Hispidulin suppresses tumor growth and metastasis in renal cell carcinoma by modulating ceramide-sphingosine 1-phosphate rheostat

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Abstract: Sphingosine 1-phosphate (S1P) rheostat is considered as a key signal that determines cell fate. This study aimed to report that hispidulin, a polyphenolic flavonoid, exerted anti-growth and anti-metastasis effects against renal cell carcinoma (RCC) by modulating the balance of ceramide-S1P. In vitro studies showed that hispidulin could effectively inhibit cell proliferation, cell migration, cell invasion, and epithelial-mesenchymal transition, and promote cell apoptosis in Caki-2 and A498 cell lines. Moreover, it also increased the ceramide/S1P ratio. Consistent with the in vitro findings, the efficacy of hispidulin in vivo showed that it effectively suppressed tumor growth and lung metastasis. Furthermore, the results revealed that hispidulin significantly suppressed the activity of sphingosine kinase 1 (Sphk1) in RCC cells; however, no significant change was observed in the mRNA or protein expression of Sphk1. The overexpression of Sphk1 could significantly abrogate the anti-growth and anti-metastasis effects of hispidulin, whereas the siRNA-targeting Sphk1 or Sphk1 inhibitor was able to augment the anticancer effects of hispidulin against RCC. Moreover, hispidulin interfered with the phosphorylation and translocation of Sphk1, leading to inhibitory effects of Sphk1 activity. In summary, the findings suggested that hispidulin suppressed tumor growth and metastasis by inhibiting the Sphk1 activity and consequently modulating ceramide-S1P rheostat. It also presented a new explanation for the antitumor mechanisms of hispidulin against RCC.

Keywords: Ceramide, hispidulin, sphingosine 1-phosphate, Sphk1, renal cell carcinoma

Introduction

Renal cell carcinoma (RCC), accounting for about 3% of adult malignancies, presents 90%-95% of neoplasms deriving from the kidney [1]. More importantly, the incidence of RCC has been increasing worldwide over the past 20 years [1]. Partial or radical nephrectomy is the mainstream treatment for patients with RCC at an early stage. However, 25%-30% patients present with metastatic disease diagnosis [2], making the treatment a challenge. Although clinical evidence has shown that the use of targeted therapies such as multikinase inhibitors can significantly improve the therapeutic outcome, short response period and resistance hamper their overall benefits, leading to a poor prognosis of patients with metastatic advanced-stage RCC [3]. Therefore, novel therapeutic agents against RCC that are able to provide long-term clinical benefits are needed.

A number of bioactive lipids, including ceramide, sphingosine, and sphingosine 1-phosphate (S1P), play a pivotal role in the development and progression of human cancers by regulating cell proliferation, apoptosis, migration, senescence, or responses to stressful conditions [4]. In cancerous cells, an increase in intracellular ceramide leads to cell growth blockade and apoptotic cell death [5]. On the contrary, S1P production is required for optimal cell proliferation induced by growth factors and suppresses ceramide-mediated apoptosis [6]. The concept of “sphingolipid rheostat”, which was first proposed in 1996, highlighted that the balance between ceramides and S1P was the key signal that determines cell fate [7]. The spin-
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golipid rheostat is commonly deregulated in cancer [8]. For instance, a metabolic shift favoring S1P at the expense of C18 ceramide in glioblastoma tumors may be a major contributor to angiogenesis [9]. Therefore, the sphingolipid rheostat has been considered a promising therapeutic target.

Epidemiological evidence shows that consumption of flavonoids, a large group of polyphenolic compounds present in foods and beverages of plant origin, is inversely correlated with the incidence of kidney cancers [10]. Hispidulin (4', 5, 7-trihydroxy-6-methoxyflavone), a polyphenolic flavonoid, has been extracted from a traditional Chinese medicinal plant *Salvia involucrata* [11, 12]. Recently, *in vivo* and *in vitro* studies have shown that hispidulin exerts its antitumor effect against a wide array of cancers, including pancreatic cancer, gastric cancer, ovarian cancer, and glioblastoma [13-16]. Previous studies have verified that hispidulin is able to expedite apoptosis in hepatocellular carcinoma and leukemia cells [17, 18]. However, the role of hispidulin in RCC remains elusive. Therefore, the present study was undertaken to determine whether hispidulin could suppress tumor growth and metastasis of RCC. The findings revealed that hispidulin induced apoptosis in RCC cells by modulating ceramide-S1P rheostat.

**Materials and methods**

**Cell culture**

Human RCC cell lines Caki-2 and A498 were purchased from ATCC (Shanghai, China). Tubule epithelial cells HK-2 were obtained from the Cell Bank of Shanghai Institute of Biological Science (Shanghai, China). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, UT, USA) with 10% (v/v) heat-inactivated FBS (Hyclone), 2 mM glutamine (Sigma, MO, USA), 1% nonessential amino acids (Sigma), and 100 U/mL streptomycin and penicillin (Sigma), at 37°C and 5% CO₂ under appropriate humidity.

**Cell counting Kit-8 assay**

Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) was used to assess the cell viability following the manufacturer’s instructions. The optical density of viable cells was measured using a spectrophotometer (Tecan Group Ltd, Männedorf, Switzerland).

**Flow cytometry analysis of apoptosis**

Cell apoptosis was determined using an FITC Annexin V Apoptosis Kit (BD Pharmingen, NJ, USA). Briefly, the cells were harvested at a density of 5 × 10⁵ cells/mL and incubated with Annexin V-FITC and propidium in the dark for 15 min before detection using a flow cytometer (Beckman Coulter Inc, FL, USA).

**Transwell migration assay**

The cells were harvested at a density of 5 × 10⁴ cells/mL in a serum-free medium, and 200 μL was added to the upper chamber of Transwell (polycarbonate filters of 8-μm porosity; Corning, NY, USA), filling the lower chamber with a culture medium containing 20% fetal bovine serum (FBS). After 24 h of incubation, cotton swab was used to scrape the residual cells in the upper chamber. The cells that penetrated through were fixed and attached to the bottom of the filter with 4% formaldehyde solution for 20 min and then stained with 0.1% crystal violet for 5 min. The number of cells that penetrated to the lower chamber was estimated in five random fields under a 200 × magnification. Each result represented an average of three individually conducted experiments.

**Transwell invasion assay**

The cell invasion assays were performed in plates with 24-well Transwells coated with Matrigel (8-μm pore size; BD Biosciences, CA, USA). After starving the cells in a serum-free medium for 24 h, the cells were trypsinized and washed with DMEM containing 1% FBS three times. The cells were resuspended in DMEM containing 1% FBS and harvested at a density of 2 × 10⁵ cells/mL; 500 μL was plated to the upper chamber of Transwell and the lower chamber was filled with minimum essential medium containing 10% FBS as chemoattractant. After 24 h of incubation, the Matrigel and the remaining cells were scraped in the upper chamber with cotton swabs. The cells attached to the lower surface of the membrane were fixed with 4% formaldehyde and then stained with hematoxylin staining solution. Five random fields were chosen to count, and the migrated cells were photographed under 200 × magnification.
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Quantitative reverse transcriptase-polymerase chain reaction analysis of the expression of sphingosine kinase 1 (Sphk1)

The total RNA was extracted from the culture cells using the TRIzol reagent (Thermo Fisher Scientific, MA, USA). In short, cDNA was acquired using a High-Capacity cDNA Archive Kit (Applied Biosystems, CA, USA). The primer for Sphk1 was synthesized based on the published sequence [33]. The first-strand cDNA was obtained using the Super M-MLV Reverse Transcriptase (BioTeke Co, Beijing, China). Polymerase chain reaction (PCR) was performed using the SYBR Green Master Mix (Solarbio Co., Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase was used to normalize the mRNA expression of Sphk1. The comparative ΔCt method (ABI Prism software, Applied Biosystems) was used to quantify the PCR results.

Western blotting

The proteins were extracted from the cells as described in a previous study [18]. They were then electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred on to polyvinylidene difluoride membrane (Millipore, MA, USA). The proteins were probed with specific antibodies following the standard protocol. Specific primary antibodies against cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), Na+–K+ ATPase, Sphk1, phospho-Sphk1, E-cadherin, N-cadherin, and β-actin were purchased from Abcam (Shanghai, China), and antibody against ceramide was procured from Sigma-Aldrich (MO, USA). The secondary antibodies used in this study were goat anti-rabbit immunoglobulin G-horse radish peroxidase (IgG-HRP) or anti-mouse IgG-HRP (Beyotime). Signals were monitored using a chemiluminescent substrate (KPL, Guildford, UK).

Ceramide and S1P assay

The ceramide and S1P levels were analyzed using commercial enzyme-linked immunosorbent assay kits (Biovol Biotech, Shanghai, China) as described in a previous study [20].

Analysis of Sphk1 activity

The activity assay of sphingosine kinase was conducted using a Sphingosine Kinase Activity Assay Kit (Echelon Research Laboratories, Inc., UT, USA) as per manufacturer’s instruction. In brief, cell lysates (20 μL) were incubated in reaction buffer, 100 μM sphingosine, and 10 μM adenosine 5’-triphosphate (ATP) for 1 h at 37°C, and a luminescent ATP detector was then added to stop the kinase reaction. The kinase activity was measured by luminescent signals [21].

Separation of the cytosolic and membrane proteins

Following treatment, the cells were resuspended in membrane protein isolation buffer (Amresco, OH, USA) to obtain cytosolic and membrane fractions of proteins following manufacturer’s protocols. The cytosolic and membrane fractions of proteins were collected for Western blotting.

Interference vector construction and transfection

The siRNA oligos for Sphk1 gene knockdown were designed and synthesized by Sangon (Shanghai, China) as described in a previous study [21]. Two distinct siRNA sequences and one scramble sequence as control were subcloned into a plasmid vector pGCsi-H1 following manufacturer’s instructions. The ccRCC cells in the logarithmic growth phase were plated in six-well plates at a density of 3 × 10⁵ cells/well, and transfection was conducted using Lipofectamine 3000 (Invitrogen, NY, USA) according to the manufacturer’s instructions. The stable transfection cell clones were validated by Western blot analysis.

Overexpression of Sphk1

The expression of Sphk1 and the control vectors were evaluated as described in a previous study [21]. Sphk1 was overexpressed through transfection with overexpressing construct using the Lipofectamine 3000 reagent (Invitrogen) following the manufacturer’s instructions.

Xenograft model

Eight-week-old male athymic BALB/c nu/nu mice were kept under pathogen-free conditions. Caki-2 cells (10⁶ cells) were injected into the left flanks of mice. Twenty-one days after the injection, the mice were randomly allocated into three groups (eight mice per group) to receive intraperitoneal injections as follows: (A) vehicle (0.9% sodium chloride plus 1% dimethyl sulfoxide), (B) hispidulin (20 mg/(kg × day), dis-
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Figure 1. Effects of hispidulin on cell survival. A: Cell viability of the Caki-2 and A498 upon hispidulin (10 µM or 20 µM) treatment for 24, 48, and 72 h using the CCK-8 assay. B: Effect of hispidulin (5 µM, 10 µM, and 20 µM) treatment on the viability of normal tubular epithelial HK-2 cells for 48 h. C: Flow cytometry analysis cell apoptosis after treatment with hispidulin (10 µM or 20 µM). D: Western blotting analyzed the expression of cleaved caspase-3 and cleaved PARP after hispidulin (10 µM or 20 µM) treatment. *P < 0.05, **P < 0.01.

solved in vehicle], and (C) hispidulin [40 mg/(kg × day), dissolved in vehicle]. Mice body weight and tumor volume were measured twice per week. The immunohistochemistry staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay were performed on the cryostat sections (4 µm/section) of Caki-2 xenograft tumors, and the protocol was described in detail in a previous study [22]. Animal experiments for this study were approved by the Institutional Animal Care and Use Committee at Qingdao University.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical comparisons between cell lines were analyzed by one-way analysis of variance followed by Dunnett t test. Experimental data were analyzed using the GraphPad Prism software (GraphPad Software Inc., CA, USA), and a P value less than 0.05 was considered to be statistically significant.

Pulmonary metastasis animal model

A498 cells (1 × 10⁶ cells) in 0.1-mL of saline were injected into the tail vein of mice to evaluate the effect of hispidulin on pulmonary metastasis of RCC. The mice were sacrificed 12 weeks after inoculation, and consecutive sections of the whole lung were subjected to hematoxylin-eosin staining. All the metastatic lesions in the lung were examined and counted to assess pulmonary metastasis. The study protocol was approved by the Institutional Animal Care and Use Committee at Qingdao University.
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Results

Hispidulin inhibited cell growth in RCC cell lines without affecting the viability of normal tubular epithelial cells

The effect of hispidulin on the cell viability of cultured RCC cells was first examined to assess the therapeutic potential of hispidulin in RCC. Hispidulin inhibited the cell growth of both RCC cell lines, Caki-2 and A498, in a time- and concentration-dependent manner (Figure 1). It was worth noting that hispidulin failed to decrease the survival of HK-2 cells, the normal tubular epithelial cells (Figure 1B). Taken together, the results suggested that hispidulin selectively exerted anti-growth effect against RCC cells without harming the healthy kidney cells.

Hispidulin induced apoptosis in RCC cells

Cell apoptosis following treatment was analyzed using flow cytometry to elucidate the mechanisms underlying the growth inhibition of hispidulin. Following treatment with hispidulin, both Caki-2 and A498 presented with a signifi-
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Hispidulin suppressed cell migration, invasion, and epithelial-mesenchymal transition

Next, the effect of hispidulin on the metastatic potential of RCC cells was examined. Hispidulin inhibited cell migration in a dose-dependent manner (Figure 2A). Then, the effect of hispidulin...
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Lin on cell invasion was determined using the Transwell migration assay. Hispidulin also exhibited inhibitory effect on cell invasion in a dose-dependent manner, and at 10 μM and 20 μM, it was able to significantly suppress cell invasion (Figure 2B). Given that epithelial-mesenchymal transition (EMT) is a dominant step in cancer metastasis, the effect of hispidulin on EMT markers was examined to further evaluate the suppressing effect of hispidulin on the metastatic potential of RCC cells. Treatment with hispidulin in both Caki-2 and A498 cells resulted in a dose-dependent elevation in the expression of E-cadherin and decrease in the expression of N-cadherin (Figure 2C). Collectively, these results suggested that hispidulin was able to significantly suppress the metastatic potential of RCC cells.

**Hispidulin increased the ceramide/S1P ratio**

The balance of ceramide-S1P rheostat has been proposed to influence the cell function of cancerous cells [23]. Therefore, the effect of hispidulin on the level of ceramide and S1P was examined to further explore the role of ceramide-S1P rheostat in the anticancer effect of hispidulin against RCC. Hispidulin treatment dose-dependently increased the cellular level of ceramide while decreasing the cellular level of S1P, leading to a remarkable increase in the ceramide/S1P ratio (Figure 3A and 3B).

**Hispidulin modulated ceramide-S1P rheostat through inhibiting Sphk1 activity**

Since SphK1 is an enzyme that plays a key role in the ceramide-S1P balance, whether hispidulin affected the activity of SphK1 was examined in this study. The activity of SphK1 was significantly suppressed by hispidulin treatment at both 10 μM and 20 μM doses in RCC cells (Figure 3C). Whether hispidulin suppressed the activity of Sphk1 though repressing its expression was investigated. Interestingly, the results showed that hispidulin treatment for 48 h did not induce any significant change in the mRNA or protein level of Sphk1 (Figure 3D), indicating that the suppressing effect of hispidulin on Sphk1 activity did not result from regulating its expression. Since the activity of Sphk1 was markedly enhanced following the phosphorylation of Sphk1 at Ser-225 [24], it was postulated that hispidulin suppressed Sphk1 activity by affecting its phosphorylation. Thus, the level of phosphorylated Sphk1 in RCC cells was examined following hispidulin treatment. Western blot analysis showed that hispidulin treatment correlated with the decreased level of phosphorylated Sphk1, suggesting that hispidulin suppressed the Sphk1 activity by affecting its phosphorylation (Figure 3E). The phosphorylation of Sphk1 at Ser-225 not only enhanced the catalytic activity of Sphk1 but also caused translocation of Sphk1 from the cytosol to the cell membrane, where it could perform its catalytic function [25]. Therefore, the cytosolic and membrane fractions of proteins were separated to further confirm the postulation. A significant increase in Sphk1 in the membrane fraction and a decrease in cytosolic fraction were observed (Figure 3F). Collectively, these findings revealed that hispidulin suppressed Sphk1 activity by affecting its phosphorylation and consequent translocation whereas it did not change the expression of Sphk1 enzyme.

**Inhibition of Sphk1 activity mediated the anticancer effect of hispidulin against RCC**

The aforementioned results showed that hispidulin modulated the ceramide-S1P rheostat through inhibiting Sphk1 activity, which contributed to its anticancer activity against RCC. Next, whether Sphk1 was the primary target of hispidulin in RCC was investigated. The off-target effect of hispidulin was demonstrated by silencing Sphk1. The expression of Sphk1 was repressed by more than 70% using siRNA in both cells (Figure 4A). Then, the effect of hispidulin on cell growth and apoptosis was examined. Hispidulin was able to further augment Sphk1 knockdown-induced growth inhibition and apoptosis (Figure 4B and 4C). Moreover, an established pharmacological inhibitor of Sphk1 was also able to exert additive effect with hispidulin in suppressing cell growth and inducing apoptosis (Figure 4B-D). Moreover, the suppressing effect of hispidulin on cell migration, invasion, and EMT was also augmented by silencing Sphk1 or Sphk1 inhibitor (Figure 4E-G). In contrast, to further verify the crucial contribution of inhibition of Sphk1 activity in the anticancer activity of hispidulin, Sphk1 was overexpressed in Caki-2 and A498 cells (Figure 5A). The CCK-8 assay showed that the antiproliferative effect of hispidulin was significantly compromised in Sphk1-overexpressed RCC cells (Figure 5B). Correspondingly, flow cytometric analysis and Western blot also showed that hispidulin-induced apoptosis and caspase-3 activation were significantly attenuated.
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Figure 4. SiRNA or inhibitor of Sphk1 enhanced the antitumor effects of hispidulin against Caki-2 and A498 cells. A: The knockdown expression of Sphk1 using siRNA; Western blotting confirmed the expression of Sphk1. B-G: Effects of hispidulin (20 µM) on the cell growth, cell apoptosis, expression of cleaved caspase-3 and cleaved PARP, cell migration, cell invasion, and expression of EMT markers in Caki-2 and A498 cells after knockdown of Sphk1 or pretreatment with Sphk1 inhibitor. *P < 0.05, **P < 0.01.
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Figure 5. Antitumor effects of hispidulin (20 µM) were abrogated by the ectopic expression of Sphk1 or pretreatment with K6PC-5, an activator of Sphk1. A: Western blotting validated the overexpression of Sphk1. B-G: Effects of hispidulin (20 µM) on the cell growth, cell apoptosis, expression of cleaved caspase-3 and cleaved PARP, cell migration, cell invasion, and expression of EMT markers in Caki-2 and A498 cells after overexpressing Sphk1 or pretreatment with Sphk1 activator K6PC-5. *P < 0.05, **P < 0.01.
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Figure 6. Anti-neoplastic activity of hispidulin (40 mg/kg) in vivo. A: Hispidulin suppressed tumor growth in the A498 xenograft model. B: Measuring the activity of Sphk1 after hispidulin treatment. C: TUNEL and immunohistochemical assay performed on cryostat sections were used to detect the cell apoptosis. D: Effect of hispidulin on the lung metastasis ability by counting the number of metastatic nodules in the lung. *P < 0.05, **P < 0.01.

ated by ectopic overexpression of Sphk1 (Figure 5C and 5D). The findings also revealed that pretreatment with K6PC-5, an Sphk1 activator, significantly reversed the antiproliferative and proapoptotic effects of hispidulin (Figure 5B-D). Furthermore, the inhibitory effect of hispidulin on the metastatic potential of Caki-2 and A498 cells was significantly abolished by the ectopic overexpression of Sphk1 or K6PC-5 (Figure 5E-G). Taken together, the findings highlighted that inhibition of Sphk1 activity mediated the anticancer activity of hispidulin against RCC.

Hispidulin suppressed tumor growth and lung metastasis of RCC in vivo

Based on the encouraging results from the in vitro studies, the therapeutic effect in vivo of hispidulin was evaluated using a mouse model. Hispidulin at both 20 and 40 mg/kg doses was
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able to significantly suppress tumor growth ($P < 0.01$ vs control; Figure 6A). Corresponding to the observation in tumor growth, the TUNEL and immunohistochemical assays showed that hispidulin treatment was associated with a dose-dependent increase in apoptosis and an increase in the expression of cleaved caspase-3, respectively (Figure 6C). Moreover, the results showed that tumor growth inhibition by hispidulin correlated with a decreased activity of Sphk1 in tumor tissue (Figure 6B), supporting the in vitro findings that hispidulin mediated apoptosis in RCC by inhibiting Sphk1 and consequently modulating ceramide-S1P balance. The effect of hispidulin on lung metastasis was also examined. Hispidulin treatment significantly decreased the number of metastatic nodules in the lung (Figure 6D). Taken together, the in vivo studies indicated that hispidulin was able to effectively suppress tumor growth and metastasis in RCC.

**Discussion**

Epidemiological evidence showed that consumption of flavonoids was associated with a reduced risk of kidney cancers [10]. The role of hispidulin, one of the flavonoid compounds, as a chemopreventive agent was first reported in 1992 [26]. In 2010, Way et al. revealed that hispidulin could induce apoptosis in ovarian cancer and glioblastoma multiforme cells through activating AMP-activated protein kinase signaling [13, 14]. The proapoptotic effect of hispidulin has also been evidenced in gastric cancer cells, pancreatic cancer cells, and hepatoma cells by different groups [16, 27, 28]. Moreover, previous studies also showed that hispidulin suppressed cell growth and induced apoptosis in hepatocellular carcinoma cells, gallbladder cancer cells, and leukemia cells [17, 18, 29]. Besides apoptosis, a previous study also showed that hispidulin suppressed hypoxia-induced EMT of colorectal cancer [30]. This study aimed to evaluate the role of hispidulin in RCC and elucidate the underlying molecular mechanisms. The findings suggested that hispidulin inhibited Sphk1 activity and modulated ceramide-S1P rheostat, leading to suppression of tumor growth and metastasis in RCC.

Besides major constituents of cell membranes, sphingolipids, such as ceramides and S1P, have been found to have multiple biological functions in cancerous cells, leading to the introduction of the concept “ceramide-S1P rheostat”. The balance of ceramide-S1P rheostat has been proposed to influence the cell function of cancerous cells [23]. A number of studies have evidenced that increasing the ceramide/S1P ratio could induce apoptosis in cancer cells, for instance, the increase in cellular ceramide and decrease in S1P by resveratrol in human leukemia cell line through transcriptional upregulation of acid sphingomyelinase, a key enzyme involved in ceramide generation [31]. Besides apoptosis, Osawa et al. also reported that ceramide-S1P rheostat was implicated in liver metastasis of colon cancer [32]. In line with these previous studies, the results of the present study also showed that the anti-growth and anti-metastatic effects of hispidulin were associated with increased ceramide/S1P ratio, further supporting that ceramide-S1P rheostat could be considered as a promising target for cancer therapy.

Sphk1 is an oncogenic sphingolipid-metabolizing enzyme that catalyzes the formation of the mitogenic second messenger S1P, while consuming proapoptotic ceramide. Therefore, Sphk1 plays a key role in modulating ceramide-S1P rheostat [33]. In fact, the aberrant overexpression of Sphk1 has been found in a variety of human cancers, and the association between the expression of Sphk1 and prognosis has been established [34]. Mechanistically, Sphk1 is involved in cell oncogenesis, survival, metastasis, and neovascularization of the tumor microenvironment [35]. In terms of RCC, inhibition of Sphk1 has been found to enhance chemosensitivity, suppressing invasion and angiogenesis in RCC [36, 37]. Moreover, a previous study showed that Sphk1 activation contributed to acquired resistance against Sunitinib in RCC cells [21]. The present study findings indicated that hispidulin exerted an anticancer effect by inhibiting the activity of Sphk1, highlighting the potential of hispidulin to overcome the resistance to Sunitinib in RCC, which remains to be further investigated.
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...genic signaling by this enzyme [25]. An early study has suggested that the translocation of Sphk1 is a calcium-dependent process mediated by calcium and integrin-binding protein 1 [38]. A recent study has proposed a feedback regulatory mechanism including vascular endothelial growth factor and calcium [39]. The results of the present study showed that hispidulin suppressed the activity by interfering with the phosphorylation and translocation of Sphk1 without affecting the mRNA and protein expression of Sphk1, suggesting that hispidulin might inhibit the activity of Sphk1 through modulating factors in the regulatory feedback. Hispidulin has been found to suppress presynaptic voltage-dependent Ca²⁺ entry in rat cerebral cortex nerve terminals [40]. However, whether hispidulin can exert a similar effect in cancerous cells remains to be elucidated.

In summary, the findings in the current study showed that hispidulin suppressed tumor growth and metastasis in RCC. Furthermore, the results also showed that hispidulin inhibited Sphk1 activity and consequently modulated ceramide-S1P rheostat, which contributed to its anticancer effect.

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Disclosure of conflict of interest

None.

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