Original Article
A novel sphingosine kinase 1 inhibitor (SKI-5C) induces cell death of Wilms’ tumor cells in vitro and in vivo

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Abstract: Sphingosine kinase 1 (SphK1) is over-expressed in many cancers and therefore serves as a biomarker for cancer prognosis. SKI-5C is a new SphK1 inhibitor, and until now its molecular function in Wilms’ tumor cells remained unknown. Here, using CCK-8 and nude mice experiments we assessed cell growth in Wilms’ tumor cell lines (SK-NEP-1 and G401) in vitro and in vivo. We demonstrated that SphK1 is highly expressed in SK-NEP-1 and G401 cells, and through annexin V/propidium iodide staining and flow cytometry analysis, we detected cell apoptosis. Treatment with SKI-5C inhibited proliferation and induced apoptosis of SK-NEP-1 and G401 cells in a dose-dependent manner. Moreover, SKI-5C treatment inhibited the growth of SK-NEP-1 xenograft tumors in nude mice, with few side effects. Our microarray analysis revealed that SKI-5C-treated SK-NEP-1 cells mostly downregulated PRKACA and significantly inhibited phosphorylation of ERK1/2 and NF-κB p65. These results imply that SKI-5C induces apoptosis of SK-NEP-1 cells through the PRKACA/MAPK/NF-κB pathway. While, further research is required to determine the underlying details, these results provide new clues for the molecular mechanism of cell death induced by SKI-5C and suggest that SKI-5C may act as new candidate drug for Wilms’ tumor.

Keywords: SKI-5C, sphingosine kinase 1 (SphK1), Wilms’ tumor, cell death

Introduction
Wilms’ tumor (WT) is the most common malignant renal tumor in children, representing approximately 6-14% of all childhood cancers and about 95% of all pediatric renal malignancies [1], with an incidence of eight cases per million. Although multimodal therapies result in high overall cure rates, relapse still occurs in approximately 15% of patients with favorable-histology WT and 50% of those with anaplastic WT [2]. The failure of some patients to respond to current treatments calls for the development of new therapeutic approaches [3].

Sphingosine kinase 1 (SphK1), an evolutionarily conserved lipid kinase, is responsible for the conversion of sphingosine to sphingosine-1-phosphate (S1P). Accumulating evidence indicates that SphK1 is associated with maintaining the balance between prosurvival and apoptotic signaling [4]. By regulating the S1P/ceramide conversion, SphK1 is considered a cellular converter determining cell fate. Also, it is well-established that SphK1 is upregulated in various cancers, including solid tumors [5, 6]. Increased expression levels correlate with poor prognosis and are associated with resistance to chemotherapy, suggesting its value as a prognostic marker [7, 8]. Overexpression of SphK1 in non-tumorigenic pro-erythroblasts increases their clonogenicity and resistance to apoptosis, and when grafted in vivo, they acquire tumorigenicity [9]. Non-transformed NIH/3T3 fibroblasts overexpressing SphK1 acquire a transformed phenotype, as determined by colony formation assay, and their ability to form tumors in nude mice [10]. These results suggest that SphK1 is an oncogene required for tumor progression. Alternatively, inhibitors of SphK1 have been shown to not only inhibit S1P production, but also increase sphingosine and ceramide, pushing cancer cells more toward cell death [3, 5], which implies a potential application of SphK1 as a novel molecular target for cancer therapy [11].
Several studies have investigated the role of S1P in Wilms' tumor. Li et al. [3] showed that in the Wilms' tumor cell line, WiT49, S1P upregulated expression of connective tissue growth factor (CTGF), a well-known negative regulator in Wilms' tumor progression, which subsequently inhibited cell proliferation. Li's group [12] also demonstrated that S1P enhanced prostaglandin E2 (PGE2) formation through S1P2, while PGE2 mediated growth and progression of Wilms' tumor. Since plenty of evidence supports a key role for S1P in Wilms' tumor growth and progression, we speculate that SphK1 (the S1P converter) may also play a crucial role in Wilms' tumor pathogenesis.

Several studies have targeted SphKs to inhibit cell survival and migration. However, there are very few established inhibitors of the enzymes of sphingolipid metabolism. In particular, the field suffers from a lack of potent and selective inhibitors of SphK [5]. N,N-dimethylsphingosine (DMS) and D,L-threo-dihydrosphingosine (DHS) are two of the earliest developed pharmacological SphK inhibitors that have tested capable of inducing growth inhibition and apoptosis in a variety of human cancer cells. One problem is that they not only inhibit SphK1 and SphK2 [4, 13], but also inhibit protein kinase C [14]. In addition, they caused severe hemolysis in mice, which greatly weakens their potential in clinical utility [14]. This highlights the need for alternative inhibitors of SphK1. Much of the current studies have developed a series of inhibitors more selective towards SphK1. Two new SphK1 inhibitors (compound V and SKI-II) have shown significant anti-tumor abilities and fewer side effects in vivo [15-19], but are still far from clinical trials.

SKI-5C is a newly developed SphK1 inhibitor. This small molecular inhibitor was first reported in 2009 by Wang et al. [20] and showed no effect on SphK2 inhibition when compared with DMS. Until then, its antitumor activity in vitro and in vivo has been rarely reported.

In this study, we investigated this newly developed SKI-5C inhibitor and evaluated its effect in Wilms' tumor. We show that SKI-5C treatment inhibits proliferation and induces apoptosis in Wilms' tumor cell lines. Our results also show SKI-5C significantly inhibits phosphorylation of ERK1/2 and NF-κB p65, indicating that SKI-5C induces apoptosis through the PRKACA/MAPK/NF-κB pathway. Our study not only confirms that SphK1 is a valuable molecule for targeting strategies, but also supports the potential application of SKI-5C as a therapeutic anticancer approach.

Materials and methods

Cell and culture conditions

SK-NEP-1 and G401 Human kidney (Wilms' Tumor) cell line obtained from the American Type Culture Collection (ATCC) was maintained in the McCoy's 5A (Life Technologies Inc., Gaithersburg, MD, USA) as introduced before [21].

Cell proliferation

SK-NEP-1 or G401 cells (2 × 10^4) were seeded in 96-well plates overnight and incubated with DMSO, or increasing concentrations of SKI-5C (0.25 μM-20 μM) for 24 hours. The volume of DMSO added to the vehicle treated wells was the same as that added to the drug treated wells. Each drug concentration was performed in four replicate wells. CCK-8 analysis was introduced before [21]. Cell proliferation was calculated as a percentage of the DMSO treated control wells with 50% inhibitory concentration (IC50) values derived after plotting proliferation values on a logarithmic curve. The IC50 of SKI-5C was calculated by GraphPad Prism software.

Cell cycle analysis

Cells were collected and washed with PBS for 5 minutes by centrifugation at 125 × g. Cells were fixed with paraformaldehyde and then resuspended in staining solution containing propidium iodide and RNase A and incubated for 30 minutes in 37°C. The samples (10000 cells) were analyzed by fluorescence-activated cell sorting with a Beckman Gallios™ Flow Cytometer.

Apoptosis assay

Apoptosis assay was according to the manual operation of BD Annexin V Staining Kit (Cat: 556420, BD Biosciences, Franklin Lakes, NJ USA) and was introduced before [21]. Briefly, wash cells twice with cold PBS and then resus-
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pend cells in Binding buffer then transfer solution (~1 x 10^5 cells) to culture tube and add Annexin V and PI 5 µl/test (better use passive tense and separate into 2-3 sentences). Apoptosis assay was analyzed by flow cytometry as soon as possible (within 1 hr).

*Hoechst 33342 staining analysis*

Cells were seeded into 6-well plates, and then treated with SKI-5C (2 µM or 5 µM) and cultured at 37°C for 24 hours, Hoechst staining was introduced before [21]. Abnormal nuclear cells were counted between the SKI-5C treatment group and DMSO control group.

*Analysis of apoptosis by TUNEL assay*

TUNEL assay was introduced before [21], TUNEL Apoptosis Detection Kit (Cat: KGA704; Ken- gent, Nanjing, China) was used. Apoptotic cells were photographed by fluorescence microscopy (OLYMPUS IX71; Olympus Corporation, Tokyo, Japan).

*Xenograft assays the treatment effect of SKI-5C in nude mice*

This study was performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocol has been approved by the Committee on the Ethics of Animal Experiments of Soochow university ( Permit Number: 2014-11-06). 1 x 10^7 SK-NEP-1 cells were subcutaneously injected into five 4-6 weeks old male nude mice each group. 10 days after injection, mice were intraperitoneal treatment with PBS, DMSO, and SKI-5C 20 mg/kg and 40 mg/kg dose two times per week. And the treatment last five weeks. During the five weeks these mice were examined for subcutaneous tumor growth and health condition two times per week. The tumor volumes were calculated according to this formula: volume = length x width^2/2. After the last treatment, the mice were killed under sodium pentobarbital anesthesia and the tumor weight was measured.

*Array analysis was performed by KangChen Bio-tech, Shanghai P. R. China. And experimental details were introduced by Yu et al. [22]. Briefly, RNA purified from total RNA after removal of rRNA was amplified and transcribed into fluorescent cRNA and cDNA was labeled and hybridized to the Human LncRNA Array v3.0 (8660 K, Arraystar). 30,586 LncRNAs and 26,109 coding transcripts which collected from the most authoritative databases such as RefSeq, UCSC, Knowgenes, Ensembl and many related literatures can be detected by the microarray.*

*Gene ontology analysis and KEGG pathway analysis*

Gene ontology (GO) analysis is a functional analysis that associates differentially expressed mRNAs with GO categories. The GO categories were derived from Gene Ontology (www.geneontology.org), which comprises three structured networks of defined terms that describe gene product attributes. The lower the p-value is, the more significant the GO term (a p-value ≤ 0.05 is recommended). Pathway analysis is a functional analysis that maps genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (http://www.genome.jp/kegg/). The lower the p-value is, the more significant the correlation (The recommend p-value cut-off is 0.05).

*Western blot analysis*

For western blot analysis, blots were blocked and then probed with antibodies against SphK1 (Cat: ab16491, 1:1000, Abcam Trading (Shanghai) Company Ltd. Pudong, Shanghai, China), Caspase 3 (Cat: 9661S 1:1000, Cell Signaling Technology, Inc. Danvers, MA), PARP (Cat: 9542S, 1:1000, Cell Signaling Technology, Inc. Danvers, MA), LC3A/B (Cat: 4108S, 1:1000, Cell Signaling Technology, Inc. Danvers, MA), PRKACA (Cat: ab175593, 1:1000, Abcam Trading (Shanghai) Company Ltd. Pudong, Shanghai, China), p44/42 MAPK (ERK1/2) (Cat: 4695S, 1:1000, Cell Signaling Technology, Inc. Danvers, MA), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cat: 4370S, 1:1000, Cell Signaling Technology, Inc. Danvers, MA), NF-kB p65 (Cat: 4764S, 1:1000, Cell Signaling Technology, Inc. Danvers, MA), phospho-NF-kB p65 (Ser536) (Cat: 3033S, 1:1000,
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Cell Signaling Technology, Inc. Danvers, MA), GAPDH (1:5000, Sigma, St. Louis, MO).

RNA interference of SphK1 in SK-NEP-1 cells

Transfection of duplex siRNAs into SK-NEP-1 cells was performed as described before and the SphK1 siRNA sequences as used before [23]. The sequences were Si-1# 5’-GGGCAA-GGCCUUGCAGCuc(TT)-3’, Si-2# 5’-GAGCUG-CAAGGCUUGCCcd(TT)-3’ and control siRNA, 5’-UUCUCCGAACGUGUCACGUd(TT)-3’. These siRNAs were transfected into SK-NEP-1 cells using Lipofectamine 2000 (Invitrogen, Thermo fisher, Massachusetts, USA), 3 days later these cells were collected for the following analysis.

Statistical analysis

At least three replicates for each experimental condition were performed, and the presented results were representative of these replicates. All values are presented as means ± SEM. Student’s paired t-test was applied to reveal statistical significances. P values less than 0.05 were considered significant. Statistical analyses were performed using SPSS Software for Windows (version 11.5; SPSS, Inc., Chicago, IL).

Results

Growth inhibitory effect of SKI-5C on SK-NEP-1 and G401 cells

SphK1 is highly expressed in SK-NEP-1 and G401 cells. Our western-blot analysis showed that expression of SphK1 is higher in SK-NEP-1 and G401 cells than in 293T and CHO (Chinese hamster ovary) cell lines, normal kidney tissues and peripheral blood mononuclear cell (PBMC; Figure 1A). To our knowledge, this is the first
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Time high expression levels of SphK1 has been reported in Wilms’ tumor cells.

Cellular immunofluorescence revealed that SphK1 is mostly expressed in the cell cytoplasm (Figure 1B). However, we noted differing expression levels of SphK1 in SK-NEP-1 and G401 cells. Some cells demonstrated significantly higher expression levels of SphK1, while others exhibited virtually no expression of SphK1.

Figure 2. SKI-5C inhibits proliferations of SK-NEP-1 and G401 cells. A. Molecular structure of SKI-5C. B. Proliferation and IC$_{50}$ analysis of SKI-5C. The IC$_{50}$ of SKI-5C for SK-NEP-1 cells was about 4.11 uM. The IC$_{50}$ of SKI-5C for G401 cells was about 9.74 uM. C. Micrographs of SK-NEP-1 and G401 cells treated with SKI-5C (2 uM and 5 uM) or DMSO. D. Proliferation analysis of SK-NEP-1 and G401 cells treated with 2 uM and 5 uM SKI-5C.
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Figure 3. Apoptosis analysis of SK-NEP-1 and G401 cells induced by SKI-5C. A. Annexin V staining of cells following 24 h treatment with SKI-5C at 2 μM or 5 μM compared with DMSO control treatment. B. The proportion of apoptotic cells in the SKI-5C-treated cells is significantly greater than that in the DMSO control group. C. Cell apoptosis analysis of SK-NEP-1 and G401 treated with 2 μM and 5 μM SKI-5C. D. Late cell apoptosis shows significant apoptosis in Wilms' tumor cells.
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**Figure 4.** SKI-5C induces cell cycle disorder in SK-NEP-1 and G401 cells. A. Cell cycle analysis of SK-NEP-1 and G401 cells treated for 24 h with SKI-5C at 2 uM or 5 uM compared with DMSO control mock treatment. B. G2 phase
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in SK-NEP-1 (SKI-5C 5 μM group: 0.20% ± 0.01% vs. DMSO 7.96% ± 0.79%, respectively; P < 0.01); and G401 cells (SKI-5C 5 μM group: 0.10% ± 0.14% vs. DMSO 7.98% ± 0.82%, respectively; P < 0.01). C. Cell apoptosis and DNA fragmentation observed. D. Significant apoptosis is shown in SK-NEP-1 (SKI-5C 5 μM group: 16.83% ± 1.93% vs. DMSO 2.23% ± 0.90%, respectively; P < 0.01); and G401 cells (SKI-5C 5 μM group: 15.2% ± 1.95% vs. DMSO 3.23% ± 0.85%, respectively; P < 0.01).

adherence (Figure 2C). Our cell proliferation analysis revealed SKI-5C significantly inhibits proliferation of SK-NEP-1 and G401 cells (Figure 2D). The inhibition rate on the fourth day in the SK-NEP-1 cells was about 71.8% (5 μM SKI-5C group: 0.37 ± 0.12 vs DMSO group: 1.31 ± 0.31, P < 0.01). The inhibition rate on the fourth day in the G401 cells was about 75.4% (5 μM SKI-5C group: 0.44 ± 0.12 vs DMSO group: 1.79 ± 0.37, P < 0.01).

**SKI-5C induces apoptosis in SK-NEP-1 and G401 cells**

To confirm whether SKI-5C induces apoptosis in SK-NEP-1 and G401 cells, we investigated annexin V binding and cell cycle assays in these two cells after treatment with SKI-5C. The results showed that cells treated with 5 μM SKI-5C for 24 h displayed clear signs of apoptosis compared with the control group (Figure 3A and 3B). The results showed a 5.1-fold increase in apoptosis of SK-NEP-1 cells upon treatment with 5 μM SKI-5C (SKI-5C 5 μM group: 20.43% ± 2.48% vs DMSO group: 4.03% ± 0.83%, respectively; P < 0.01). We saw a 9.4-fold increase in apoptosis of G401 cells in the SKI-5C 5 μM treatment group (SKI-5C 5 μM group: 22.57% ± 5.08% vs DMSO group: 2.4% ± 0.66%, respectively; P < 0.01).

After 24 h of SKI-5C treatment, Hoechst 33342 staining analysis revealed DNA fragmentation increases significantly with SKI-5C treatment compared with mock treatment in both SK-NEP-1 and G401 cells. A. DNA fragmentation increases significantly with SKI-5C treatment compared with mock treatment in both SK-NEP-1 and G401 cells. B. Results show the percentages of TUNEL positive cells in SK-NEP-1 (SKI-5C 10 μM group: 56.5% ± 9.49% vs. DMSO 2.2% ± 1.99%, respectively; P < 0.01); and G401 cells (SKI-5C 10 μM group: 39.8% ± 8.13% vs. DMSO 2.4% ± 1.67%, respectively; P < 0.01). **P < 0.01.

**Figure 5.** Micrographs showing TUNEL staining of cells treated with SKI-5C (2 μM and 5 μM) for 24 h. Red cells demonstrates the induction of DNA fragmentation. A. DNA fragmentation increases significantly with SKI-5C treatment compared with mock treatment in both SK-NEP-1 and G401 cells. Results show the percentages of TUNEL positive cells in SK-NEP-1 (SKI-5C 10 μM group: 56.5% ± 9.49% vs. DMSO 2.2% ± 1.99%, respectively; P < 0.01); and G401 cells (SKI-5C 10 μM group: 39.8% ± 8.13% vs. DMSO 2.4% ± 1.67%, respectively; P < 0.01). **P < 0.01.
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We assessed the inhibition impact of SKI-5C on the growth of SK-NEP-1 cells in nude mice. Figure 6A shows that SKI-5C significantly inhibited the growth of SK-NEP-1 xenografts (SKI-5C 20 mg/kg: 1.64 ± 0.67 cm; SKI-5C 40 mg/kg: 1.12 ± 0.45 cm) compared with the DMSO group (DMSO: 2.56 ± 1.99 cm) or the PBS group (PBS: 2.89 ± 1.90 cm), ANOVA P < 0.05 (Figure 6B and 6C). SKI-5C treatment decreased the weight of the tumors (SKI-5C 20 mg/kg: 1.39 ± 0.22 g; SKI-5C 40 mg/kg: 0.89 ± 0.26 g) compared with the DMSO group (DMSO: 1.78 ± 0.25 g) or the PBS group (PBS: 1.60 ± 0.20 g, ANOVA P < 0.05; Figure 6D). The body mass curve of the nude mice treated with SKI-5C was almost identical to the control group. At the end of experiment, the body mass for the 20 mg/kg SKI-5C group was 22.00 ± 2.63 g; and for the 40 mg/kg SKI-5C group was 20.536 ± 2.54 g, compared to the DMSO group (DMSO: 22.44 ± 1.97 g) or PBS group (PBS: 23.10 ± 2.59 g, ANOVA P > 0.05 Figure 6D). These studies support the view that SKI-5C has a significant role with few side effects in the treatment of SK-NEP-1 xenograft tumors.

Microarray analysis of differentially expressed genes in SKI-5C-treated SK-NEP-1 cells

We submitted our microarray analysis to the Gene Expression Omnibus (GEO) repository and assigned the GEO accession number: GS-E72980. We identified 2309 differently expressed mRNAs in the SK-5C-treated SK-NEP-1 cells (Figure 7A, Supplemental Data). Compared with the DMSO-treated control cells, 27 mRNAs were significantly upregulated and 19 mRNAs were significantly downregulated (> 8 fold) in the SKI-5C-treated SK-NEP-1 cells. We applied cluster analysis to visualize the relationship between the mRNA expression patterns present in the samples (fold changes ≥ 8; Figure 7B). In the IncRNA expression profiling data, we analyzed 33327 IncRNAs and 4946 IncRNAs differentially expressed in SKI-5C-treated SK-NEP-1 cells (Figure 7C, Supplemental Data). We used hierarchical cluster analysis to show 59 differently expressed IncRNAs with a fold change ≥ 10, which is presented in Figure 7D.

Gene ontology and KEGG pathway analysis of differentially expressed mRNAs in SKI-5C-treated SK-NEP-1 cells

We performed gene ontology (GO) enrichment analysis for the differently expressed genes and gene products with particular attention to GO biological processes and molecular function. We used Fisher’s exact test to determine whether the overlap between the differentially expressed genes list and the GO annotation list was greater than that expected by chance (a P-value ≤ 0.05 is recommended).

The most enriched GOs targeted by the upregulated transcripts were involved in a variety of functions including cellular macromolecule metabolic process (P value 2.99433E-09), nucleic acid metabolic process (P value 2.34638E-08), DNA conformation change (P value 1.32661E-07), regulation of cellular metabolic process (P value 1.39102E-07). The most enriched GOs...
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**Molecular mechanism analysis of SKI-5C in SK-NEP-1 cells**

Our results showed that expression of SphK1 was inhibited when SK-NEP-1 cells were treated with SKI-5C. To validate apoptosis in these cells, we analyzed expression levels of apoptosis-regulated genes in the SKI-5C-treated SK-NEP-1 cells using poly (ADP-ribose) polymerase (PARP) and caspase-3. We observed cleavage of PARP and caspase-3 in the SKI-5C treatment group. Our gene microarray analysis showed mRNA expression of protein kinase cAMP-activated catalytic subunit alpha (PRKACA) was mostly downregulated after SKI-5C treatment; our western blot analysis corroborated these findings. As PRKACA encodes one of the catalytic subunits of protein kinase A (PKA), cAMP-dependent phosphorylation of proteins by PKA is important to many cellular processes, including differentiation, proliferation, and apoptosis.

The specificity of small molecule inhibitors is a continued challenge. In order to confirm the molecular function of Sphk1 in SK-NEP-1 cells, we knocked down the expression of SphK1 using the RNA interference system (Figure 9B). Our results showed that cell proliferation was inhibited when the expression of SphK1 was downregulated (Si-1# group: 0.91 ± 0.24; Si-2# group: 0.72 ± 0.23 vs DMSO group: 1.23 ± 0.33, P < 0.05; Figure 9C). Moreover, cell apoptosis was significantly upregulated in the SphK1 interference group (Si-1# group: 7.7 ± 0.67; Si-2# group: 13.1 ± 0.71 vs DMSO group: 3.87 ± 0.25, P < 0.05; Figure 9D).

**Discussion**

SphK1, an enzyme of sphingolipid metabolism, is emerging as a key player in tumor pathology and a promising new target for cancer therapy [24-27]. SphK1 has been found to be upregulated in various types of human malignancies, playing a crucial role in tumor development and progression. SphK1 exhibits excellent tumor targeting characteristics for many types of
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progression and poor survival. As SphK1 inhibits doxorubicin- or docetaxel-induced apoptosis, it is therefore a potential pharmacologic target for the treatment of NSCLC [28]. SphK1 is also involved in tumor development of cervical cancer and presents a potential prognostic marker for this cancer [29]. Another example where SphK1 is markedly elevated is in multiple myeloma (MM) cells. Here, downregulation of SphK1 has been shown to potentiate the apoptotic effects of green tea polyphenol epigallocatechin-3-O-gallate (EGCG), therefore, targeting SphK1 may represent a novel therapeutic strategy against MM [30]. All head and neck squamous cell carcinoma (HNSCC) stages exhibit overexpression of SphK1, which is associated with depth of tumor invasion, metastasis and clinical failure, underscoring the importance of SphK1 in HNSCC pathology. As SphK1 sits at the hub of multiple key signaling cascades, all which have been implicated in the regulation of invasiveness, SphK1 is undoubtedly an attractive target for the development of HNSCC therapies [31].

Our previous study identified SphK1 as an invasion and metastasis-related gene of esophageal cancer. In esophageal cancer cells, downstream mediators of SphK1 may mediate enhanced malignant behavior and therefore useful as therapeutic targets [23]. Although SphK1 has gained increasing prominence as an important enzyme in cancer biology, its potential as a therapeutic target in Wilms’ tumor remains unknown.

Figure 7. Cluster analyses of differentially expressed genes and lncRNAs in SKI-5C-treated SK-NEP-1 cells. A. Hierarchical clustering analysis of mRNA expression in SKI-5C-treated SK-NEP-1 cells. B. Hierarchical clustering analysis of the 59 differently expressed lncRNAs with a fold change ≥ 10-folds in SKI-5C-treated SK-NEP-1 cells. C. Scatter-Plot assessing mRNA expression variation between DMSO-treated control cells and SKI-5C-treated SK-NEP-1 cells. The green lines are fold change lines (the default fold change value given is 2.0). mRNAs above the top green line and below the bottom green line indicate more than 2.0 fold change of mRNAs between the two compared samples. D. Scatter plot assessing the IncRNAs expression variation between DMSO-treated control cells and SKI-5C-treated SK-NEP-1 cells. The lncRNAs above the top green line and below the bottom green line indicate more than 2.0-fold change of lncRNAs between the two compared samples.
In this study, we showed that SphK1 is highly expressed in SK-NEP-1 and G401 cells. Noteworthily, this is the first report of high expression of SphK1 in Wilms’ tumor cells. Our western-blot analysis demonstrated that expression of SphK1 is higher in SK-NEP-1 and G401 cells than normal cell line 293T and CHO and normal kidney tissues and PBMC. Our cellular immunofluorescence results showed SphK1 is mostly expressed in cell cytoplasm. Knocking down the expression of SphK1 with RNA interference system revealed cell proliferation was significantly inhibited when expression of SphK1 was downregulated and apoptosis of cells was upregulated in the SphK1 treatment group. Altogether, our results implicate SphK1 as a useful therapeutic target for Wilms’ tumor.

Researchers have previously developed many inhibitors against SphK1. For example, a sphin-
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Figure 9. Molecular mechanism analysis of SKI-5C in SK-NEP-1 cells. A. Western blot analysis of apoptosis-regulated genes in SKI-5C-treated SK-NEP-1 cells. B. Knocking down the expression of SphK1 with RNA interference system to confirm the molecular function of SphK1 in SK-NEP-1 cells. C. CCK-8 analysis shows that cell proliferation is inhibited when the expression of SphK1 was downregulated in SK-NEP-1 cells. D. Annexin V staining shows apoptosis of cells is significantly upregulated in SphK1 interference group.

gosine analogue, FTY720 (fingolimod), has been shown to induce apoptosis in prostate cancer. Here, HeLa and SiHa cervical cancer cells were treated with FTY720 from a patient-derived xenograft (PDX) model of cervical cancer, and FTY720 was shown to significantly
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reduce viability and increased apoptosis in cervical cancer cells in vitro and in vivo [29]. SKI-II is another inhibitor against SphK1. In acute myeloid leukemia (AML), SKI-II inhibited both growth and survival of human AML cell lines HL-60 and U937. SKI-II was generally safe to normal cells including human PBMCs. In vivo, SKI-II suppressed the growth of U937 leukemic xenograft tumors, suggesting that SKI-II is a promising anti-AML agent [17].

SKI-5C is a new inhibitor against SphK1 and the anti-cancer effects has only been reported in triple-negative breast cancer (TNBC). In this instance, treatment with SKI-5C sensitized TNBCs to chemotherapeutic drugs [32]. In our study, we provide the first evidence that SKI-5C treatment results in inhibition of cell proliferation of Wilms’ tumor cell lines, SK-NEP-1 and G401, in a dose-dependent manner. We also show, in vivo, that SKI-5C treatment inhibits the growth of SK-NEP-1 xenograft tumors. Notably, the unwanted toxicity of SKI-5C appears to be low as the body weight of treated mice was well-kept.

The side effects of SphK1 and its inhibitor have been observed by others previously. In one study, SphK1-KO mice analysis showed that SphK1 deficiency ameliorated Ang II-induced acute hypertension, and these mice fared better than WT mice treated with celecoxib [33]. Moreover, FTY720 is a recently approved drug for the treatment of multiple sclerosis and exhibits inhibitory effects on sphingosine kinase. FTY720 increases vascular tone and blood pressure only in hypertensive rats, most likely due to its inhibitory effect on sphingosine kinase [34]. We hypothesize that SphK1 may be a potential target for cancer chemoprevention because of its safer role in cardiovascular functions.

To glean mechanistic insight into the role of SphK1, we analyzed expression levels of apoptosis-regulated genes in SKI-5C-treated SK-NEP-1 cells. We observed cleavage of PARP and caspase-3 in the SKI-5C treatment group. In order to comprehensively understand the effect of SKI-5C on Wilms’ tumor cells, we used microarray analysis to identify differentially expressed genes and IncRNAs in SKI-5C-treated SK-NEP-1 cells and compared them with DMSO-treated control cells. Our gene microarray analysis showed mRNA expression of PRKACA was mostly downregulated after SKI-5C treatment and phosphorylation of ERK1/2 and NF-κB p65 was significantly inhibited in the SKI-5C treatment group. NF-κB and ERK1/2 pathways are important downstream regulators of PKA [35-37]. NF-κB plays an important role in the development of malignant phenotypes through multiple signaling pathways. A prominent mechanism linking NF-κB signaling to cancer progression is the abrogation of apoptosis. Numerous anti-apoptotic proteins, such as Bcl-xL, c-IAPs, TRAFs, and c-FLIP6, are transcriptionally regulated by the NF-κB. Therefore, these results imply that SKI-5C induces the apoptosis of SK-NEP-1 cells through PRKACA/MAPK/NF-κB pathway and PRKACA may be related to the function of SKI-5C.

In conclusion, our studies demonstrate that the SphK1 inhibitor, SKI-5C, is a new promising therapy for Wilms’ tumor. SKI-5C has a significant role with few side effects in the treatment of SK-NEP-1 xenograft tumors. Our results suggest that SKI-5C induces the apoptosis of SK-NEP-1 cells through PRKACA/MAPK/ NF-κB pathway. These results altogether provide new clues of molecular mechanism of cell death induced by SKI-5C.

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Disclosure of conflict of interest

None.

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