Discovery and Evaluation of Inhibitors of Human Sphingosine Kinase[1]

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ABSTRACT

Sphingolipid-metabolizing enzymes control the dynamic balance of the cellular levels of bioactive lipids, including the proapoptotic compound ceramide and the proliferative compound sphingosine 1-phosphate. Accumulating evidence indicates that sphingosine kinase (SK) plays a pivotal role in regulating tumor growth and that SK can act as an oncogene. Despite the importance of SK for cell proliferation, pharmacological inhibition of SK is an untested means of treating cancer because of the current lack of nonlipid inhibitors of this enzyme. To further assess the involvement of SK in human tumors, levels of RNA for SK in paired samples of cDNA prepared from tumors and normal adjacent tissue were analyzed. Expression of SK RNA was significantly elevated in a variety of solid tumors, compared with normal tissue from the same patient. To identify and evaluate inhibitors of SK, a medium throughput assay for recombinant human SK fused to glutathione *S*-transferase was developed, validated, and used to screen a library of synthetic compounds. A number of novel inhibitors of human SK were identified, and several representative compounds were characterized in detail. These compounds demonstrated activity at sub- to micromolar concentrations, making them more potent than any other reported SK inhibitor, and were selective toward SK compared with a panel of human lipid and protein kinases. Kinetic studies revealed that the compounds were not competitive inhibitors of the ATP-binding site of SK. The SK inhibitors were antiproliferative toward a panel of tumor cell lines, including lines with the multidrug resistance phenotype because of overexpression of either P-glycoprotein or multidrug resistance phenotype 1, and were shown to inhibit endogenous human SK activity in intact cells. Furthermore, each inhibitor induced apoptosis concomitant with tumor cell cytotoxicity. Methods for the synthesis of a series of aurone inhibitors of SK were established, and a prototypical dihydroxyaurone was found to have moderate antitumor activity *in vivo* in the absence of overt toxicity to the mice. These compounds are the first examples of nonlipid inhibitors of SK with *in vivo* antitumor activity and so provide leads for additional development of inhibitors of this important molecular target.

INTRODUCTION

The mechanisms and effects of the sphingolipid interconversion have been the subjects of a growing body of scientific investigation (1–3). As indicated in Fig. 1, sphingomyelin is not only a structural component of cellular membranes but also serves as the precursor for the potent bioactive lipids ceramide and S1P.[3] A ceramide, S1P rheostat has been hypothesized to determine the fate of the cell such that the relative cellular concentrations of ceramide and S1P determine whether a cell proliferates or undergoes apoptosis. Ceramide is produced by the hydrolysis of sphingomyelin in response to stresses,

including exposure to chemotherapeutic drugs. Ceramide induces apoptosis in proliferating cells by a mechanism that remains to be elucidated (4, 5). Alternately, ceramide can be additionally hydrolyzed by the action of ceramidase to produce sphingosine. Sphingosine is then rapidly phosphorylated by SK to produce S1P. Ceramidase and SK are activated by a number of growth factors and intracellular oncoproteins, leading to rapid increases in the intracellular levels of S1P and depletion of ceramide levels. This situation promotes cell proliferation and inhibits apoptosis in tumor cells.

Because the balance between the cellular concentrations of ceramide and S1P determines whether a cell proliferates or undergoes apoptosis, the enzymes in this pathway provide potential targets for the development of new anticancer drugs (6). However, there are very few known inhibitors of sphingolipid-metabolizing enzymes, and so pharmacological manipulation of these pathways remains an untested approach toward cancer chemotherapy. In particular, inhibition of SK should have profound antiproliferative effects on tumor cells because S1P is the most direct mitogenic messenger in this pathway. This focus is substantiated by a recent demonstration that SK can directly transform cells (7). Pharmacological studies, to date, have used three compounds to inhibit SK activity: DMS; D,L-*threo*-dihydrosphingosine; and *N,N,N*-trimethylsphingosine. However, these compounds are not specific inhibitors of SK as they are known to affect protein kinase C (8), sphingosine-dependent protein kinase (9), 3-phosphoinositide-dependent kinase (10), and casein kinase II (11). Very recently, a few natural product inhibitors of SK have been isolated (12–14). Although these inhibitors are moderately potent, with *in vitro* K_is ranging from 2 to 38 μM, their biological activities and large-scale production capabilities remain unknown. Clearly, potent inhibitors of SK that can be easily synthesized would be highly desirable for evaluating this enzyme as a target for cancer therapy.

In this study, we demonstrate that SK mRNA is frequently overexpressed in a variety of human solid tumors, additionally supporting the consideration of SK as a new molecular target for cancer chemotherapy. Additionally, we developed an assay for recombinant human SK activity and screened a library of synthetic compounds, resulting in the identification of a panel of inhibitors of this enzyme. These compounds are selective toward SK in comparison with other lipid and protein kinases and are not competitive inhibitors of the ATP-binding site of SK. Furthermore, the compounds are antiproliferative toward a panel of human tumor cell lines. Antiproliferation is concomitant with induction of apoptosis. The compounds inhibit S1P formation in intact cells and maintain activity toward cells that express the drug transport proteins P-glycoprotein or MRP1. A prototypical SK inhibitor was found to inhibit tumor growth *in vivo*. Overall, we have identified a series of potent, structurally novel inhibitors of SK that have potential utilities as antiproliferative drugs.

MATERIALS AND METHODS

Materials. Unless otherwise noted, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). The chemical library was purchased from Chembridge Corporation (San Diego, CA), and compounds were provided as solutions at a concentration of 1 mg/ml in DMSO.

Synthesis of Compound V. Benzofuran-3-one exists in equilibrium between the keto- and enol tautomers and underwent an aldol condensation with
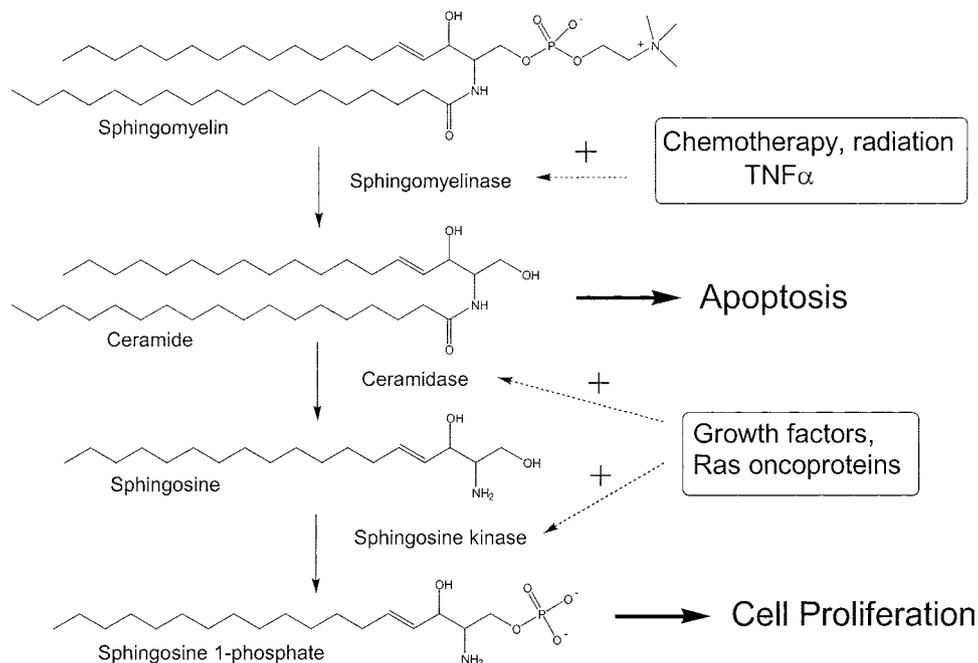
Fig. 1. Metabolism of sphingolipids. (Adapted from Macchia, *et al.*, J. Med. Chem. 44: 3994, 2001.)

3,4-dihydroxy-benzaldehyde in acetic anhydride to form 3,4-diacetoxyaurone (**1**). Hydrolysis of **1** in a mild hydrochloric acid solution gave 2-(3,4-dihydroxy-benzylidene)-benzofuran-3-one (**compound V**). For these reactions, solvents were dried and distilled before use, and reactions requiring anhydrous conditions were conducted under an atmosphere of nitrogen. Purifications were done by column chromatography on silica (Merck, silica gel 60, 230–400 mesh). The identities of **1** and **compound V** were verified by NMR spectroscopy on a Bruker 200-MHz instrument. Chemical shifts relative to trimethylsilane for ^{1}H- and ^{13}C-NMR spectra follow. Mass spectral data were provided by Mass Consortium (San Diego). For 3,4-diacetoxyaurone (**1**): ^{1}H NMR(200 MHz, CDCl$_3$) δ 2.32–2.35(s,s, 6H, 2CH$_3$), 6.82 (s, 1H, HC =), 7.24–7.36 (m, 3H, Ar-H), 7.67–7.83 (m, 4H, Ar-H); ^{13}C NMR(200 MHz, CDCl$_3$) δ 20.65, 20.69, 111.0, 113.0, 123.7, 123.9, 124.8, 126.0, 129.9, 130.9, 131.2, 137.1, 142.5, 147.2, 166.2; MS *m/z* (rel intensity) 339 (MH$^+$, 30), 297 (100), 255 (40). For 2-(3,4-dihydroxy-benzylidene)-benzofuran-3-one (**compound V**): ^{1}H NMR(200 MHz, Acetone-d$_6$) δ 6.75 (s, 1H, HC =), 6.94–6.98 (d, J = 8 Hz, 1H, Ar-H), 7.31–7.49 (m, 3H, Ar-H), 7.63 (s, 1H, Ar-H), 7.74–7.81 (m, 2H, Ar-H), 8.32 (s, 1H, OH), 8.65 (s,1H, OH); ^{13}C NMR(200 MHz, CDCl$_3$) δ 114.5, 114.6, 117.4, 119.8, 125.1, 125.6, 126.9, 138.3; MS *m/z* (rel intensity) 255 (MH$^+$, 100). We have also completed the synthesis of compound IV; however, the S atom appears to favor the enol tautomer in the starting material, and this dramatically reduces the rate of the aldol condensation.

Analysis of SK RNA Expression. SK mRNA levels were examined using the Cancer-Profiling Array from Clontech, which consists of 241 paired samples of cDNA made from the tumor and adjacent normal tissue from individual patients. The array was hybridized with a ^{32}P-labeled cDNA probe corresponding to a 1-kb fragment of the open reading frame of human SK. The membrane was then washed to remove nonspecific binding, and bound probe was visualized using a phosphorimager. The relative intensities of bound probe were quantified using Pathways 4 software, and the ratios of the relative intensities of tumor samples and the corresponding nontumor samples were determined.

Recombinant Human SK and Cell Lines. Previous studies have demonstrated that functional human SK can be expressed in *Escherichia coli* (15). The open reading frame of human SK cDNA was subcloned into a pGEX bacterial expression vector (Pharmacia), which results in expression of the enzyme as a fusion protein with GST. The fusion protein was then expressed and purified on a column of immobilized glutathione (Glutathione-Sepharose 4B; Pharmacia) according to the manufacturer's specifications. T-24 human bladder carcinoma cells and JC mouse mammary adenocarcinoma cells were obtained from American Type Culture Collection. MCF-7 human breast adenocarcinoma cells and NCI/ADR, a P-glycoprotein-overexpressing cell line

(16), were obtained from the Division of Cancer Treatment of the National Cancer Institute. MCF-7/VP cells that overexpress MRP1, but not P-glycoprotein (17), were obtained from Drs. Schneider and Cowan. The cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum and 50 μg/ml gentamicin sulfate.

SK Assay. A medium-throughput assay suitable for screening for inhibitors of recombinant human SK has been established. The activity assay is based upon the method of Louie *et al.* (18). Briefly, 5 μg of purified GST-SK fusion protein were combined with 12 nM sphingosine, which contained a 100-fold dilution of [3-^{3}H]sphingosine (20 Ci/mmol; American Radiolabeled Chemicals), 1 mM ATP, 1 mM magnesium chloride, and 200 μl of assay buffer [20 mM Tris HCl (pH 7.4), 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 20 mM zinc chloride, 1 mM sodium orthovanadate, 15 mM sodium fluoride, and 0.5 mM 4-deoxypyridoxine]. Assays were run for 30 min at 25°C with shaking and contained either 1% DMSO or 5 μg/ml test compound, which corresponds to concentrations of 10–25 μM. The reactions were terminated with 50 μl of concentrated ammonium hydroxide, followed by extraction of the assay mixture with chloroform:methanol (2:1). The aqueous portion was transferred to scintillation vials and radioactivity was quantified as a measure of [^{3}H]]S1P formation using a Beckman LS 3801 Scintillation Counter. The intra-assay coefficient of variation was <10%, whereas interassay variation was ~20%.

ATP Competition Assays. For the ATP competition assays, SK activity was determined according to the method of Spiegel (19). Briefly, 5 μg of purified human GST-SK fusion protein was incubated with 10 μM sphingosine, 5 mM MgCl$_2$, vehicle (DMSO), or 2 μM test inhibitor in assay buffer. Reactions were initiated by the addition of ATP containing 1 μCi of [γ-^{32}P]ATP (EasyTides, 3000 Ci/mmol; New England Nuclear) and incubated with shaking for 30 min at 37°C. ATP concentrations were varied from 50 to 1000 μM. Reactions were terminated by the addition of 1 N HCl and then extracted with 200 μl of chloroform:methanol:HCl (100:200:1). The organic layer was loaded onto a G60 silica gel TLC plate (Whatman), and S1P was resolved from ATP using a mobile phase consisting of 1-butanol:methanol:water:acetic acid (8:2:2:1). Plates were exposed to radiography film for 2 h, and S1P bands were scraped and counted for activity.

Cellular S1P Formation Assay. MDA-MB-231 human breast cancer cells were grown to confluency in 24-well tissue culture plates and deprived of serum overnight. Cells were then pretreated with 1% vehicle (DMSO), 20 μM DMS, or 20 μg/ml of each inhibitor for 5 h. Next, each well was dosed with 1 μM sphingosine containing 250 nCi [3-^{3}H]sphingosine and incubated for 15 min. Cells were washed three times with PBS and lysed with 50 μl of ammonium hydroxide. PBS (200 μl) was added to each well, and lysate was transferred to Eppendorf tubes followed by the addition of 600 μl of chloro-

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form:methanol (2:1) and vortexing. The organic phase containing [3H]]sphingosine and the aqueous phase containing [3H]]S1P were individually transferred to scintillation vials and counted.

Cell Proliferation Assay. T24, MCF-7, MCF-7/VP, and NCI/ADR cells were plated into 96-well tissue culture plates at ~15% confluency. After 24 h, cells were treated with various concentrations of inhibitors. After an additional 48 h, cell survival was assayed using the sulforhodamine B assay (20).

Apoptosis Assay. T24 cells, which were shown previously to undergo apoptosis in response to anticancer drugs (21), were incubated with vehicle alone (DMSO) or 10 μM SK inhibitor for 24 h and assayed for apoptosis using the fluorescein-labeled TUNEL Cell Death Detection Kit (Roche) according to the manufacturer's specifications. TUNEL-positive cells were identified by fluorescence microscopy and quantified based upon multiple randomly chosen views for each set of treated wells. The percentage of TUNEL-positive cells in each field was determined by dividing the number of TUNEL-positive cells by the total number of cells viewed in the bright field mode. The results shown were confirmed by triplicate wells in duplicate experiments.

ERK2, PKC, and PI3k Assays. The kinase activity of recombinant human ERK2 was measured using the mitogen-activated protein kinase indirect ELISA assay kit from Upstate Signaling. The assay measures myelin basic protein phosphorylation in the presence of vehicle (DMSO) or various concentrations of each inhibitor and was performed according to the manufacturer's specifications. Inhibition of human recombinant purified PKCα (Upstate) was tested using the Creb kinase indirect ELISA assay kit (ImmuneChem, Burnaby, BC, Canada) and performed as per specifications. PI3K immunoprecipitation and activity assays were performed according to the methods of Guan *et al.* (22, 23).

Antitumor Evaluation. A syngenic mouse tumor model that uses a transformed murine mammary adenocarcinoma cell line (ATCC CRL-2116) and Balb/C mice was recently developed in our laboratory.[4] Animal care and procedures were in accordance with guidelines and regulations of the Institutional Animal Care and Use Committee of the Penn State College of Medicine. Animals were housed under 12-h light/dark cycles, with food and water provided *ad libitum*. BALB/c female mice (Charles River), 6–8 weeks old, were injected s.c. with 1×10^6 JC cells suspended in PBS. After palpable tumor growth, ~2–3 weeks after injection, tumor volume was determined (day 1) using calipers measuring the length (*L*) and width (*W*) of the tumor. Tumor volume was calculated using the equation: $(L \times W^2)/2$. Animals were randomized into four groups ($n = 5$/group). Treatment was then administered on days 1, 5, 9, and 15 and consisted of i.p. administration of either 100 μl of DMSO (control) or compound V in DMSO at a dose of 75 mg/kg. On day 18, animals were euthanized, and tumors were excised and weighed. Whole body weight and tumor volume measurement were performed each day of treatment. *P*s were determined using unpaired *t* tests using GraphPad InStat (San Diego, CA).

RESULTS

Expression of SK RNA in Human Tumors. SK mRNA levels were analyzed using the Cancer Profiling Array from Clontech. This array consists of 241 paired samples of cDNA made from the tumor and adjacent normal tissue from individual patients. Consequently, it allows rapid comparison of the levels of gene expression in tumor and normal tissues from the same patient. A broad range of tumor types is represented. As shown in the top panel of Fig. 2, the expression of SK message was generally higher in breast tumors compared with adjacent normal breast epithelium. Increases in expression up to 4-fold were found in ~80% of the individuals, whereas decreases in the tumor compared with the normal specimen were rare. There were insufficient numbers of samples to allow stratification of the tumors by stage of disease, but the overall pattern appears to hold for all stages. Similar increases in expression of SK in tumors were observed in all of the tissue types analyzed, and the pooled data are presented in the bottom panel of Fig. 2. Statistically significant differences were found for most of the tumor types (*P*s ranged from 0.09 to 0.00007 for
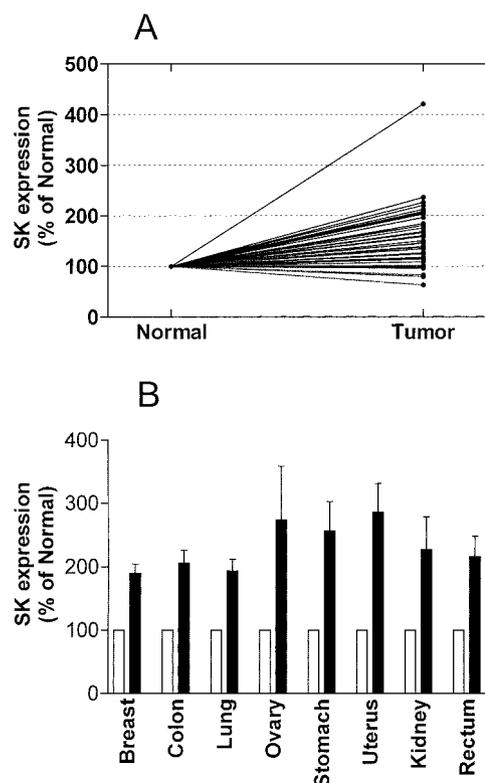

Fig. 2. Expression of SK mRNA in human tumors. *A*, tumor data are expressed as the percentage of SK expression found in the normal tissue for the individual patient with breast cancer. Each line connects data from an individual patient's normal tissue and breast tumor. *B*, the average ± SD of SK expression in various types of tumors compared with corresponding normal tissue is indicated. As above, the data are normalized for each individual tumor based on the patient's level of SK expression in the adjacent normal tissue. The numbers of paired samples studied ranged from 14 to 50 for different types of tumors.

different tissues), indicating that up-regulation of SK may be a common property of tumors.

Screen for Inhibitors of Human SK. Recombinant human SK was expressed in *E. coli* as a fusion protein with GST and purified by chromatography on immobilized reduced glutathione. Induction of recombinant protein expression by treatment with isopropyl-1-thio-β-D-galactopyranoside was somewhat variable, but yields of ~3 mg of recombinant protein/liter of culture were typical. The SK assay uses [3H]sphingosine as the substrate and is based on partitioning of [3H]S1P into the aqueous phase with [3H]sphingosine remaining in the organic phase. As a negative control, GST without fused SK was expressed in *E. coli* and purified. As demonstrated in Fig. 3, the GST control did not increase the appearance of [3H] in the aqueous phase. In contrast, recombinant hSK-GST caused increases of at least 3-fold after 30 min of incubation, indicating that all of the increased radioactivity in the aqueous phase derives from SK activity. Inclusion of 2% DMSO, the solvent for the chemical library, did not affect the SK activity, whereas 25 μM DMS totally suppressed SK activity.

A chemical library consisting of ~16,000 compounds was screened for inhibitors of human SK. The screening assays were conducted at a fixed concentration of 5 μg/ml compound, which corresponds to concentrations of 10–25 μM. The library was screened using pools of eight compounds/sample followed by deconvolution to identify individual active compounds. The hit rate, defined as at least 80% inhibition of SK activity, was ~0.8%. Therefore, sufficient numbers of hits were identified and verified to provide confidence that low molecular weight, nonlipid SK inhibitors could be identified by these methods.

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[4] B. D. Lee, K. J. French, Y. Zhuang, and C. D. Smith, submitted for publication.

Potency of Novel SK Inhibitors. Additional analysis of the SK inhibitors detected in the screen described above has revealed several active chemotypes. An active compound from each of four of these chemotypes was selected for additional characterization (Fig. 4). Compounds I, II, III, and IV (at 5 μg/ml) inhibited SK activity by 99, 85, 99, and 89%, respectively, and serve as prototypes for nonlipid SK inhibitors. Although the screen provided rapid identification of compounds that inhibit SK activity, additional studies were necessary to determine their potencies. Therefore, the effects of compounds I–IV were determined at multiple concentrations, and IC$_{50}$s for each compound were calculated. As summarized in Table 1, the compounds demonstrated IC$_{50}$s in the sub- to low micromolar range, making them more potent inhibitors of SK than any previously reported compound.

Mechanism of Action of Novel SK Inhibitors. An important issue for these compounds relates to the mechanism for their inhibition of SK. Specifically, we were interested in determining if these compounds are competitive inhibitors at the ATP-binding site. Com-
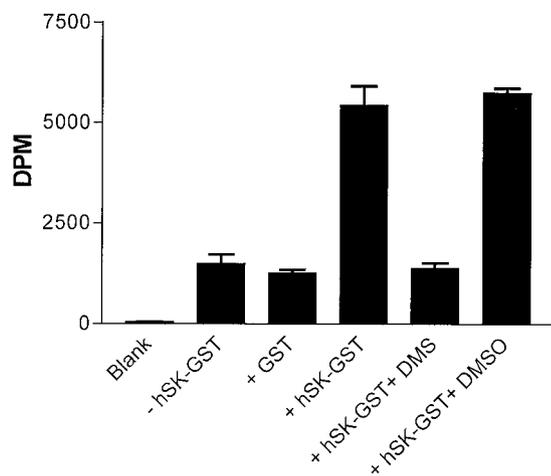
Table 1 *In vitro potencies of SK inhibitors against various kinases*

Potencies of each inhibitor were determined for human isoforms of ERK2, PI3k, and PKC-α. GST-hSK inhibition data is provided for comparison. Data represent the concentrations required to inhibit enzyme activity by 50% and are the mean $\pm$ SD of triplicate experiments. None: no inhibition detected up to 60 μM.

Compound	GST-hSK	hERK2	hPI3k	hPKCα
I	1.2 $\pm$ 0.3	11 $\pm$ 1	None	None
II	0.5 $\pm$ 0.3	None	None	None
III	2.5 $\pm$ 0.7	26 $\pm$ 2	42 $\pm$ 10	None
IV	0.6 $\pm$ 0.1	40 $\pm$ 5	7 $\pm$ 2	None
V	2.0 $\pm$ 0.2	80 $\pm$ 4	6 $\pm$ 2	None

Table 2 *Effects of SK inhibitors on Michaelis-Menten parameters for ATP*

Varying concentrations of ATP were incubated with purified human GST-SK, 10 μM sphingosine and DMSO (control) or 2 μM of the indicated SK inhibitor for 30 min at 37°C. The resulting K$_M$ for ATP (μM) and the V_{max} (nmol S1P/min) for each condition was determined as described in "Materials and Methods." Data represent the mean $\pm$ SD of duplicate experiments. *$P < 0.001$ *versus* DMSO as determined with unpaired t test.

Compound	K$_M$	V_{max}
DMSO	111 $\pm$ 16	0.633 $\pm$ 0.024
I	82 $\pm$ 5	0.335 $\pm$ 0.087*
II	124 $\pm$ 15	0.114 $\pm$ 0.018*
III	12 $\pm$ 3	0.249 $\pm$ 0.019*
IV	46 $\pm$ 14	0.318 $\pm$ 0.021*

Fig. 3. An example of data from the SK assay. Triplicate samples containing the indicated assay components and positive (25 μM DMS) and negative (2% DMSO) controls were analyzed as indicated in "Materials and Methods." Values represent the mean $\pm$ SD for these replicates and are typical of multiple experiments.

Compound I: 306301-68-8

Compound II: 312636-16-1

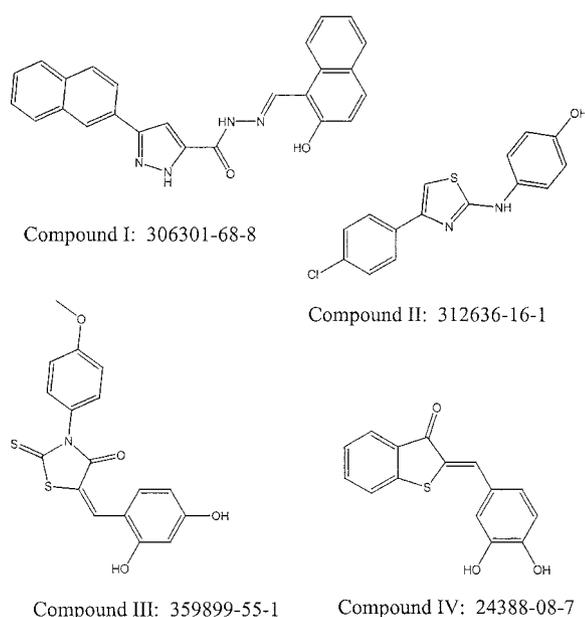Compound III: 359899-55-1

Compound IV: 24388-08-7

Fig. 4. Structures of identified SK inhibitors. Several compounds with replicated activity in the SK assay are indicated. The CAS numbers for the compounds are indicated. These will be referred to as compounds I, II, III and IV in this article.

pounds that bind to this site may not be specific for SK, causing undesired inhibition for other ATP-dependent enzymes. A common problem with kinase inhibitors is their tendency toward nonselectivity because the majority of these inhibitors interact with the highly conserved nucleotide binding site. Therefore, we performed competition assays in which sphingosine and SK concentrations were held constant, whereas ATP concentrations were varied. For each inhibitor, the K$_M$ for ATP and the V_{max} for S1P formation was determined. Compounds that are competitive inhibitors for the ATP-binding site would be expected to increase the K$_M$ for ATP without affecting the V_{max} of the reaction. The data for compounds I–IV are summarized in Table 2. The V_{max}s show significant decreases with all of the test compounds *versus* vehicle alone. In contrast, K$_M$s were not increased such that ATP concentrations up to at least 10 times the K$_M$ were unable to overcome inhibition by the compounds. Therefore, these compounds are not competitive inhibitors at the ATP-binding site of SK.

Selectivity of SK Inhibitors. To establish proof-of-concept of SK inhibitors as therapeutic agents and for additional characterization, we evaluated their effects on a small panel of human protein kinases (ERK2 and PKC-α) and a lipid kinase (PI3K). The results are summarized in Table 1. As shown, all five compounds demonstrated highest potency toward SK, with most having no inhibition of the other kinases at any concentration tested. Compound I showed some activity toward ERK2 but only at 10-fold higher concentrations than those required to inhibit SK. Compound II was the most selective as no inhibition was seen with any of the kinases tested. Compound III was 10–20-fold less potent toward ERK2 and PI3k than SK. Compound IV and its analogue compound V had weak activity toward ERK2 and moderate activity toward PI3k. None of the compounds inhibited PKC-α. Thus, although modest inhibition of certain other kinase was observed, these compounds demonstrate a reasonable degree of selectivity toward SK.

Inhibition of Endogenous SK in Intact Cells. Although the SK inhibitors demonstrated potency toward the purified enzyme, it was important to determine their ability to inhibit endogenous SK in an intact cell model. A survey of several human tumor cell lines indicated that the breast cancer cell line MDA-MB-231 expresses high levels of SK activity (data not shown). As shown in Fig. 5, the positive control DMS as well as each of the inhibitors decreased S1P formation. TLC
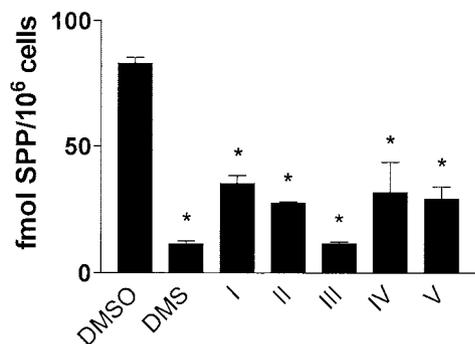
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Fig. 5. Sphingosine-1-phosphate levels in MDA-MB-231 cells. Sphingosine-1-phosphate formation was assayed in MDA-MB-231 cells in the absence (vehicle alone) or presence of DMS or compounds I–IV (20 μg/ml). Values represent the mean $\pm$ SD generation of S1P by endogenous SK. *, $P < 0.01$ as determined by unpaired t tests *versus* DMSO-treated control cells.

Table 3 *Cellular potencies of SK inhibitors*

Varying concentrations of the indicated SK inhibitors were evaluated for inhibition of proliferation of the indicated cell lines. Data represent the concentrations required to inhibit cell proliferation by 50% and are the mean $\pm$ SD of triplicate experiments.

Compound	Cytotoxicity toward			
	T24[a]	MCF-7[a]	NCI/ADR[a]	MCF-7/VP[a]
I	1.1 $\pm$ 1.2	0.4 $\pm$ 2.3	0.8 $\pm$ 1.3	2.9 $\pm$ 2.0
II	4.6 $\pm$ 1.6	1.2 $\pm$ 1.3	1.3 $\pm$ 1.1	0.9 $\pm$ 1.8
III	5.8 $\pm$ 1.5	6.8 $\pm$ 1.5	6.2 $\pm$ 1.5	24.6 $\pm$ 1.9
IV	7.8 $\pm$ 2.0	1.0 $\pm$ 1.7	1.2 $\pm$ 2.4	1.8 $\pm$ 1.1

[a] IC$_{50}$ (μM).

analysis confirmed the decrease in activity was attributable to decreased S1P formation (data not shown). In all cases, activity was decreased by at least 50% when compared with vehicle-treated cells. Compound III was the most potent, with SK activity levels inhibited to <15%. These results demonstrate that the compounds inhibit not only purified but endogenous SK in intact cells.

Cytotoxicity of Novel SK Inhibitors. According to the hypothesis that S1P drives proliferation and inhibits ceramide-induced apoptosis, it is expected that inhibitors of SK will be antiproliferative and induce apoptosis in replicating cells. To assess their efficacies in intact cells, the SK inhibitors were evaluated for cytotoxicity using a panel of human cancer cell lines. Cell lines tested included T-24 human bladder carcinoma cells, MCF-7 human breast adenocarcinoma cells, a subline of MCF-7 cells, MCF-7/VP, which is resistant to several anticancer drugs because of overexpression of the transport protein MRP1 (17), and NCI/ADR, a cell line resistant to many anticancer drugs because of overexpression of the drug transporter P-glycoprotein (16). We have used these cell lines extensively in other projects relating to the development of compounds to overcome drug resistance (24, 25). The cytotoxicities of the SK inhibitors are summarized in Table 3. The IC$_{50}$s (in μM) indicate the concentration of a compound that inhibits tumor cell proliferation by 50%. As the data show, these compounds are antiproliferative at sub- to low micromolar concentrations that correspond quite well with the IC$_{50}$s for inhibiting SK activity *in vitro*. An important finding is that these SK inhibitors are effective against tumor cells demonstrating the MRP because of overexpression of either P-glycoprotein or MRP1. As these transporters are often overexpressed in tumor cells, particularly after the patient has been treated with cytotoxic drugs, the SK inhibitors may be effective therapeutic agents for resistant tumors.

Induction of Apoptosis in Tumor Cells. Although the compounds demonstrated antiproliferative effects, it was unknown whether the SK inhibitors caused the tumor cells to undergo apoptosis.

Therefore, we treated T24 cells with the inhibitors and assayed for apoptosis using the TUNEL method. T24 cells had been previously demonstrated to undergo apoptosis upon exposure to Taxol (21) and thus are an appropriate model for testing. As shown in Fig. 6, all of the SK inhibitors induced apoptosis, manifested as a significant increase in TUNEL-positive cells compared with vehicle-treated controls. The known SK inhibitor DMS also induced apoptosis as previously described (26). Thus, all of the SK inhibitors inhibit cancer cell proliferation and induce apoptosis consistent with the hypothesized consequence of reducing S1P levels.

Synthesis and Antitumor Activity of Compound V. Additional proof-of-principle studies required the synthesis of sufficient quantities of a SK inhibitor for *in vivo* testing in a tumor model. Consideration of the synthetic routes to compounds I–IV indicated that the chemotype of compound IV was the most easily synthesized of the four classes of compounds. Initially, an analogue of compound IV was synthesized in which a furan heterocycle is substituted for the thiophene moiety to take advantage of the commercial availability of the starting benzofuran-3-one (Fig. 7). This bioisosteric replacement was expected to produce a compound with comparable activity and resulted in compound V. Biological evaluations demonstrated that the IC$_{50}$s for inhibition of SK and tumor cell proliferation by compound V were $\sim$2 μM, indicating that it is somewhat less potent than
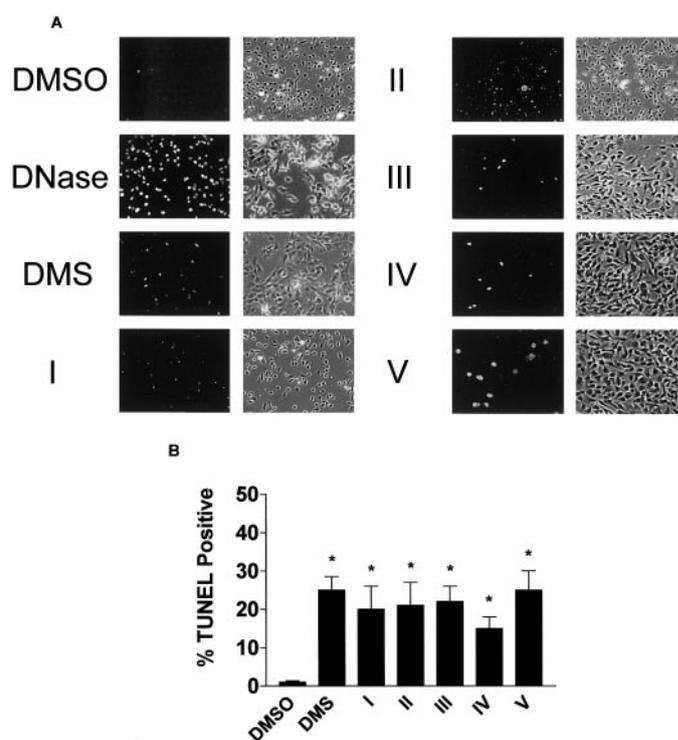
Fig. 6. Induction of apoptosis in human cancer cells. Subconfluent T24 cells were incubated for 24 h with vehicle (DMSO), 10 μM DMS or 10 μM compounds I–V. Apoptosis was determined via the TUNEL assay with fluorescein-labeling. *A*, fluorescent microscopy identifies TUNEL-positive cells (*left images*), whereas bright field microscopy (*right images*) of the same fields are shown for comparison. *B*, the percentage of TUNEL-positive cells were quantified for each treatment group. DMS and compounds I–V all significantly increased apoptosis above control levels. * indicates $P < 0.05$.

Fig. 7. Synthesis of compound V.

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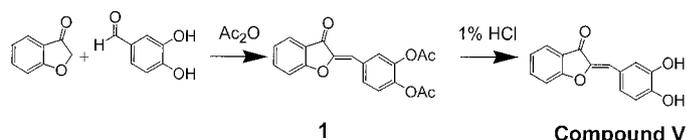compound IV. i.p. administration of compound V to normal female Swiss-Webster mice did not result in any immediate or delayed toxicity at doses up to at least 75 mg/kg. Repeated injections of compound V into the same mice over a period of 15 days also failed to induce overt toxicity, indicating that this compound was suitable for additional *in vivo* studies. Antitumor activity was assessed by determining the effects of compound V in a syngenic tumor model that uses the mouse JC mammary adenocarcinoma cells growing s.c. in immunocompetent BALB/c mice. These cells express elevated levels of SK activity relative to nontransformed cells, as well as the MRP attributable to P-glycoprotein activity. As indicated in Fig. 8, tumor growth in animals treated with compound V was significantly lower (>50% decreased at day 18) than tumor growth in control animals. No significant difference in the body weights of animals in the two groups was observed, indicating the lack of overt toxicity from compound V.

DISCUSSION

The sphingolipid metabolites S1P and ceramide are being increasingly recognized as critical regulators of tumor cell proliferation and apoptosis, respectively. Specifically, S1P has been shown to induce DNA synthesis in fibroblasts (27–31), endothelial cells (32), smooth muscle cells (33), intestinal epithelial cells (34), glioma cells (35), and mouse oocytes (36). Additionally, S1P has been shown to effectively inhibit ceramide-induced apoptosis in association with decreased caspase activation (37) and to attenuate radiation-induced apoptosis in prostate cancer cell lines (38). Importantly, ceramide appears to induce apoptosis in tumor cells without disrupting quiescent normal cells (39, 40). Furthermore, ceramide enhances apoptosis in response to anticancer drugs, including Taxol (41, 42) and etoposide (43). These studies, in a variety of cell lines, consistently indicate that S1P is able to induce proliferation and protect cells from apoptosis. Although the elucidation of downstream targets of S1P remains an interesting problem in cell biology, sufficient validation of these pathways has been established to justify their evaluation as targets for new types of anticancer drugs. As S1P appears to be the most direct

mitogenic messenger, inhibition of its production should have anti-proliferative effects on tumor cells.

Phosphorylation of sphingosine by SK is the only known mechanism for the production of S1P in cells. The M_r 49,000 enzyme was initially isolated from rat kidney and demonstrated K_Ms 5 and 93 μM for sphingosine and ATP, respectively (44). The human isoform was recently cloned and displays similar physical and biochemical characteristics (15). Shortly thereafter, a second SK isoform was cloned; however, this species displays much lower activity and different kinetic profiles than the type 1 enzyme (45). RNA-encoding SK can be detected in most tissues, with higher levels in lung and spleen (46). Interestingly, a number of studies have shown that a variety of proliferative factors, including PKC activators (37), FCS and platelet-derived growth factor (47), epidermal growth factor (48), and tumor necrosis factor α (49) rapidly elevate cellular SK activity. Although the signaling links between mitogenic factors and SK remain unclear, these results have led to consideration of the role of SK in cancer.

Recently, an oncogenic role of SK has been directly demonstrated (7). In these studies, transfection of SK into NIH 3T3 fibroblasts was sufficient to promote foci formation and cell growth in soft agar and to allow these cells to form tumors in NOD/SCID mice. Additionally, inhibition of SK by transfection with a dominant-negative SK mutant or by treatment of cells with the nonspecific SK inhibitor DMS blocked transformation mediated by oncogenic H-Ras. Because elevated activation of Ras proteins, either by mutation of *ras* genes or increased stimulation by upstream activators, frequently occurs in cancer, it is likely that SK plays a significant role in this disease. Another study showed that EDG4, a receptor that specifically binds S1P, is a marker for ovarian cancer cells (50). S1P has also been implicated in angiogenesis because it induces motility and mitogenesis in smooth muscle cells and promotes endothelial cell differentiation (51). Results presented herein provide the first indications that SK may be commonly overexpressed in a variety of human tumors. Specifically, levels of mRNA encoding SK were found to be ~2-fold higher in tumors of the breast, colon, lung, ovary, stomach, uterus, kidney, and rectum compared with normal tissue from the same patient. Overall, these studies suggest that inhibition of SK may be an effective therapy for cancer and other hyperproliferative diseases.

Despite the high level of interest in sphingolipid-derived signaling, there are very few demonstrated inhibitors of the enzymes of this pathway. In particular, the field suffers from a lack of potent and selective inhibitors of SK. Pharmacological studies to date have used sphingosine analogues, especially DMS; however, as indicated above, these lipids are known to inhibit several protein kinases (8–10). Therefore, selective and potent inhibitors of SK are required for both basic research and as lead compounds for developing novel anticancer agents. Very recently, a few natural product inhibitors of SK have been isolated (12–14). Although these inhibitors are moderately potent, with *in vitro* K_is ranging from 2 to 58 μM, their selectivity and large-scale production capabilities remain unknown. Clearly, potent inhibitors of SK that can be easily synthesized would be highly desirable for evaluating this enzyme as a therapeutic target for cancer therapy. To this end, we initiated a program to identify and evaluate potent and structurally novel inhibitors of SK.

Because no structural data for SK is currently available to enable a computational docking approach to identifying SK inhibitors, we screened a large library of diverse synthetic compounds using purified recombinant human SK fused to GST. This identified a series of low molecular weight, drug-like molecules that inhibit this enzyme. Although too few and diverse for pharmacophore mapping, several of these hits segregated into four chemotypes, and a representative of each of these chemotypes, *i.e.* compounds I–IV, was additionally
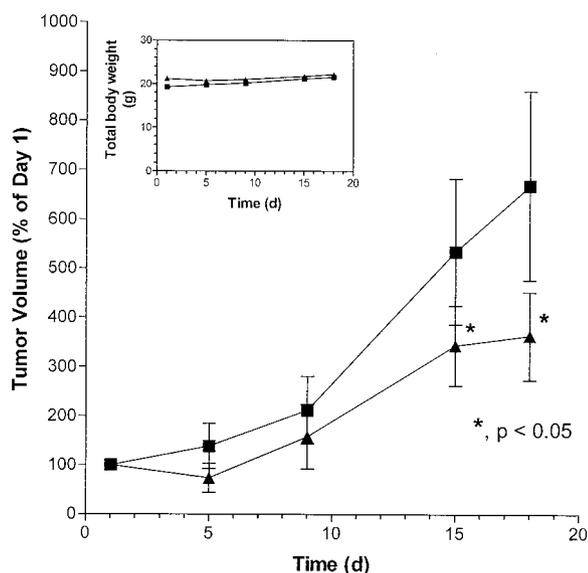
Fig. 8. Antitumor activity of compound V. BALB/c mice, 6–8 weeks old, received s.c. injections of 10^6 JC cells suspended in PBS. DMSO (control, ■) or compound V at a dose of 75 mg/kg (▲) was administered i.p. on days 1, 5, 9, and 15. Tumor growth is expressed as the volume relative to day 1 for each animal. ∗ indicates Ps <0.05, as determined by unpaired *t* tests. The insert indicates the body weight of the animals during this experiment.

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characterized. Each compound was highly potent toward purified SK with IC_{50}s lower than any compounds described in the literature.

An important question to address is the mechanism of inhibition of SK by these compounds. Because most inhibitors of other kinases act by competing with ATP for the highly conserved nucleotide-binding domain, it has been difficult to identify selective kinase inhibitors. A recent study by Pitson *et al.* (49) demonstrated that the amino acid sequence of the putative nucleotide-binding domain of human SK-1 [SGDGX(17-21)K] is significantly different from all other known nucleotide-binding sites. In our screen for inhibitors of SK, we used high levels of ATP ($\sim$10 times the K_M) in the hopes of identifying compounds that bind at sites other than the nucleotide-binding site. Importantly, competition experiments demonstrated that excess levels of ATP did not remove the inhibition of recombinant human SK, indicating that these compounds are not competitive inhibitors at the ATP-binding site.

We further explored potential interactions with nucleotide-binding domains by determining the effects of the SK inhibitors on a small panel of protein and lipid kinases. None of the compounds inhibited PKC-α at doses up to at least 100 times their IC_{50} for SK, indicating that they do not act as generic kinase inhibitors. Compound I has some activity toward ERK2, whereas compounds IV and V were reasonably potent inhibitors of PI3K. It is important to note that although these compounds are not totally selective for SK, the purpose of lead optimization is to enhance the potency and selectivity of screening hits. In particular, compound II is the most selective SK inhibitor and so may be the most attractive candidate for additional medicinal chemistry efforts.

Compounds I–IV demonstrated cytotoxicities toward human cancer cell lines that closely parallel their potencies for inhibition of purified human SK. The sulforhodamine B staining assay determines the extent of cell survival upon drug exposure (19). Concordant with the S1P:ceramide rheostat model for cell survival, we hypothesized that these compounds enter cells and inhibit SK activity, thereby decreasing S1P levels, increasing ceramide levels, and resulting in apoptosis. Our hypothesis was affirmed by detecting elevated levels of apoptosis as determined by TUNEL staining upon exposure of T24 cells to each of the SK inhibitors. Additionally, cells treated with the SK inhibitors demonstrated morphological features of apoptosis that paralleled cytotoxicity. We have demonstrated that each chemotype does indeed decrease S1P production in intact cells, indicating that endogenous SK is sensitive to these compounds. The antiproliferative activities of these compounds toward drug-resistant cell lines suggest that they may be effective agents for cancers that are unresponsive to many current drugs. The issue of multidrug resistance is important because numerous established and experimental therapeutics, including tyrosine kinase inhibitors (52), provide excellent examples of target-based drug development that resulted in suboptimal clinical activity because the compounds interact with the multidrug transporters P-glycoprotein and/or MRP1. During the process of selecting candidates for additional drug development, priority is often now given to compounds that are not substrates for these transporters.

To test whether these inhibitors could serve as chemotherapy agents, an analogue of compound IV was synthesized and tested for antitumor activity. Compound V was selected for synthesis because an expedient synthetic route was apparent. The methods can be used with a variety of commercially available aldehydes to generate a focused combinatorial library. Compound V was found to have moderate antitumor activity *in vivo* under conditions that were not toxic to the animals. Administering the compound on a more frequent dosing schedule or by an alternate route may increase this antitumor effect. It should be recognized that compound V is not expected to be the most effective of the SK inhibitors. It was simply used to provide proof-of-principle in these initial studies, and this was successful in providing the first evidence that a SK inhibitor can have antitumor activity *in vivo* in the absence of toxicity to the animal. We do note the presence of the conjugated cathecol ring in compounds IV and V that may be readily metabolized by cytochrome P450. The acetylated intermediate of compound V (compound 1, Fig. 7) had similar potency toward purified SK and similar cytotoxicity as compound V (data not shown), indicating that the free hydroxyls are not required for activity. Additional structure-activity analyses of these series of SK inhibitors are in progress.

In conclusion, SK is critically important in the regulation of tumor cell proliferation and apoptosis and so represents a potentially important target for the development of new anticancer drugs. We have now demonstrated that SK expression is significantly elevated in a variety of solid tumors and have identified several low molecular weight compounds that potently inhibit this enzyme in close relationship with their antiproliferative activities. The identification of an SK inhibitor with antitumor activity *in vivo* additionally substantiates the hypothesis that SK is an attractive target for new therapeutics. Additional development of these compounds through medicinal chemistry efforts and early-stage ADME (Absorption, Distribution, Metabolism and Elimination) profiling will be critical in lead optimization. Analysis of their cellular and *in vivo* effects will also be crucial for evaluating the clinical potential of this new class of targeted compounds.

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