation catalyzed by S1P phosphatase; ref. 1). S1P binds to S1P-specific G-protein-coupled receptors termed SIP₁₋₅ (1). S1P also binds to intracellular protein targets (see later).

There is evidence of a major role for sphingosine kinase in human cancers. For instance, there is elevated *SK1* mRNA transcript and/or SK1 protein expression in stomach, lung, brain, colon, kidney, and breast cancers and non-Hodgkin lymphoma (1). Indeed, we reported that high tumor expression of SK1 is correlated with poor patient survival rates and induction of tamoxifen resistance in patients with estrogen receptor (ER)-positive breast cancer ($n = 304$; refs. 2, 3). Moreover, S1P promotes migration of ER⁺ MCF-7 breast cells via an SK1-dependent mechanism, and this might suggest a role for SK1 in metastasis (2). Ectopic overexpression of SK1 in MCF-7 cells also induces resistance to tamoxifen (for review, see ref. 1). In addition, SK1 expression is higher in ER⁻ than in ER⁺ breast tumors and this is correlated with a poorer prog-

nosis (for review, see ref. 1). Similarly, high expression of SK1 in astrocytoma correlates with poor prognosis, and knockdown of SK1 reduces glioblastoma cell proliferation (for review, see ref. 1). Therefore, SK1 appears to play a role in 2 major hallmarks of cancer, namely, enhanced proliferation and metastasis/invasion. In addition, the overexpression of SK1 in fibroblasts induces their transformation to fibrosarcoma (for review, see ref. 1). S1P is also involved in regulating angiogenesis and creation of a tumor microenvironment. This is exemplified by the use of the sphingosine analogue, FTY720, which is converted to (*S*)-FTY720 phosphate *in vivo* by SK2 and has recently been approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of relapsing multiple sclerosis (4). (*S*)-FTY720 phosphate binds to SIP_{1,3,4,5} and is a functional antagonist of SIP₁ (4), whereas FTY720 is a direct inhibitor of SK1 activity (5). Moreover, FTY720 reduces tumor metastasis in a mouse melanoma model and decreases tumor cell proliferation and increases apoptosis due to inhibition of neovascularization (for review, see ref. 1). FTY720 also decreases metastasis in a breast cancer mouse model which is associated with deformed and decreased filopodia formation in the cancer cells (for review, see ref. 1). Additional *in vivo* evidence supports a role for SK1 as a chemotherapeutic "sensor" for promotion of tumorigenesis. Large vascularized resistant tumors are formed when cancer cells overexpressing SK1 are injected or implanted into mice (for review, see ref. 1).

Multiple mechanisms regulate the expression of SK1. For instance, the *SK1* gene is regulated by AP2, Sp1, SMAD4 (6), and HIF2 α (for review, see ref. 1), suggesting that SK1 expression might be controlled by mitogen-activated protein kinase signaling, cytokines, and hypoxia (in solid tumors). Moreover, a number of growth factors and steroid hormones regulate the expression of SK1, such as TGF- β , estrogen, and progesterone (1, 7, 8). SK1 expression in cells is also regulated by proteolysis.

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For instance, cathepsin B has been implicated in regulating lysosomal degradation of SK1 in podocytes (9). SK1 expression is also regulated by the ubiquitin-proteasomal pathway in LNCaP prostate cancer and MCF-7 breast cancer cells (5, 10), raising the possibility that this route of degradation might be deregulated in certain cancers. In summary, altered expression of SK1 underlies the major cancer-promoting properties of this enzyme. Cancer cells that overexpress SK1 appear to exhibit a nononcogenic addiction for SK1 (for review, see ref. 1). This is defined by a positive selection of cancer cells because elevated SK1 expression confers a survival and growth advantage to these cells.

SK2 also has a role in cancer. Thus, siRNA knockdown of SK2 in breast or colon cancer cells reduces doxorubicin-induced expression of p21 (a cyclin-dependent kinase inhibitor) and G₂-M arrest and enhances doxorubicin-induced apoptosis. Moreover, breast or colon cancer progression is reduced upon knockdown of SK2 (for review, see ref. 1). In addition, epidermal growth factor stimulates the extracellular signal-regulated kinase 1-catalyzed phosphorylation of SK2 on Ser 351 and Thr 578, which is required for the migration of MCF-7 breast cancer cells in response to this growth factor (for review, see ref. 1).

The Need for S1P Therapeutics

The major objective of drug discovery has focused on new molecules that are capable of agonizing/antagonizing S1P₁₋₅. A prominent example is FTY720, which, via transformation to (S)-FTY720 phosphate has been developed as a functional S1P₁ antagonist (4). Another approach is to reduce the bioavailability of S1P at its receptors using S1P-neutralizing antibodies. Formulation of humanized S1P-neutralizing antibody is currently being evaluated in phase I clinical trials for solid tumors (11).

However, in addition to acting on cell surface S1P receptors, S1P also binds to and modulates the activity of intracellular proteins. Novel intracellular targets of S1P include histone deacetylase (HDAC; ref. 12), TRAF2 (13), p21-activated protein kinase 1 (PAK1; ref. 14), and prohibitin 2 (15). S1P formed by SK2 binds to and inhibits HDAC1 and HDAC2 within repressor complexes that are enriched at the promoters of genes encoding p21 (a cyclin-dependent kinase inhibitor) and c-fos (a transcriptional regulator). S1P inhibition of HDACs increases histone acetylation, thereby promoting expression of p21 and c-fos (12). The identification of TRAF2 as a target for S1P formed by SK1 (13) is particularly important given its role in regulating NF- κ B in inflammation and cancer. S1P activates TRAF2, which is an E3 ligase that catalyzes the ubiquitination of RIP1 (Lys63-signaling ubiquitination), required for NF- κ B activation (13). Indeed, NF- κ B is linked with the acquisition of resistance of prostate cancer cells to docetaxel (16). S1P formed by SK1 has also been shown to directly activate PAK1 (14). Heregulin-induced migration requires SK1 and is contingent on a heregulin-induced colocalization of SK1 with filamin A in lamellipodia in A7 melanoma cells. Downregulation of SK1 markedly reduces activation of PAK1 and phosphorylation of filamin A (14). S1P formed by SK2 also binds directly to prohibitin-2 (PHB2) at the inner mitochondrial membrane

and induces the correct assembly of the cytochrome *c* oxidase complex (respiratory complex IV) required for oxidative phosphorylation (15).

Sphingosine Kinase Inhibitors

The studies described above highlight the importance of limiting the intracellular action of S1P in cancer by developing inhibitors of SK1 and SK2. Indeed, there has been a concerted effort to produce inhibitors. One of the first to be identified was SKi [or SKI-II, 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl)thiazole; Fig. 1], which inhibits both SK1 and SK2 activity. SKi reduces intracellular S1P, inhibits proliferation, and induces apoptosis in various cancer cell lines (17). *In vivo* studies showed good oral bioavailability and inhibition of tumor growth (18). A water-soluble sphingosine analogue, BML-258 [SK1-I; (2*R*,3*S*,4*E*)-*N*-methyl-5-(4'-pentylphenyl)-2-aminopent-4-ene-1,3-diol; Fig. 1], is an SK1-selective inhibitor (19) that is effective both *in vitro* and *in vivo* (20). ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide; Fig. 1] is a selective competitive (with sphingosine) SK2 inhibitor (21), which is an effective orally bioavailable anticancer agent, and inhibits tumor proliferation and migration *in vitro* (21, 22). ABC294640 induces autophagic cell death in PC-3 prostate, MDA-MB-231 breast, and A-489 kidney tumor cells (22). (R)-FTY720-OME is a new analogue in which one of the prochiral hydroxyl groups of FTY720 has been replaced by a methoxy group (Fig. 1; ref. 23). The rationale for replacing one of the hydroxyl groups with a methyl ether was to block the site that is phosphorylated by SK2. (R)-FTY720-OME is a specific, competitive (with sphingosine) inhibitor of SK2 (with no effect on SK1) with a K_{ic} of 16 μ mol/L (23). The enantiomer of (R)-FTY720-OME does not inhibit SK2. (R)-FTY720-OME also induces growth arrest of MCF-7 breast cancer cells, thereby providing additional means of reducing proliferation of these cells. (R)-FTY720-OME also inhibits S1P-induced actin rearrangement in MCF-7 cells, thereby preventing formation of a migratory phenotype, suggesting an additional application to preventing metastasis (23).

Recent enzyme kinetic studies revealed that FTY720 is a novel competitive (with sphingosine) inhibitor of SK1 with a K_{ic} of 2 μ mol/L (5, 24). The K_M of SK2 for FTY720 is 18.2 μ mol/L (13.8 μ mol/L for sphingosine; ref. 25). However, the V_{max} for FTY720 is appreciably lower than for sphingosine (FTY720, 5.1 nmol/min/mg protein; sphingosine, 43 nmol/min/mg protein), thus, FTY720 is also an effective inhibitor of SK2 activity where sphingosine is the substrate (unpublished data, S. Pyne and N.J. Pyne). SKi is a mixed inhibitor of SK1 (with sphingosine) with a K_{ic} of 17 μ mol/L and a K_{iu} of 48.3 μ mol/L and thus, at low micromolar concentration, favors competitive inhibition (24). We have synthesized multiple analogues of FTY720 and established that minor modifications in chemical scaffold have profound effects on effectiveness and selectivity (Fig. 1). Inhibitor characterization studies reveal that (S)-FTY720 vinylphosphonate inhibits SK1 in an uncompetitive manner (with sphingosine) with K_{iu} of 14.5 μ mol/L (24). This mode of inhibition is indicative of allosterism contingent on

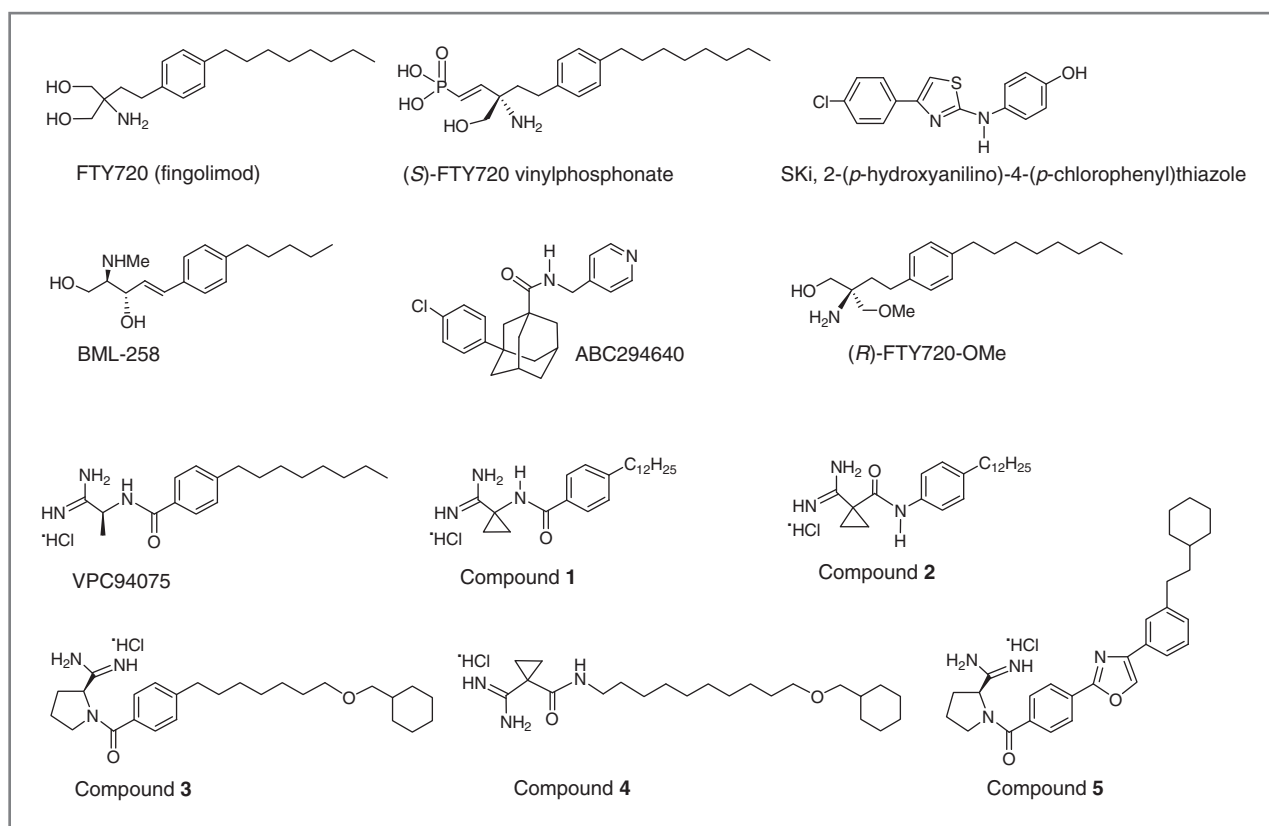


Figure 1. Structural formulas of FTY720 (fingolimod), (S)-FTY720 vinylphosphonate, SKI, BML-258, (R)-FTY720-OMe, ABC294640, and 6 amidine-based analogues that have been shown to inhibit SK1 and/or SK2 activity.

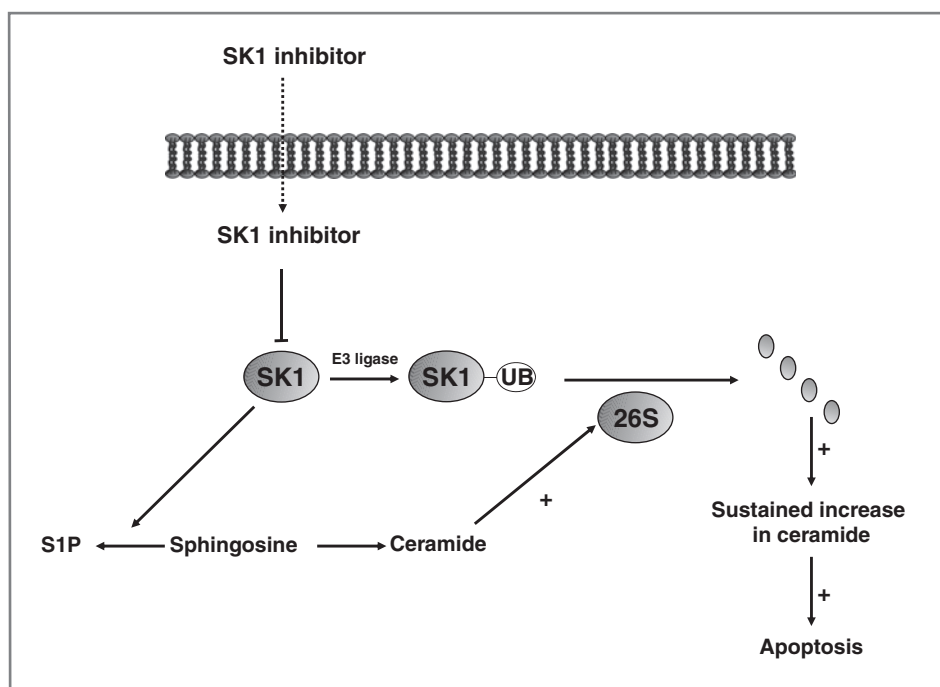
formation of the sphingosine–SK1 complex. Two new FTY720 analogues, a conjugate of sphingosine with a fluorophore [Bodipy-sphingosine (Bdp-So)] and (S)-FTY720 regioisomer (in which the position of the amino group and a hydroxyl group are interchanged in the molecule), stimulate SK1 activity (24), thereby providing additional evidence for the presence of allosteric site(s). SK1 is an oligomeric protein (minimally a dimer) containing noncooperative catalytic sites and that the allosteric site(s) exert an autoinhibition of the catalytic site. We have proposed a model in which the allosteric site exists in an "on" and "off" state in equilibrium (24). Thus, the binding of (S)-FTY720 vinylphosphonate to SK1 stabilizes the "on" state, which is predicted to have a longer lifetime in terms of autoinhibiting activity than SK1 with an unbound allosteric site. Conversely, activators of SK1, such as Bdp-So and (S)-FTY720 regioisomer, stabilize the "off" state to relieve the inhibition of SK1 activity by the allosteric site and to therefore stimulate the enzyme activity.

An Issue of Effectiveness: Ability to Induce the Proteasomal Degradation of SK1

We have proposed an entirely novel mechanism for the interaction of inhibitors with SK1 that offers additional opportunities for drug discovery. SK1 inhibitors activate the ubiquitin-proteasomal degradation pathway to remove SK1 from

MCF-7 breast cancer and LNCaP prostate cancer cells (Fig. 2; refs. 5, 10, 24). The SKI-induced proteasomal degradation of SK1 is reduced by the proteasomal inhibitor MG132. In podocytes, SKI also induces the proteolytic degradation of SK1, but this is mediated by a lysosomal- and cathepsin B (CA074Me sensitive)–dependent pathway (9). The treatment of prostate cancer cells with the cathepsin B inhibitor CA074Me has no effect on the SKI-induced degradation of SK1, suggesting that this route of degradation does not operate in these cells (10). The proteasomal degradation of SK1 is partly dependent on the inhibition of SK1 activity, the accumulation of ceramide, and the subsequent ceramide-induced activation of the proteasome (10). SK1 is polyubiquitinated, and SK1 inhibitors enhance the rate at which this modified version of the enzyme is degraded by the proteasome (10). Evidence to support a role for ceramide was obtained using the mycotoxin fumonisin B1, which inhibits the conversion of dihydrosphingosine to dihydroceramide and sphingosine to ceramide catalyzed by ceramide synthase. Fumonisin B1 treatment partially reversed the effect of SKI on the proteasomal degradation of SK1 (10). The creation of an SK1-null cancer cell leads to the onset of apoptosis (5, 10). This is associated with a reduction in intracellular S1P levels and an elevation of C22:0-ceramide (10). Moreover, these effects are recapitulated by siRNA knockdown of SK1 in MCF-7 cells (for review, see ref. 1). We also found that FTY720 and (S)-FTY720 vinylphosphonate induce

Figure 2. A schematic representation of the effect of SK1 inhibitors in promoting the ubiquitin (UB)-proteasomal degradation of SK1. Acute inhibition of SK1 by the SK1 inhibitor appears to promote a ceramide-dependent proteasomal degradation of SK1 that is polyubiquitinated under basal conditions. 26S represents the proteasome.



the proteasomal degradation of SK1 in MCF-7 cells (5, 24), suggesting that a wide range of structurally diverse SK1 inhibitors use this common pathway to remove SK1 from cancer cells. FTY720 itself also has additional targets (for review, see ref. 1), including activation of PP2A (26), which might have a significant effect on cell-cycle progression.

An Issue of Potency: Designing New Nanomolar Inhibitors

To date, most inhibitors of SK1 exhibit K_i or IC_{50} values in the micromolar range, which largely preclude their use as therapeutic agents. This places constraints on bioavailability and increases the potential for off-target effects, even though some of these inhibitors are effective in *in vivo* cancer models. This has, therefore, hampered progress to the clinic. However, recent advances in this area have generated optimism that new inhibitors may become available that can be translated to the clinic. For example, Kennedy and colleagues have recently synthesized amidine-based SK1 inhibitors with nanomolar potency that reduce endogenous S1P levels in human leukemia U937 cells (27). The lead compound was the amidine analogue VPC94075 [(*S*)-*N*-(1-amino-1-iminopropan-2-yl)-4-octylbenzamide hydrochloride; Fig. 1], which exhibits selectivity for sphingosine kinases (competitive with sphingosine), with no activity against diacylglycerol kinase and protein kinase C. Selectivity for SK1 over SK2 was dependent on the length of the apolar tail. Compounds with a short (C8 and C10) apolar tail inhibited both SK1 and SK2, whereas those with a longer tail length (C12) such as compounds 1 and 2 (Fig. 1) were selective for SK1. This can be explained by a model in which the catalytic

binding pocket of SK1 is larger than for SK2. The SK1/SK2 selectivity of compound 2 was higher than that of compound 1, suggesting that the orientation of the carboxamide moiety (phenyl-NHCO- vs. phenyl-CONH-) affects ligand binding to SK1 and SK2. Analogues containing a pyrrolidine-2-carboximidamide head group were synthesized; for example, compounds 3 and 4 (Fig. 1) that exhibited K_i values of 75 and 110 nmol/L for SK1 with an 80- and 470-fold selectivity for SK1 over SK2, respectively (27). An SK1 homology model based on the crystal structure of diacylglycerol kinase was used in which linkers were docked into the SK1 model. A class of heteroaromatic compounds with 6 fewer rotatable bonds was also synthesized. Of these, oxazole 5 (Fig. 1) was identified as an SK1 inhibitor with a K_i of 47 nmol/L and 180-fold selectivity over SK2. Therefore, a major barrier to translation of SK1 inhibitors, with respect to potency, has been removed.

An Attack on Cancer

S1P formed by SK1 can be released from cancer cells and has the potential to bind to S1P receptors on the cell surface to inhibit apoptosis. This process is called "inside-out" signaling (for review, see ref. 1). Indeed, high membrane S1P₁ expression in tumors of patients with ER⁺ breast cancer ($n = 304$) is associated with the acquisition of resistance to tamoxifen (3). S1P released from cancer cells also has the potential to bind to S1P receptors on endothelial cells to promote their proliferation, leading to neovascularization of the tumor. S1P can also act on fibroblasts within tumors, which might also function to sustain cancer progression through growth factors released from the fibrotic cells (28). Thus, a compound that combines

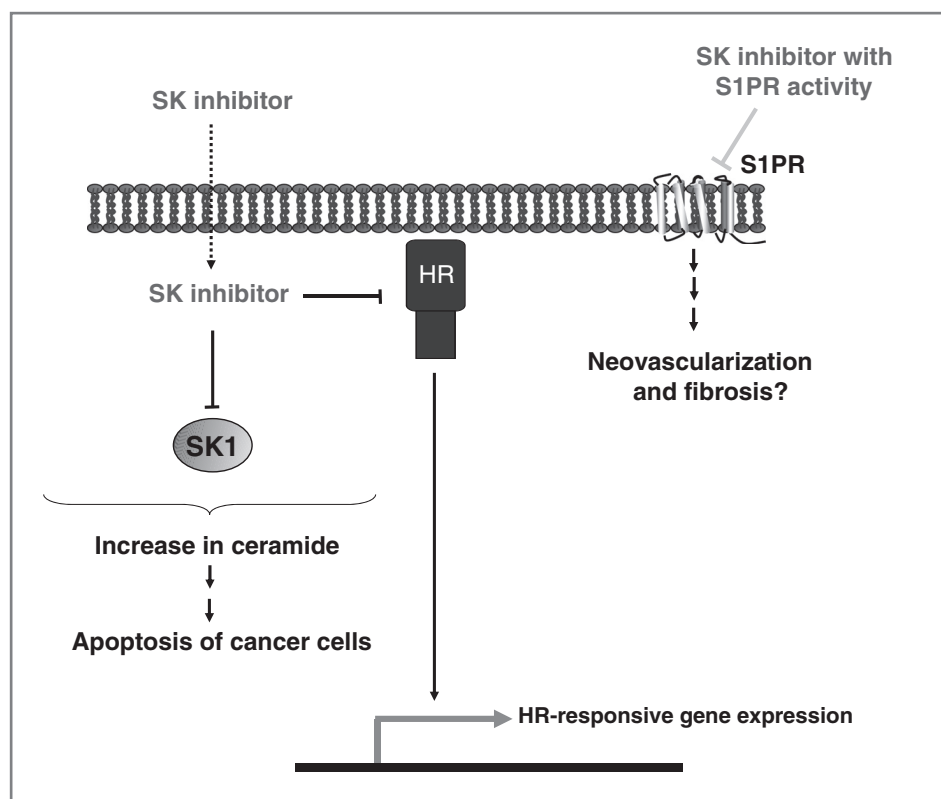


Figure 3. A schematic model of the "Hydra effect" of sphingosine kinase (SK) inhibitors. Inhibitors, such as (S)-FTY720 vinylphosphonate, that exhibit multiple actions on S1P-related proteins, including S1P₁, SK1, and SK2, might be usefully exploited to launch a multiprong attack on cancer. HR represents hormone receptor corresponding to androgen or estrogen receptors. S1PR, S1P receptors.

properties that block these cellular interactions would be highly advantageous in the treatment of cancer. Antagonism of S1P receptors might be effective at blocking neovascularization, fibrosis, and cancer cell proliferation, whereas binding of the compound to SK1 might promote its proteasomal degradation to induce apoptosis of fibroblasts and cancer cells (Fig. 3). Multiple effects may also underlie the action of SKi and ABC294640, which in addition to inhibiting SK1/SK2 and SK2, respectively, can bind to and antagonize the estrogen receptor in breast cancer cells (29, 30). SKi, FTY720, or (S)-FTY720 vinylphosphonate also reduce the expression of the androgen receptor in androgen-independent LNCaP-AI cells (5), thereby offering an additional therapeutic option, as the constitutively active androgen receptor is responsible for driving androgen-independent growth of these cells.

(S)-FTY720 vinylphosphonate also inhibits SK2 (23) and is a full antagonist of S1P_{1,3,4} ($K_i = 208, 15,$ and $1,190$ nmol/L, respectively) and a partial antagonist of S1P₂ and S1P₅ (31). The fact that (S)-FTY720 vinylphosphonate exhibits a K_i of 14.5 μ mol/L for SK1 inhibition (24) is not incompatible with a dual action at both S1P receptor and SK1, even under conditions where the extracellular concentration of (S)-FTY720 vinylphosphonate is in the nanomolar range. We draw an analogy with the action of FTY720. In this regard, nanomolar concentrations of FTY720 are clinically effective in the treatment of multiple sclerosis. FTY720 is phosphorylated by SK2 to provide the clinically effective agent (S)-FTY720 phosphate, which acts on 4 of the 5 S1P receptors. SK2 exhibits a K_M of 18 μ mol/L for FTY720 (25). Therefore, from an extracellular nanomolar con-

centration of FTY720, it seems likely that the cells concentrate the inhibitor, such that an intracellular concentration can accumulate into the range where it can be efficiently phosphorylated by SK2. This would also bring the concentration of FTY720 into a range that inhibits and induces proteasomal degradation of SK1 in cancer cells.

(S)-FTY720 vinylphosphonate (which is a putative allosteric inhibitor; ref. 24) induces the proteasomal degradation of an N-terminal variant of SK1 (86 amino acid N-terminal variant) in androgen-independent LNCaP-AI cells (10). This is significant because the N-terminal SK1 variant is resistant or less sensitive to proteasomal degradation induced by SKi and FTY720, respectively, in these cells (10). Therefore, allosteric inhibitors might force SK1 to adopt a conformation that improves susceptibility to proteasomal degradation. These findings are important because resistance to chemotherapeutic agents is correlated with high expression of SK1. This includes prostate cancer cells resistant to camptothecin, pancreatic cancer cells to gemcitabine, and chronic myeloid leukaemia cells to imatinib (for review, see ref. 1). Therefore, targeting SK1 to the proteasome opens avenues for restoring sensitivity of cancer cells to chemotherapeutic agents and provides a rationale for combination therapies with SK1 inhibitors.

Conclusions

The evidence presented here suggests that SK1 inhibitors have the potential to be the "golden sword" of Hercules

because they may cut off the many heads of cancer. Thus, inhibitors that can kill cancer cells by this "Hydra effect" (named after the Greek mythological monster with 9 heads) offer the potential for translation to the clinic. Several additional important questions have been raised by recent advances. For instance, can new sphingosine analogues be developed to generate a wider structurally diverse library of compounds with nanomolar potency? Is the enhanced potency of amidine SK1 inhibitors augmented by the proteasomal degradation of SK1? In addition to these questions, we suggest that sphingosine kinase allosterism offers new drug discovery opportunities. Indeed, the elucidation of the structure of sphingosine kinases by x-ray crystallography may provide the means by which development of high

potency novel catalytic and allosteric inhibitors can be accelerated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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