A Novel Sialyltransferase Inhibitor Suppresses FAK/Paxillin Signaling and Cancer Angiogenesis and Metastasis Pathways

Jia-Yang Chen, Yen-An Tang, Sin-Ming Huang, Hsueh-Fen Juan, Li-Wha Wu, Ying-Chieh Sun, Szu-Chi Wang, Kuan-Wei Wu, Gopula Balraj, Tzu-Ting Chang, Wen-Shan Li, Hung-Chi Cheng, and Yi-Ching Wang

Abstract

Increased sialyltransferase (ST) activity promotes cancer cell metastasis, and overexpression of cell surface sialic acid correlates with poor prognosis in cancer patients. To seek therapies targeting metastasis for cancer treatment, we developed a novel ST inhibitor, Lith-O-Asp, and investigated its antimetastatic and antiangiogenic effects and mechanisms. We found that cells treated with Lith-O-Asp showed a reduction of activity on various ST enzymes by in vitro and cell-based activity analyses. Lith-O-Asp inhibited migration and invasion abilities in various cancer cell lines and showed inhibitory effect on the angiogenic activity of human umbilical vein endothelial cells. Indeed, Lith-O-Asp treatment consequently delayed cancer cell metastasis in experimental and spontaneous metastasis assays in animal models. Importantly, Lith-O-Asp decreased the sialic acid modification of integrin-β1 and inhibited the expression of phospho-FAK, phospho-paxillin, and the matrix metalloprotease (MMP) 2 and MMP9. Lith-O-Asp attenuated the Rho GTPase activity leading to actin dynamic impairment. In addition, 2DE-MS/MS and immunoblotting analyses showed that Lith-O-Asp altered the protein expression level and phosphorylation status of various proteins involved in crucial metastasis and angiogenesis pathways such as vimentin and ribonuclease/angiogenin inhibitor RNH1. Furthermore, Lith-O-Asp treatment significantly inhibited the invasive ability exerted by ectopic overexpression of various ST enzymes catalyzing α-2,3- or α-2,6-sialylation. Our results provide compelling evidence that the potential pan-ST inhibitor, Lith-O-Asp, suppressed cancer cell metastasis likely by inhibiting FAK/paxillin signaling and expressing antiangiogenesis factors. Lith-O-Asp is worthy for further testing as a novel antimetastasis drug for cancer treatment.

Introduction

Metastasis is a major cause of death in cancer patients (1). Traditional anticancer drugs mostly focus on inhibiting tumor cell growth or killing tumor cells directly or indirectly; however, there are only a few drugs that specifically inhibit metastasis of tumor cells (2). Although there are still questions on mechanism of cancer cell metastasis, some clues pertaining to developing antimetastasis compounds for the treatment of cancer metastasis have been revealed (3). For example, overexpression of sialyltransferase (ST) has been reported to positively correlate with cancer metastasis and poor prognosis (4). Sialyltransferases belong to the glycosyltransferase family (5), members of which localize at trans-Golgi apparatus and are responsible for specifically adding mono- or polysialic acids at nonreducing termini of the glycoproteins or glycolipids. Sialic acid–containing glycoconjugates are important in cellular functions (3). It is believed that alteration in cell surface sialylated antigens changes many cellular properties: for example, cell–cell adhesion, cell–extracellular matrix adhesion, immune defense, cell metastasis, and invasion abilities (4, 6–9). Importantly, ST overexpression and cell surface sialylated antigen overpresentation have been reported to positively correlate with cancer metastasis and poor prognosis in leukemia (4, 10), colon (11), breast (12, 13), gastric (14, 15), bladder (16), and ovarian cancers (17). In addition, ST6 β-galactosamide α-2,6-sialyltransferase I...
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(ST6Gal I) knockout mice enhance epithelial tumor differentiation through reduced focal adhesion signaling (18). Blockade of sialyl Lewis antigens by antibody treatment in vivo shows the reduction of tumor burden and angiogenesis in tumor nodules (19). Therefore, targeting of ST to inhibit cancer cell metastasis could be applied to cancer therapy.

Soyasaponin I, which is a natural compound purified from soybean (20), has been shown to be an STβ-galactoside α-2,3-siaiyltransference I (ST3Gal I) inhibitor. It effectively inhibits breast cancer cell MDA-MB-231 and murine melanoma cell, B16F10, from metastasis in vivo (21, 22). However, soyasaponin I is difficult to be obtained in a large amount for cancer treatment. Therefore, we have developed a series of lithocholic acid analogues derived from soyasaponin I by chemical synthesis in high purity and good yield (23). One of our compounds named AL10 has been found to be cell permeable and could effectively attenuate α-2,3-sialylation on cell surface. AL10 suppresses lung metastasis of A549 in tail-vein-injected animal model (24). To further explore the cellular effects of these lithocholic acid analogues, the present study used various cell models to examine the cell surface sialylated antigen expression profile, cell migration, invasion, and angiogenesis inhibition of a lithocholic acid analogue named Lith-O-Asp. Western blot analyses were conducted to identify the signaling pathways affected by Lith-O-Asp revealed by 2DE-MS/MS proteomic assays. In addition, the ant metastasis effect of Lith-O-Asp was performed by both spontaneous and experimental metastasis assays in animal models to test its ant metastasis effects in vivo.

Materials and Methods

Cell line used and cell culture

Normal human lung epithelial cells, BES-6, and human lung cancer cell lines, CL1-0, CL1-1, and CL1-5F4, were obtained from Dr. P.C. Yang (Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan; ref. 25). The human lung cancer cell lines, H1299 and A549, were obtained from Dr. M.L. Kuo (Institute of Toxicology, National Taiwan University Hospital, Taipei, Taiwan). This experiment was independently repeated 3 times.

Compounds used and in vitro activity assay

The synthesis, purification, and in vitro activity assay of ST inhibitor, Lith-O-Asp, were done as previously described (23). The enzymes analyzed included rat ST3Gal I (CalBiochem), rat ST3Gal III (CalBiochem), and human ST6Gal I (CalBiochem). The modified disaccharides used as UV-labeled acceptors were Galβ1,3GalNAcα-PNP for ST3Gal I, Galβ1,4Glcβ-4,5-dimethoxy-2-nitrobenzyl for ST3Gal III, and Galβ1,4GlcNAcβ-4,5-dimethoxy-2-nitrobenzyl for ST6Gal I.

Ectopic expression of sialyltransferases

Vectors including pCMV-SPORT6-ST3Gal I and pCMV-SPORT6-ST6Gal I (both from Thermo Scientific) were used for ectopic expression of ST3Gal I and ST6Gal I, respectively. Cells were transfected using Lipofectamine 2000 (Invitrogen). The pCMV-SPORT6 empty plasmid was used as a control vector.

Western blot, immunoprecipitation, immunocytochemistry staining, and flow cytometry analyses

All antibodies used and their experimental conditions are provided in Supplementary Table S1.

Wound-healing assay

Cancer cells were treated with different concentration of Lith-O-Asp or DMSO for 48 hours. A cell-free gap of 500 μm was created after removing the Culture-Insert (Ibidi). The cells that migrated into the wound area were calculated as follows: 500 μm × (5- to 12-hour area × 500 μm)/0-hour area. Four independent experiments were photographed and quantified under a microscope.

Transwell invasion assay

Transwell insert (8-μm pore; Falcon, BD Biosciences) were precoated with 20-μg Matrigel (BD Bioscience) in DMEM medium. The cells were treated with different doses of Lith-O-Asp or DMSO for 48 hours. After 2 hours of recovery in medium with Lith-O-Asp, 5 × 10^5 cells were seeded onto precoated transwell insert with serum-free medium containing Lith-O-Asp, and the fitted culture dishes filled with DMEM containing 20% FBS and Lith-O-Asp. Invaded cells were fixed and stained after 16 hours. Four random views were photographed and quantified under a microscope.

Tube formation assay

Human umbilical vein endothelial cells (HUVEC) were propagated in endothelial growth medium 2 (EGM2) supplemented with 2% FBS. HUVECs (2 × 10^5) were seeded onto 48-well culture dishes coated with Matrigel followed by treatment with EGM2 containing DMSO or 1 to 10 μmol/L of Lith-O-Asp. Peak tube formation was observed at 12 hours posttreatment, and 4 random fields were observed under a microscope. The length of tubes for each treatment was quantified using software developed by Dr. Y-N. Sun (Department of Computer Science and Information Engineering, National Cheng Kung University, Tainan, Taiwan). This experiment was independently repeated 3 times.

In vivo spontaneous metastasis assay

The BALB/c mice were acquired from the animal center of the National Cheng-Kung University Medical College Laboratory Animal Center after obtaining appropriate institutional review board permission and were raised in a specific pathogen-free environment. Highly metastatic murine 4T1-Luc breast cancer cells (5 × 10^5) were subcutaneously injected into the mammary fat pad of mice. Mice received 3 mg/kg of Lith-O-Asp intraperitoneally on every other day. DMSO treatment was used as control. The growth and the spontaneous metastasis of the tumors were observed under IVIS50 in vivo imaging system (Xenogen) with endotoxin-free luciferase substrate (VivoGlo, Promega) injection. The metastasized
tumor tissues were dissected on day 26, and hematoxylin and eosin (H&E)-stained for further pathologic confirmation.

**In vivo experimental metastasis assay**

The BALB/c mice were acquired and raised after obtaining appropriate institutional review board permission as described above. 4T1-Luc cells were treated with 10 μmol/L of Lith-O-Asp or DMSO as control for 48 hours, and then trypsinized and recovered for 2 hours in 20% FBS-containing media with 10 μmol/L of Lith-O-Asp or DMSO control. Cells (1 × 10^5 cells/200 μL) were subsequently resuspended in serum-free medium, and intravenously injected into tail-vein of BALB/c mice. These mice were then given luciferase substrate (Promega) and photographed by IVIS50 in vivo imaging system (Xenogen) at day 7 and day 9 for the observation of in vivo cancer cell metastasis. The metastasized tumor nodules in lung tissue were dissected and H&E-stained for further confirmation.

**Results**

**Lith-O-Asp inhibits both α-2,3- and α-2,6-sialyltransferase activities**

By semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blot analyses, more mRNA and protein of ST genes (ST3Gal I, ST3Gal III, ST3Gal IV, and ST6Gal I) were expressed in lung cancer cells with higher metastatic potential (Supplementary Fig. S1A and B), suggesting that overexpression of STs contributed to the metastatic ability in the lung cancer cells, in addition to other cancer cells reported (12, 13, 16). Interestingly, there was no significant difference in mRNA expression level between untreated and treated cells with various concentrations of Lith-O-Asp for 48 hours (Supplementary Fig. S1C), whereas Lith-O-Asp treatment decreased the ST3Gal I protein level by 20% to 25% in H1299, CL1-5, and A549 cells as quantified by flow cytometry (Supplementary Fig. S1D).

By in vitro activity assay, we revealed the ability of Lith-O-Asp to inhibit the activities of ST3Gal I, ST3Gal III, and ST6Gal I. The IC₅₀ values ranged from 12 to 37 μmol/L (Fig. 1A). By cell-based flow cytometric analysis, expression of α-2,3-sialylated antigens was found to be significantly inhibited after 48 hours of treatment with Lith-O-Asp detected using SLeα and SLeβ monoclonal antibodies to sialyl-Lewis antigens (Fig. 1B).

**Table 1.** Proteins with differential expression and altered phosphorylation level after Lith-O-Asp treatment in CL1-5 cells analyzed by 2DE-MS/MS

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Abbreviations: EMT, epithelial-mesenchymal transition; TGF, transforming growth factor; TNF, tumor necrosis factor.
treatment with Lith-O-Asp in lung cancer cells (Fig. 1C and D).

The results indicated that Lith-O-Asp decreased the activity of both $\alpha$-2,3- and $\alpha$-2,6-sialyltransferases, and thus inhibited the transfer of sialic acids to the targeted glycoproteins.

To examine whether Lith-O-Asp could form stable structure with mammalian ST, computational molecular docking was conducted. The docking computation located the acid end of Lith-O-Asp compound at the disaccharide-binding site and the amine end leaning toward CMP. Major stabilizing forces of ST were in close contact with acid end of Lith-O-Asp by Val318 and Tyr233, the nonpolar 4-ring segment of Lith-O-Asp was in contact with hydrophobic micropocket of the enzyme, and the amine end of Lith-O-Asp interacted with the polar environment (Supplementary Fig. S2). Lith-O-Asp also formed a hydrogen bond with Tyr269 at its acid end, and Val318 may also contribute to the hydrophobic stabilization interaction. The predicted binding conformation supported that the compound inhibits ST activity.

Lith-O-Asp inhibits migration and invasion abilities of cancer cells

To investigate the effect of Lith-O-Asp on cancer cell motilities, human lung cancer cell line, A549, H1299, and murine breast cancer cell line, 4T1-Luc, were treated with...
10 or 30 µmol/L of Lith-O-Asp prior to wound-healing assay. Lith-O-Asp at such doses did not affect cell proliferation (Supplementary Fig. S3), so its anticancer metastasis effects can be further studied unambiguously at noncytotoxic doses. The results showed that the noncytotoxic Lith-O-Asp treatment significantly decreased cell migration in 5 to 12 hours during wound-healing assay of various cancer cell lines (Fig. 2A).

Matrigel-coated transwell was used to test the invasion inhibition of Lith-O-Asp. Cells were pretreated with 10 or 30 µmol/L of Lith-O-Asp and allowed to invade for 16 hours. The results showed that the invasive ability of H1299, CL1-5, A549, and 4T1-Luc cells was inhibited significantly (Fig. 2B).

**Lith-O-Asp exhibits in vivo spontaneous and experimental metastasis inhibition**

Because migratory and invasive activities are essential for the cancer cells in the primary sites and the extravasated cancer cells in distant organs to successfully establish final metastasis (26), in vivo spontaneous metastasis assay was performed to investigate the antimetastasis ability of Lith-O-Asp. Murine 4T1-Luc mammary adenoma cells were orthotopically injected into the mammary fat pad of the BALB/c mice. Mice received 3 mg/kg of Lith-O-Asp intraperitoneally on every other day starting from day 1. DMSO was used as solvent control. A significant amount of secondary metastatic cancer cells were observed in lung tissues of DMSO control mice detected using IVIS in vivo imaging system after 26 days of fat pad inoculation. However, Lith-O-Asp–treated mice showed fewer lung metastases (Fig. 3A). Reduction of metastasized cancer mass and number of tumor nodules in lung tissues was further confirmed by H&E staining (Fig. 3B). All DMSO-treated mice confirmed secondary lung metastasis, but only 3 of 8 Lith-O-Asp–treated mice showed lung metastasis. Average tumor nodules per mouse were 11 ± 9 nodules in DMSO-treated group, and 2 ± 4 nodules in Lith-O-Asp–treated group.
The hematology and biochemistry tests of blood from mice showed that Lith-O-Asp had limited toxic effects on the treated mice (Supplementary Fig. S4). To further validate the anti-metastatic effect of Lith-O-Asp, we performed experimental metastasis assays. 4T1-Luc cancer cells were treated with 10 μmol/L of Lith-O-Asp or DMSO, and then intravenously injected into tail-vein of BALB/c mice. Detected and judged with the IVIS50 in vivo imaging system, significantly stronger 4T1-Luc illumination signals were shown in control mice injected with Lith-O-Asp–treated cancer cells on days 7 and 9 (Fig. 3C). H&E-stained lung tissue sections from those mice.

Figure 3. In vivo metastasis assays. A, about 5 × 10^5 4T1-Luc cells were orthotopically injected into mammary fat pad of BALB/c mice. Lith-O-Asp (3 mg/kg) was injected intraperitoneally into each mouse on every other day. DMSO served as solvent control. The luciferase signals of mice were detected and photographed by IVIS50 on day 26. DMSO-treated mice showed more secondary metastases, but only a few Lith-O-Asp-treated mice showed secondary metastases. B, the H&E staining of dissected lung tissues from the representative mice orthotopically inoculated with 4T1-Luc cells (original magnification ×100). C, about 1 × 10^5 4T1-Luc cells, pretreated with 10 μmol/L of Lith-O-Asp, were tail-vein injected into BALB/c mice. The luciferase signals of mice were detected and photographed by IVIS50 in vivo image system at days 7 and 9 (left). Synchronized images were quantified (right). *, P < 0.05. D, the H&E staining of lung tissue from 4T1-Luc–injected mice through the tail vein. The red arrows indicate the sites of tumor nodules in the lung tissue.
Lith-O-Asp decreases cell migration ability through inhibition of integrin sialylation and FAK/paxillin signaling pathway

Recent reports have shown evidence that the clustering of monosialyl-glycosphingolipid initiates focal adhesion kinase (FAK)/paxillin/Rac/MMP downstream signaling pathway and activates cell invasion ability (27, 28). In addition, Lith-O-Asp inhibited the sialylation of integrin-β1 (Supplementary Fig. S5). Therefore, we investigated whether the decreased migration ability of cells treated with Lith-O-Asp resulted from inhibition of integrin/FAK/paxillin signaling pathway and its downstream effectors. The results demonstrated that after 10 or 30 µmol/L of Lith-O-Asp treatment for 48 hours, the active forms of FAK (p-FAK/Y397) and paxillin (p-paxillin/Y31) were decreased, whereas there was no change in the expression of total FAK and paxillin proteins. The downstream matrix metalloprotease (MMP) 2 and MMP9 were consistently decreased in a dose-dependent manner after Lith-O-Asp treatment (Fig. 5A). The FAK downstream small GTPase, Rho, which acts as an actin modulator in cell motility, also showed a dose-dependent decrease of activity after Lith-O-Asp treatment (Fig. 5B) along with the loss of actin stress fibers in Lith-O-Asp–treated cells (Fig. 5C).

Lith-O-Asp takes part in cancer metastasis suppression in multiple ways revealed by proteomic studies

To identify whether there were other differentially expressed proteins and proteins with altered phosphorylation status influenced by Lith-O-Asp, in addition to FAK/paxillin/Rac/MMP signaling proteins, 2D-MS/MS proteomic analyses were performed. CyproRuby stain and Pro-Q Diamond PhosphoProtein gel stain were used for total protein and phosphoprotein staining, respectively, from whole-cell lysates of CL1-5 lung cancer cell, followed by in-gel digestion and MS/MS analysis (Supplementary Fig. S6). The proteins showing significant difference in expression or phosphorylation status are listed in Table 1. The results indicated that after Lith-O-Asp treatment, decreased expression at total protein level of tumor necrosis receptor–associated protein 1 (TRAP1), WD-repeated protein 1 (WDR1), and chaperonin containing TCP1 subunit 6A isoform a (CCT6A1) was observed; whereas, the total protein levels of the RNH1, PGK1, and cofilin 1 (CFL1) were increased. In phosphoprotein-staining gels, Ran-GTPase activating protein 1 (RANGAP1), vimentin, heat shock protein β1 (HSPB1), and β-galactoside α-2,3-sialyltransferase 4 (ST3Gal IV) increased in their phosphorylation statuses after Lith-O-Asp treatment. To confirm the Lith-O-Asp–affected proteins identified by 2DE-MS/MS, the expression levels of RNH1, PGK1, WDR1, CCT6A1, and cofilin proteins were detected by Western blot (Figs. 4C and 5A). The phosphorylation status of vimentin and ST3Gal IV proteins was confirmed by immunoprecipitation (IP)-Western assay (Fig. 5D).

Reduction of metastasis by Lith-O-Asp is mediated through inhibition of sialyltransferases

To reveal the causal relationship between reduced metastatic potential and inhibition of ST activity by Lith-O-Asp,
invasion assay was performed on cells ectopically overexpressing ST3Gal I or ST6Gal I with 30 μmol/L of Lith-O-Asp treatment. The results showed that Lith-O-Asp treatment significantly inhibited the invasive ability exerted by ectopically overexpressed ST3Gal I or ST6Gal I (Fig. 6). In addition, inhibition of FAK activity and transwell invasion ability were similar in ST3Gal I or ST6Gal I knocked down and Lith-O-Asp-treated cells (Supplementary Fig. S7). These results demonstrated a causal relationship between blockade of ST activity and altered cell behavior by Lith-O-Asp.

**Discussion**

Metastasis is the major cause of death in cancer patients. However, effective antimetastasis compounds for cancer treatment remain to be developed. In this study, we provide compelling evidence from cell and animal studies that Lith-O-Asp, a potential pan-ST inhibitor (Fig. 1), is an antimetastasis compound. Lith-O-Asp repressed cell migration in various cancer cell lines (Fig. 2) and inhibited the angiogenic activity of HUVECs (Fig. 4). The results of in vivo spontaneous and experimental metastasis assays further confirmed that Lith-O-Asp suppressed tumor metastasis likely through inhibition of migration, invasion, and colonization during early and late stages of cancer progression (Fig. 3). Lith-O-Asp inhibited sialylation of integrin-β1 and FAK/paxillin/Rho/MMP signaling pathway (Fig. 5 and Supplementary Fig. S5). In addition, Lith-O-Asp altered the protein expression levels and phosphorylation statuses of various proteins involved in crucial metastasis and angiogenesis pathways such as vimentin and RNH1 (Table 1). These results suggested that Lith-O-Asp may be a promising lead compound for antimetastatic treatment.

Our data showed that Lith-O-Asp inhibited the metastasis potential of cancer cell through inhibiting both α-2,6- and α-2,3-sialylations (Figs. 1 and 2). In addition, the immunocytochemistry staining and IP-Western blot further confirmed the correlation between decrease of α-2,6- and α-2,3-sialylation of integrin-β1 after Lith-O-Asp treatment (Supplementary Fig. S5). Importantly, Lith-O-Asp treatment significantly inhibited the invasive ability exerted by ectopically overexpressed ST3Gal I or ST6Gal I (Fig. 6). Furthermore, siRNA knockdown of ST3Gal I or ST6Gal I caused inhibition in FAK signaling and impeded the invasive ability of A549 lung cancer cells, similar to what was observed on Lith-O-Asp treatment (Supplementary Fig. S7). Collectively, these results demonstrated a causal relationship between blockade of ST activity and invasion inhibition of Lith-O-Asp.

Activated integrins control downstream signaling pathway activation through non-receptor tyrosine kinase FAK (27, 29) and correlate with cancer metastasis (28). Integrin-β1 has been genomically identified and shown to clinically promote cancer metastasis (26). In addition, the Rho/Rac/CDC42 small-G proteins, which are downstream effectors of FAK, control cell motility through WASP (Wiskott–Aldrich syndrome protein) and actin-related protein (ARP) 2/3 complex signaling pathway to regulate the extension and branching of actin filament and cell protrusion (30). Importantly, the integrin-β1 downstream signaling player, RhoA, is known to regulate the actin stress fiber-coordinated fibronectin matrix assembly (31), which is capable of promoting cancer metastasis in the lungs (32, 33). We confirmed that Lith-O-Asp treatment decreased α-2,6- and α-2,3-sialylation of integrin-(1; decreased p-FAK(Y31), p-paxillin(Y397), MMP2, MMP9 protein expression; and decreased Rho activity (Fig. 5 and Supplementary Fig. S5). Lith-O-Asp may inhibit cancer cell
motility partly through suppression of sialylation of integrin-β and further attenuation of downstream FAK/paxillin/Rho/MMP signaling pathway. Further studies on WASP and ARP2/3 complex level upon Lith-O-Asp treatment are under way.

Several differentially expressed proteins and proteins with altered phosphorylation status induced by Lith-O-Asp were revealed by 2DE-MS/MS assay (Table 1). For example, CCT6A, which is a chaperonin and stimulates TGF-β-mediated migration and invasion through maturation of sphingosine kinases 1 (34), and WDR1, which is essential for actin dynamics through interaction with cofilin (35), showed decreased expression after Lith-O-Asp treatment. Interestingly, we observed an increase in p-cofilin (Fig. 5A), which represents inhibition of actin dynamics (36). Collectively, Lith-O-Asp may suppress cell migration by inhibiting several pathways controlling cytoskeleton dynamics. It has been shown that sialylation of cell surface adhesion molecules correlates with cancer–endothelial cell adhesion, proliferation, and angiogenesis (19, 37). We observed that RNH1, which inhibits in vivo angiogenesis and tumor growth through angiogenin inhibition (38) and PGK1, which acts as endogenous angiogenesis inhibitor (39), were overexpressed after Lith-O-Asp treatment. Lith-O-Asp dose dependently inhibited tube formation of HUVEC cells, suggesting that Lith-O-Asp may participate in antiangiogenic process through RNH1 and PGK1 expression (Fig. 4). Interestingly, phosphorylation of vimentin, which decelerates intermediate filament formation during epithelial–mesenchymal transition (40; (Fig. 5D) and phosphorylation of HSPβ1, which mediates its antiadhesive activity through cytoskeleton dynamic control (41), were increased after Lith-O-Asp treatment (Table 1). Our 2DE-MS/MS and IP-Western results confirmed that α-2,3-ST isotype, ST3Gal IV, was phosphorylated after Lith-O-Asp treatment (Table 1 and Fig. 5D). ST3Gal IV can be phosphorylated by protein kinase C leading to its inactivation (42), and the protein kinase C can be activated by secondary bile acids such as lithocholic acid (43). Whether our lithocholic acid derivative, Lith-O-Asp, activates protein kinase C needs further confirmation. Nevertheless, the proteins revealed by proteomic studies suggested that Lith-O-Asp inhibited cancer cell migration/metastasis through impaired angiogenesis and cytoskeleton responses in multiple ways. Further protein–protein interaction analyses will be performed to dissect the mechanism in relation to pathways affected by Lith-O-Asp.

We proposed that Lith-O-Asp inhibited tumor cell metastasis partly through inhibition of ST activity to attenuate sialylation modification of cell surface proteins, such as
Disclosure of Potential Conflicts of Interest

The authors indicated no conflicts of interest involved in this study.

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