Blockade of Four-Transmembrane L6 Family Member 5 (TM4SF5)-Mediated Tumorigenicity in Hepatocytes by a Synthetic Chalcone Derivative

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We previously reported that the four-transmembrane L6 family member 5 (TM4SF5) was highly expressed in hepatocarcinoma, induced morphological elongation and epithelial-mesenchymal transition, and caused abnormal cell growth in multilayers *in vitro* and tumor formation *in vivo*. In this study, we identified a synthetic compound, 4′-(*p*-toluenesulfonylamido)-4-hydroxychalcone (TSAHC) that antagonized both the TM4SF5-mediated multilayer growth and TM4SF5-enhanced migration/invasion. TSAHC treatment induced multilayer-growing cells to grow in monolayers, recovering contact inhibition without accompanying apoptosis, and inhibited chemotactic migration and invasion. Tumor formation in nude mice injected with TM4SF5-expressing cells and the growth of cells expressing endogenous TM4SF5, but not of TM4SF5-null cells, was suppressed by treatment with TSAHC, but not by treatment with its analogs. The structure-activity relationship indicated the significance of 4′-*p*-toluenesulfonylamido and 4-hydroxy groups for the anti-TM4SF5 effects of TSAHC. Point mutations of the putative *N*-glycosylation sites abolished the TM4SF5-specific TSAHC responsiveness. *Conclusion*: These observations suggest that TM4SF5-enhanced tumorigenic proliferation and metastatic potential can be blocked by TSAHC, likely through targeting the extracellular region of TM4SF5, which is important for protein-protein interactions. (HEPATOLOGY 2009;49:1316-1325.)

Epithelial monolayer integrity is maintained by integrin-mediated cell adhesion between the cell and the extracellular matrix (ECM) and by E-cadherin–mediated contact between adjacent cells.[1] Epithelial-mesenchymal transition (EMT) through loss of cell-cell contacts disrupts monolayer integrity and alters cell-ECM interactions.[2] Tumor cells disseminated from primary tumors via loss of cell adhesion and contact can migrate to and invade distal tissues.[3] We recently reported that TM4SF5-mediated EMT results in a loss of contact inhibition and multilayer growth.[4] Therefore, altered cell adhesion and contact may lead to both a loss of contact inhibition and a dissemination of metastatic cells from the primary tumor.[5]

Integrin-mediated cell adhesion reorganizes actin filaments[6] through activation of diverse intracellular signaling molecules, including focal adhesion kinase (FAK), Rho guanosine triphosphatases (RhoA, Rac1, and CDC42), and others,[7] which are critical for cellular morphology and migration.[8]

TM4SF proteins (i.e., tetraspanins or tetraspans) are a group of membrane proteins with four transmembrane

Abbreviations: DMSO, dimethylsulfoxide; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; SNU449Cp, SNU449 hepatocytes stably infected with control retrovirus encoding pLNCX; SNU449Tp, SNU449 hepatocytes stably infected with retrovirus encoding pLNCX-TM4SF5; TERM, tetraspanin-enriched microdomain; TM4SF5, four-transmembrane L6 family member 5; TSAHC, 4′-(p-toluenesulfonylamido)-4-hydroxychalcone.

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domains, two extracellular loops, and two short cytoplasmic tails.[9] TM4SFs form massive tetraspanin-enriched microdomains (TERMs) and function collaboratively with integrins in cell adhesion and motility.[10] TM4SF5 is a homolog of tumor-associated antigen L6 (TM4SF1 or L6-Ag) and forms the four-transmembrane L6 superfamily.[11] TM4SF5 is highly expressed in pancreatic tumor,[12] hepatocarcinoma,[4] and colon carcinoma (S.-A. Lee and J.W. Lee, unpublished data). Ectopic expression of TM4SF5 in Cos7 cells results in integrin α2–dependent actin reorganization and focal adhesion turnover, which can be inhibited by serum treatment.[13] TM4SF5 expression in epithelial cells causes EMT, loss of contact inhibition, and aberrant proliferation.[4] Therefore, the identification of an anti-TM4SF5 reagent is important for development of a therapeutic reagent against TM4SF5-mediated tumorigenesis.

Although the antitumoral properties of chalcones are intensively investigated,[14,15] we found in this study that a toluenesulfonylamido-chalcone, 4′-(*p*-toluenesulfonylamido)-4-hydroxychalcone (TSAHC), antagonized TM4SF5-mediated tumorigenic effects. Our findings suggest that TSAHC functions as a specific anti-TM4SF5 reagent which may be clinically useful in the treatment of diverse tumor types.

Materials and Methods

Cells. Stably TM4SF5-null SNU449Cp or TM4SF5-expressing SNU449Tp cells, SNU398, SNU368, HepG2, and Huh7 cells were previously described.[13] HepG2 or Huh7 cells stably transfected with control scrambled short, hairpin RNA (shRNA) or TM4SF5 shRNA (shTM4SF5),[13] or SNU449 cells stably-expressing TM4SF5 wild-type or mutant (N138A, N155Q, or N138A/N155Q) were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium/10% fetal bovine serum (FBS)/0.25 μg/mL gentamycin with 200 μg/mL G418 at 37°C in 5% CO_2.

Western Blots. Cells were transfected with the indicated plasmids, control shRNA or shTM4SF5, or adenovirus–small interfering RNA against $p27^{Kip1}$ or a control scrambled sequence[16] for the indicated periods. Cells were treated with dimethylsulfoxide (DMSO) or 4′-(*p*-toluenesulfonylamido)-4-hydroxychalcone (TSAHC) at different concentrations for the indicated periods. Lysates were immunoblotted using anti-phospho-Y^{577}FAK (BioSource International Inc.), RhoA, $pS^{10}p27^{Kip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA), caspase 3 (BD Bioscience), FAK, $p27^{Kip1}$ (BD Transduction Laboratories), α-tubulin, α-smooth muscle actin (Sigma), or TM4SF5.[4] Treatment of PNGase F or Endo H glycosidase (New England Biolabs) to cell lysates was performed for 18 hours, following the manufacturer's protocols.

Immunofluorescence. Cells on coverslips were transfected or infected with the indicated genetic reagents. Immunofluorescent staining was performed using primary antibodies against bromodeoxyuridine (BrdU; Oncogene Research Products), $p27^{Kip1}$ (BD Transduction Laboratories), β-catenin, or E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), as described.[4] Mounted samples were analyzed with a fluorescent (BX51; Olympus) or a confocal microscope (LSM 5 PASCAL; Carl Zeiss).

Mouse and Tumor Xenografts Animal procedures were performed in accordance with the Seoul National University Laboratory Animal Maintenance Manual and Institutional Review Board agreement. Mice aged 5-6 weeks (n = 6 for each condition) were injected subcutaneously in the flank with 1×10^7 viable cells. Tumor volumes were calculated as previously explained.[4] Intraperitoneal injection of TSAHC (5 or 50 mg/kg body weight), 4′,4-dihydroxychalcone, or 4′-amino-4-hydroxychalcone (50 mg/kg) in mice with TM4SF5-driven tumors (~200 mm^3 of calculated tumor volume) was performed every other day for 3 weeks. Alternatively, the cells were directly injected into livers with or without TSAHC treatment (50 mg/kg × 6 times at 2-day intervals after cell injection), before counting survival days (n = 6 for each condition).

TM4SF5 Mutation. The pcDNA3.1-myc-$(His)_6$-TM4SF5[13] was mutated (N138A, N155Q, or N138A/N155Q) via the QuikChange polymerase chain reaction method (Stratagene), before direct sequencing.

Migration and Invasion Analysis. Trans-well chambers (Corning Costar) with 8 μm porosity were used in the migration assay. The filters of chambers were coated with matrigel (1.2 mg/mL; BD Biosciences) at 80 μL/well in 48-well trans-well chambers for invasion assays. A half million cells with DMSO or 20 μM TSAHC were placed in the upper chamber, and the lower chamber was filled with RPMI-1640 containing 10% FBS and incubated for 24 hours at 37°C in 5% CO_2. After removing nonmigrated or noninvaded cells, cells on the bottom filter surface were fixed and stained with Diff-Quik solution (Scientific Products) before imaging.[13] Cells from at least five random fields were counted for mean ± standard deviation values. Alternatively, Cytodex-3 microcarrier beads (Sigma Aldrich) confluently coated with cells were resuspended in 10× RPMI-1640/20% FBS and placed in collagen I gels (PureCol; INAMED) to evaluate cell invasion by monitoring outgrowth from microcarrier beads by phase-contrast microscopy (Olympus).[17]

TSAHC Synthesis. TSAHC ($C_{22}H_{19}NO_4S$), synthesized as described,[18] is yellowish solid (melting point,

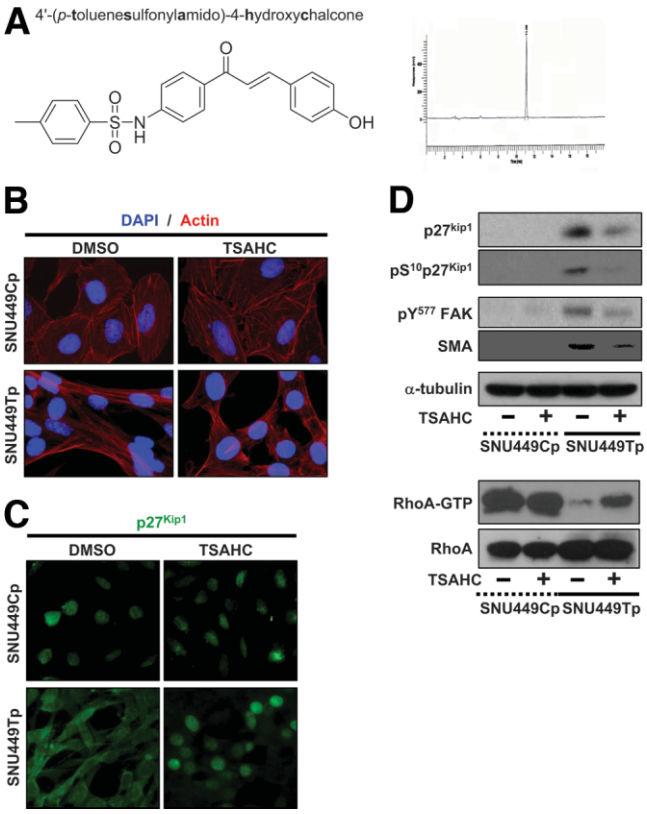

Fig. 1. TSAHC inhibits TM4SF5-mediated effects. (A) Chemical structure (left) and HPLC chromatogram (right) of TSAHC. (B-D) Cells treated with DMSO (-) or 20 μM TSAHC (+) for 24 hours. The cells were (B) stained with 4′,6-diamidino-2-phenylindole (DAPI) for DNA (blue) and with phalloidin for actin (red), (C) immunostained for $p27^{Kip1}$, or (D) harvested for standard western blot or RhoA-guanosine triphosphate analysis.[4] (B) Note that SNU449Tp cells have piled-up nuclei, but this was abolished by TSAHC treatment. Images were originally observed at 40× (B) or 10× (C) magnifications. Data shown represent three independent experiments.

145°C) and stable (>98%) in DMSO for 8 weeks at room temperature and 45°C. Its purity was confirmed by high-performance liquid chromatography (HPLC) analysis using a Spheri-5 in RP-18 column (PerkinElmer). TSAHC structure was deduced with ^{1}H-nuclear magnetic resonance spectrum (of δH 7.94, 7.74, 7.60, 7.30, 7.26, and 6.84 ppm) and X-ray structural analysis (CCDC No. 703762; Supporting Fig. 1).

Statistical Methods. Student *t*-tests were performed for P values < 0.05 to be significant.

Viability, DNA content, RhoA activity assay, wheat-germ agglutinin (WGA) pull down, caspase 3 assay, and gelatin zymography are described in Supporting Information.

Results

We previously reported that ectopic expression of TM4SF5 in SNU449 hepatocytes resulted in elongated cell morphology through cytosolic $p27^{Kip1}$ stabilization, Rho guanosine triphosphatase inactivation, and aberrant actin bundling (Supporting Fig. 2). These cellular phenomena led to EMT (Supporting Fig. 3), abnormal S-phase progression even in confluent conditions (Supporting Fig. 4), and eventually multilayer growth (Supporting Fig. 5),[4] serving as introductory information for this study.

TSAHC Reverses the TM4SF5-Mediated Cellular Phenotypes. Given the robust phenotypes of TM4SF5-expressing cells, we sought to identify chemical inhibitors of TM4SF5 by screening synthetic compounds that could reverse these phenotypes. The screening was performed with 24 synthesized flavonoids, including TSAHC, which had been selected for strong cytotoxic effects on diverse tumor cell lines from an initial library of ~300 compounds. Among the compounds, TSAHC (Fig. 1A) inhibited TM4SF5-mediated effects without significant effects on SNU449Cp cells. SNU449Tp cells treated with TSAHC showed changes in morphology from elongated to polygonal, control cell–like ones without overlapping nuclei (Fig. 1B). TM4SF5-enhanced cytosolic $p27^{Kip1}$ stabilization in SNU449Tp cells was blocked by TSAHC treatment, leading to undetectable cytosolic $p27^{Kip1}$ levels (Fig. 1C) and suggesting that cytosolic $p27^{Kip1}$ may not be stabilized after TSAHC treatment, despite the presence of TM4SF5. TM4SF5-enhanced $p27^{Kip1}$ and $pS^{10}p27^{Kip1}$, which are responsible for cytosolic $p27^{Kip1}$ stabilization,[19] were inhibited by TSAHC (Fig. 1D). Reduced cytosolic $p27^{Kip1}$ stabilization in TSAHC-treated SNU449Tp cells restored RhoA activity and decreased TM4SF5-enhanced smooth muscle actin expression, indicating an inhibition of EMT (Fig. 1D).

TSAHC-Mediated Recovery of Contact Inhibition. TM4SF5 expression causes EMT and multilayer growth,[4] and TSAHC treatment blocked TM4SF5-mediated cytosolic $p27^{Kip1}$ stabilization and RhoA inactivation in SNU449Tp cells (Fig. 1). Therefore, we examined whether TSAHC treatment blocked EMT and multilayer growth, and restored contact inhibition to SNU449Tp cells. We treated SNU449Tp cells with TSAHC and then monitored the localization of molecules involved in cell-cell contact and observed patterns of cell growth. TSAHC treatment restored the localization of E-cadherin and β-catenin to cell-cell contact correlating with monolayer growth (Fig. 2A). When we examined the S-phase progression of confluent SNU449Tp cells after TSAHC treatment, we found that TSAHC suppressed the abnormal S-phase progression mediated by TM4SF5. BrdU incorporation assays showed that TSAHC suppressed S-phase progression in confluent SNU449Tp cells, indicating that contact inhibition had been restored (Fig. 2B). We next examined the effects of TSAHC on cell growth.

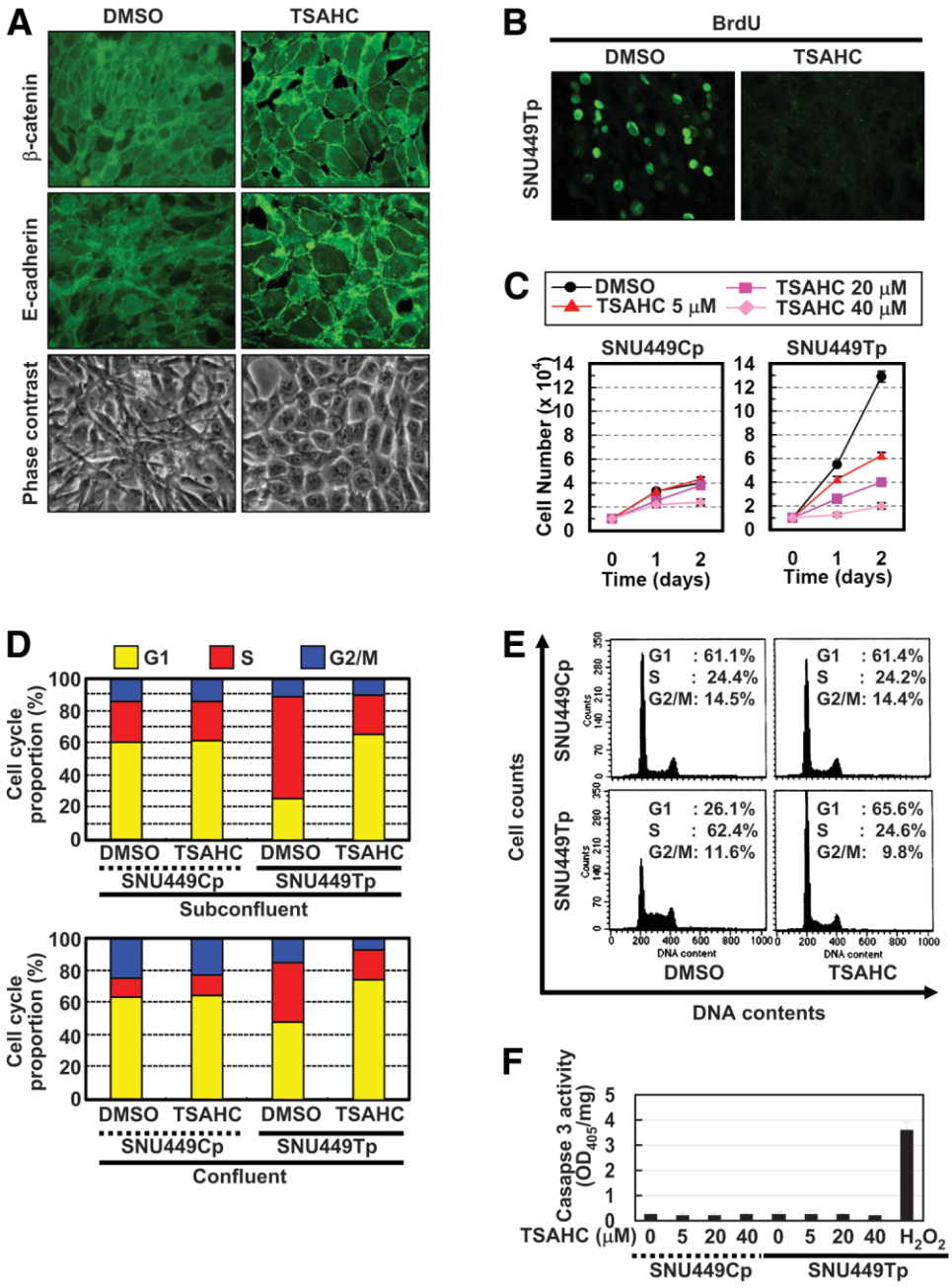

Fig. 2. TSAHC blocks TM4SF5-mediated multilayer growth without causing apoptosis. (A) SNU449Tp cells on coverslips in normal serum-containing media were treated with DMSO or 20 μM TSAHC for 48 hours prior to recording images or immunostaining for E-cadherin or β-catenin. (B) Confluent SNU449Tp cells treated with DMSO or 20 μM TSAHC were cultured with BrdU for 24 hours, before BrdU incorporation analysis. (C) One day after cell seeding (10^4 cells/well of six-well plate) in triplicates, DMSO or TSAHC at diverse concentrations was added to the culture media ($t = 0$). Viable cells were counted after trypan blue staining as time passed. (D, F) Subconfluent [top of (D) and (E)] or confluent (F) cells treated with DMSO or TSAHC (20 μM for 24 hours) were analyzed for cell cycle status via DNA content analysis (D, E) or examined for caspase 3 activity (F). (E) Note no significant sub-G_1 population in conditions. Data shown represent three independent experiments.

TSAHC inhibited proliferation of SNU449Tp cells in a dose-dependent and time-dependent manner, whereas SNU449Cp cell growth was not significantly affected after treatment with TSAHC concentrations up to 20 μM (Fig. 2C). These data suggest that TSAHC inhibits TM4SF5-mediated multilayer growth, a characteristic of tumor cell growth.

We next examined the effects of TSAHC treatment on cell cycle population. TSAHC-untreated SNU449Tp cells showed a higher S-phase but lower G_1-phase population both in subconfluent and confluent conditions, compared to SNU449Cp cells (Fig. 2D). However, the G_2/M phase in confluent SNU449Tp cells was slightly less than that in confluent SNU449Cp cells, indicating that the TM4SF5-mediated effect on the cell cycle can include an abnormally enhanced S-phase even in confluent conditions (Fig. 2D). Meanwhile, TSAHC treatment did not alter SNU449Cp cell cycle populations, whereas TSAHC treatment decreased S-phase and slightly decreased G_2/M phase of SNU449Tp cells but increased G_1 phase, indicating TSAHC-mediated G_1 arrest presumably for contact inhibition (Fig. 2D,E). Being consistent, G_1-phase and S-phase cyclins and pRb phosphorylation dominant even in confluent TM4SF5-expressing cells were decreased upon TSAHC treatment (Supporting Fig. 6). Furthermore, TSAHC treatment did not cause any

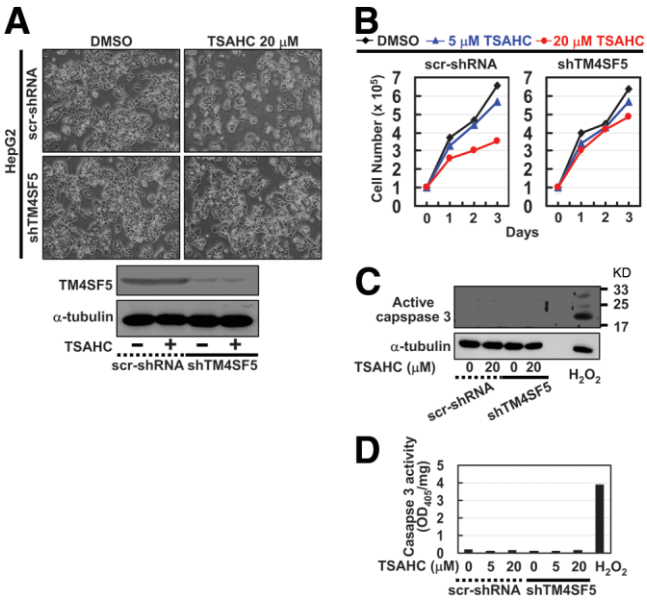

Fig. 3. TSAHC affected growth of endogenously TM4SF5-expressing cells without causing apoptosis. Endogenously TM4SF5-expressing HepG2 cells were stably transfected with scrambled (scr) shRNA or shTM4SF5. The stable cells were treated with DMSO or TSAHC (20 μM for 24 hours), before recording representative images, immunoblots for TM4SF5, α-tubulin (A) or caspase 3 (C), or caspase 3 activity assay (D). H_2O_2 was treated for positive control at 1 mM for 16 hours. Data shown represent three isolated experiments.

significant sub-G_1 population, representing apoptotic cells (Fig. 2E), and did not activate caspase 3 (Fig. 2F), suggesting that the TSAHC-mediated recovery of monolayer growth of SNU449Tp cells did not involve apoptosis. Cells expressing endogenous TM4SF5 were then tested for TSAHC sensitivity. HepG2 (Fig. 3) or Huh7 (Supporting Figure 7) cells expressing endogenous TM4SF5 were growth-arrested by TSAHC treatment, but stable transfection of shTM4SF5 made the cells insensitive to TSAHC without any caspase 3 activation (Fig. 3A-D).

Inhibition of TM4SF5-Mediated Cell Growth and Tumorigenesis by TSAHC. We next examined the effects of TSAHC on TM4SF5-mediated tumorigenicity. Injection of SNU449Tp cells into nude mice resulted in prominent tumors, whereas injection of SNU449Cp cells did not (Fig. 4A). To examine if TSAHC can antagonize TM4SF5-dependent tumor formation in nude mice, TSAHC was injected intraperitoneally every other day into mice with TM4SF5-derived tumors (~200 mm^3 calculated tumor volume). Whereas TM4SF5-derived tumors (n = 6, 7051.5 ± 534.6 mm^3) grew continuously, intraperitoneal injection of 5 or 50 mg/kg TSAHC reduced the tumor growth to 48.9% (n = 6, 3444.8 ± 1980.8 mm^3) or 12.2% (n = 6, 857.2 ± 502.4 mm^3), respectively, with normal body weight increases suggesting no significant side effects (Fig. 4A,B). Mice whose livers had been directly injected with SNU449Tp cells before TSAHC administration (50 mg/kg × six times at a 48-hour interval after cell injection), survived for 51.8 ± 5.17 (mean ± standard deviation) days, whereas SNU449Tp cell–injected mice without TSAHC treatment lived for 32.4 ± 2.19 days (Fig. 4C). However, mice injected with SNU449Cp cells were healthy (Fig. 4C).

Second, we examined whether TSAHC treatment affected the growth of diverse cell lines that either lack or endogenously express TM4SF5. TM4SF5-null SNU398 hepatocytes were not significantly affected, whereas HepG2, Huh7, or SNU368 hepatocytes endogenously expressing TM4SF5 showed clear growth suppression when treated with 20 μM TSAHC (Fig. 4D).

We examined the chemical characteristics of TSAHC required for the blockade of TM4SF5-mediated multilayer growth. Because typical chalcones are known to have antitumoral properties,[20] we examined the effects of chalcones with or without the *p*-toluenesulfonylamido group on TM4SF5-mediated multilayer growth. Interestingly, the compounds lacking the *p*-toluenesulfonylamido group failed to restore monolayer growth, indicating its significance (Fig. 5A). Furthermore, treatment with other substituents or derivatives showed that the 4′-*p*-toluenesulfonylamido group was critical for the effects, and substitution of the 4-hydroxy group with a 4-methoxy group resulted in loss of specificity (Supporting Fig. 8). Anti-TM4SF5 effects were specific for TSAHC, but not for general chalcones. Furthermore, chalcones or aminochalcone did not significantly affect TM4SF5-derived tumor formation (Fig. 5B). TSAHC significantly inhibited tumor formation by HepG2 hepatocarcinoma cells expressing TM4SF5 (Fig. 5C).

Blockade of TM4SF5-Mediated Migration and Invasion by TSAHC. TM4SF5 likely enhances cell migration and invasion, because TM4SFs cooperate with integrins to regulate these cellular functions.[10] We thus explored the effects of TSAHC on TM4SF5-mediated migration and invasion. SNU449Tp cells showed increased chemotactic migration during the trans-well migration assay, compared to SNU449Cp cells (Fig. 6A). However, following TSAHC treatment, TM4SF5-enhanced chemotaxis was inhibited, whereas chemotaxis of SNU449Cp cells was not affected (Fig. 6A). During invasion assays, SNU449Tp cells invaded through matrigel more efficiently than SNU449Cp cells, and TM4SF5-enhanced invasion was inhibited by TSAHC treatment (Fig. 6B). Similarly, we found that SNU449Tp cells grew invasively through a three-dimensional collagen I gel, which was again blocked by TSAHC treatment, but SNU449Cp cells did not show invasive activity (Fig. 6C).

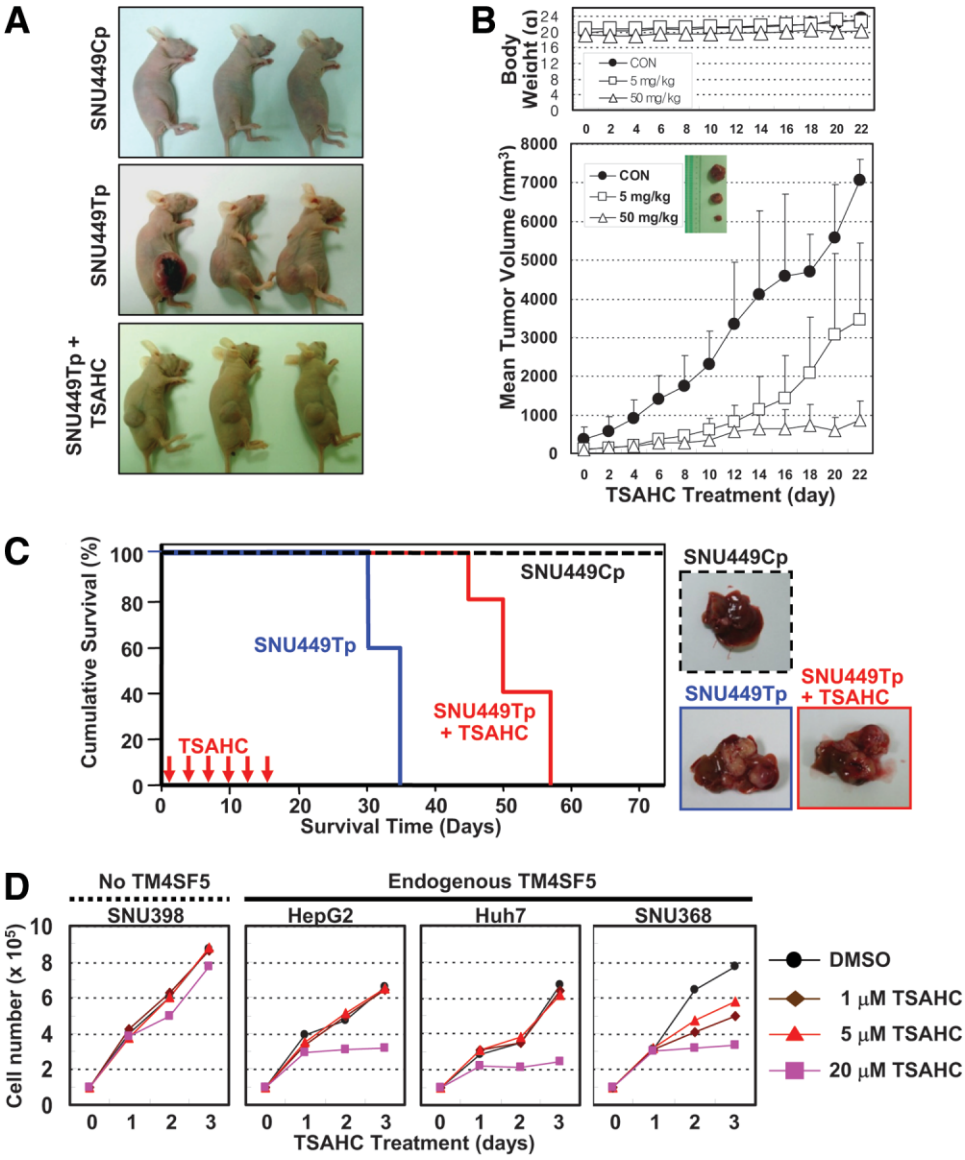

Fig. 4. Suppression of TM4SF5-mediated cell proliferation and tumor formation in nude mice by TSAHC. (A-C) Cells were (A,B) injected subcutaneously into nude mice (10^7 cells/mouse) or (C) directly into their livers. Significant tumorigenesis in the SNU449Tp-injected mice was observed and imaged. TSAHC administration at 5 or 50 mg/kg was performed every other day after TM4SF5-mediated tumors had reached about 200 mm^3 (A,B) or six times at a 2-day interval after cell injection (SNU449Tp + TSAHC) (C). Tumor volumes were calculated and body weights were measured, as explained in Materials and Methods, and were plotted as mean ± standard deviation. (Inset) Images depict representative tumors driven from nude mice injected with SNU449Tp, which were then administered with DMSO or 5 or 50 mg/kg TSAHC (B). Survival of mice was counted and representative livers were dissected and imaged (C). (D) Diverse hepatoma cells were seeded in triplicate. One day later, DMSO or TSAHC at the indicated concentrations was administered ($t = 0$) before cell number counting at the indicated times for graph presentations (mean ± standard deviation). Data shown represent three independent experiments.

Matrix metalloproteinases (MMPs) play critical roles in cell invasion.[21] We examined MMP activities by gelatin zymography on conditioned media with or without TSAHC treatment. MMP2 and MMP9 activities were higher in SNU449Tp cells compared to those in SNU449Cp cells. TSAHC treatment blocked their enhanced activities (Fig. 6D).

TSAHC Affects the EC2 Loop of TM4SF5. We next examined how TSAHC affected TM4SF5 function. TM4SFs interact with other membrane receptors including integrins, and their EC loops are involved in protein-protein interactions[22] by virtue of *N*-glycosylation.[23] To determine if TSAHC altered the pattern of TM4SF5 *N*-glycosylation, the lysates from SNU449Tp cells treated with either DMSO or TSAHC were incubated with glycosidases, and the motility of TM4SF5 on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels was monitored. TSAHC appeared to affect the configuration of *N*-linked carbohydrates on TM4SF5, because TM4SF5 in TSAHC-treated SNU449Tp cells was more susceptible to carbohydrate cleavage by peptide-N-glycosidase F (PNGase F), but not by endo-glycosidase H (Fig. 7A).

We next examined whether mutation of putative *N*-glycosylation sites abolished the activities of TM4SF5 sensitive to TSAHC treatment. We expressed point mutants of putative *N*-glycosylation sites (N138A, N155Q, or N138A/N155Q) in EC loop 2 (EC2) of TM4SF5. The mutants were not pulled-down by wheatgerm agglutinin (WGA)-agarose beads, unlike wild-type TM4SF5 (Fig. 7B). The cellular morphology, cell-cell contacts, and absence of piled-up nuclei in stable TM4SF5 mutant-expressing cells were similar to those observed in the TM4SF5-negative control cells (Fig. 7C and Supporting Figs. 2, 3, and 5). Furthermore, the mutant TM4SF5-

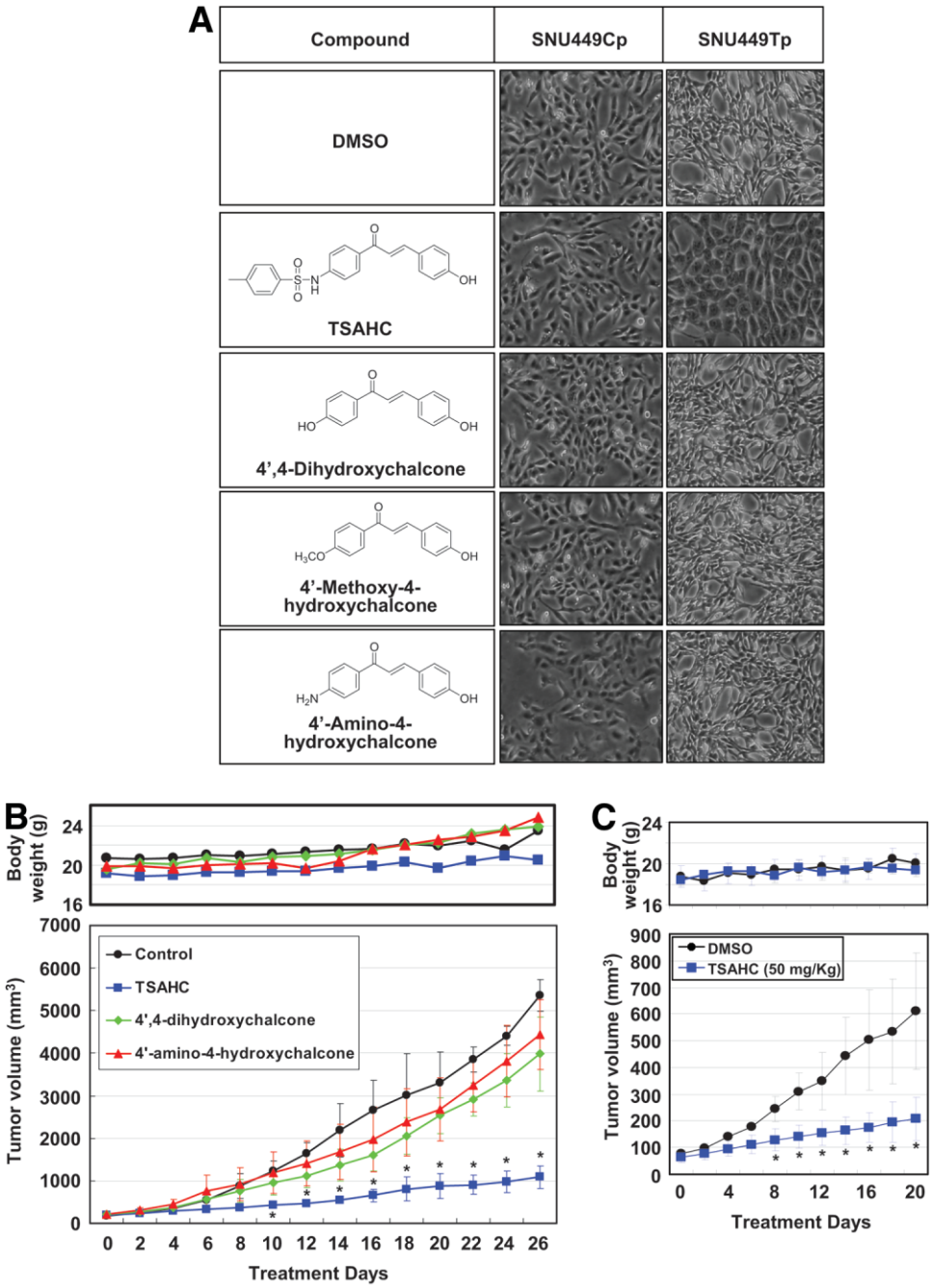

Fig. 5. Structural characteristics of TSAHC for the anti-TM4SF5 effects. (A) Cells were treated with DMSO, TSAHC, or TSAHC derivatives for 48 hours, before recording images. Note that only TSAHC blocked multilayer growth of SNU449Tp cells, but not SNU449Cp cells. Images were originally captured at 40× magnification. Data shown represent three different experiments. (B,C) DMSO (vehicle control), TSAHC, and control analog compounds (50 mg/kg) were injected every other day to mice with approximately 100∼200 mm^3 tumors via (B) SNU449Tp or (C) HepG2 cell injection, as explained in Materials and Methods. The values for control analogs were statistically insignificant ($P \geq 0.05$). Asterisk (*) depicts $P < 0.05$ for a significant difference.

expressing cells grew more slowly than the wild-type TM4SF5-expressing cells (Fig. 7D). The growth rates of the mutant cells were similar to SNU449Cp cells (Fig. 2C). Interestingly, growth of wild-type TM4SF5-expressing cells was inhibited by TSAHC in a dose-dependent manner, but the growth of the TM4SF5 mutant-expressing cells, like SNU449Cp cells, was unaffected (Figs. 7E, 2C, and 4D). These observations suggest that TSAHC may target the *N*-glycosylation status and/or structural integrity of the TM4SF5 EC2 loop, inhibiting the tumorigenic activities of TM4SF5.

Discussion

We previously observed that TM4SF5 protein is highly expressed in clinical liver tumor tissues compared to normal tissues.[4] When we screened compounds to identify an anti-TM4SF5 reagent for an extension of a previous study, we found that TSAHC suppressed TM4SF5-mediated tumorigenicity, suggesting that TSAHC represents a promising novel antitumor drug candidate, particularly against TM4SF5-overexpressing tumors, such as hepatocellular carcinoma (HCC).

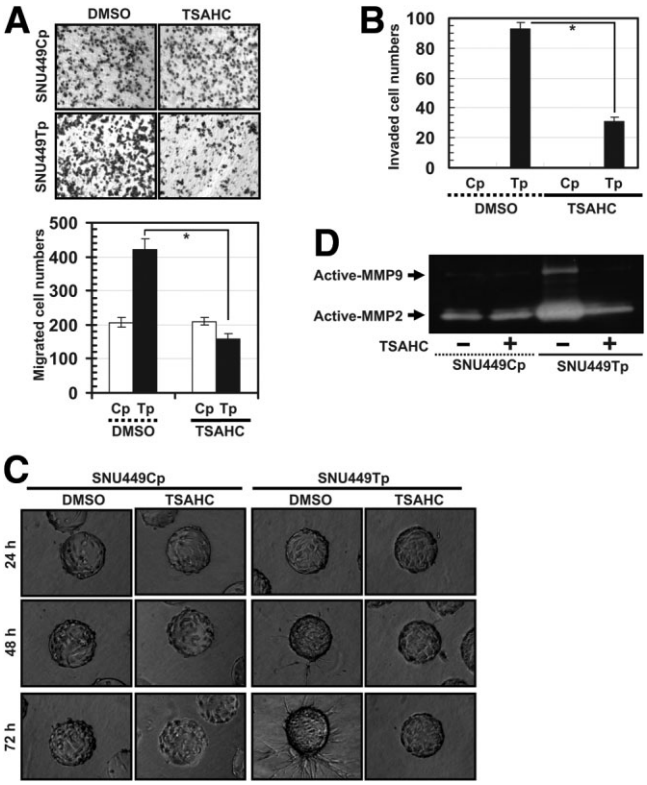

Fig. 6. TSAHC blocks TM4SF5-mediated cell migration and invasion. (A) A half million cells were treated with DMSO or 20 μM TSAHC for 24 hours during chemotactic trans-well migration assays. Migrated cells were imaged (upper) and counted for mean $\pm$ standard deviation values (lower). (B) A half million cells per well were examined for the Matrigel (1.2 mg/mL, 80 μL/well of 48-well plate) invasion assay with DMSO or 20 μM TSAHC treatment for 24 hours. (C) Confluent cell-coated Cytodex-3 microcarrier beads were resuspended in 10$\times$ RPMI-1640 containing 20% FBS and then placed in three-dimensional collagen I gels and incubated for 72 hours with DMSO or 20 μM TSAHC before imaging of outgrowth through collagen I gel from microcarrier beads. (D) Conditioned media collected from cells treated with DMSO (-) or 20 μM TSAHC for 24 hours were analyzed for MMP2 and MMP9 activities by gelatin zymography. Asterisk (*) depicts $P < 0.05$ for a significant difference. Data shown represent three independent experiments.

TSAHC is a chalcone functionalized with a *p*-toluenesulfonylamido group, which is critical for its anti-TM4SF5 effects. Typical chalcones lacking the 4′-(*p*-toluenesulfonylamido) group have antitumoral activities,[20] however, these compounds did not antagonize TM4SF5. Removal of the 4′-(*p*-toluenesulfonylamido) group abolished the inhibitory effect on TM4SF5-mediated multilayer growth and tumor formation in nude mice. Changing the $-CH_3$ of the *p*-toluene group to $-OCH_3$ or $-OH$ also did not result in an anti-TM4SF5 effect. In addition, removal or change of the 4-hydroxy group eliminated the specificity against TM4SF5-mediated multilayer growth and showed strong general cytotoxicity. Therefore, both the 4′-(*p*-toluenesulfonylamido) and 4-hydroxy groups are critical for the specific anti-TM4SF5 effects. Meanwhile, TSAHC treatment did not cause apoptosis and TSAHC-mediated recovery of contact inhibition did not involve apoptosis.

HCC occurs via chronic liver damage resulting from exposure to hepatotoxic chemicals, viruses, and alcohol in addition to numerous genetic and epigenetic alterations.[24] Diverse extracellular cues, including growth factors, cytokines, and ECMs promote hepatocellular proliferation and migration/invasion. TM4SF5 expression appears to be regulated by transforming growth factor-β signaling (S. Choi and J.W. Lee, unpublished data), which is implicated in HCC.[25] TM4SF5 coop-

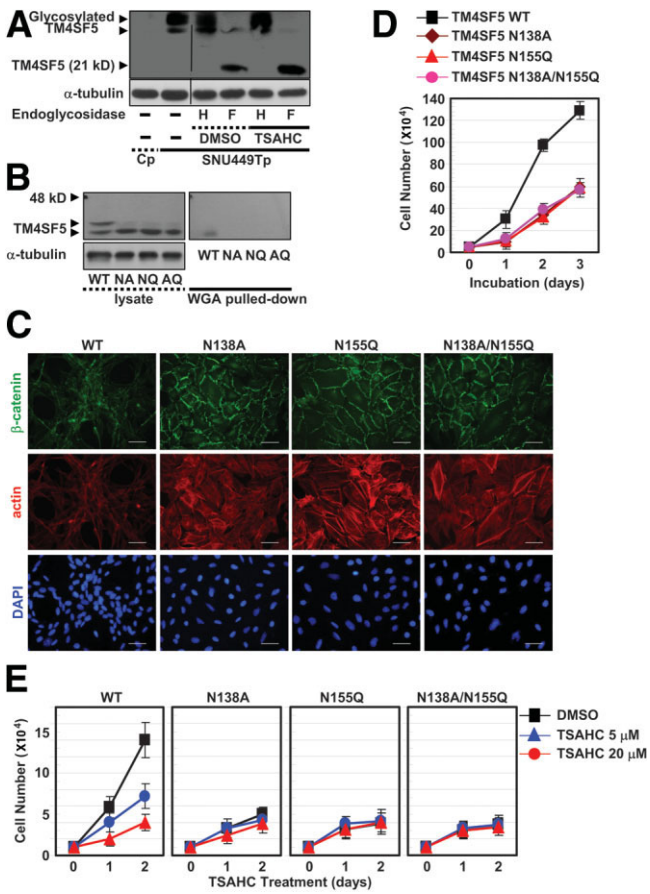

Fig. 7. TSAHC targets the extracellular loop 2 (EC2) of TM4SF5. (A) Cell lysates were prepared using the lysis buffer included in the PNGase F (F) or Endo H (H) glycosidase analysis kit, and an equal amount of protein was treated with or without the endoglycosidase for 18 hours, before standard Western blots for TM4SF5 and α-tubulin. (B) Cell lysate and WGA–agarose bead pull-downs were immunoblotted using mouse anti-TM4SF5 polyclonal antibody. NA, N138A; NQ, N155Q; AQ, N138A/N155Q. (C-E) Confluent SNU449 cells stably expressing TM4SF5 wild-type (WT), N138A, N155Q, or N138A/N155Q on coverslips were immunostained for β-catenin, or stained for actin or nuclei. Scale bars = 20 μm (C). Alternatively, 1 day after seeding of cells without (control, C) or with DMSO or TSAHC treatment ($t = 0$), viable cells were counted by trypan blue staining as time passed (D, E). Data shown represent three independent experiments.

erates with integrins to reorganize actin depending on serum treatment,[13] and causes morphological changes, EMT, and abnormal multilayer growth.[4] TM4SF5 may have a function in the organization of membrane receptors into TERMs, as do other genuine TM4SFs.[10] These collaborations may allow abnormal hepatic cells to communicate more efficiently with microenvironment, during HCC progression.

TSAHC appears to disrupt the interaction between the EC2 loop of TM4SF5 and the extracellular region of other tetraspanins and/or integrins.[10] TSAHC appeared to affect the configuration of *N*-linked carbohydrates on TM4SF5, because TSAHC treatment of SNU449Tp cell extracts increased carbohydrate cleavage from TM4SF5 by PNGase F, which cleaves glycans of both the high mannose and the complex type linked through asparagine residues to the protein backbone. TM4SF5 has two putative *N*-glycosylation residues within the EC2 loop (Asn^{138} and Asn^{155}).[12] Mutations in these residues abolished the tumorigenic activities and recapitulated the antagonistic effects of TSAHC. Cells expressing the TM4SF5 mutants were not sensitive to TSAHC. Another TM4SF5 homolog, IL-TMP (intestinal and liver tetraspan membrane protein), is involved in the regulation of density-dependent proliferation. IL-TMP activity is dependent upon *N*-glycosylation of asparagines within its EC2 loop.[26]

TSAHC was originally found to inhibit α-glucosidase (median inhibitory concentration [IC_{50}] = 0.98 μM)[18] more strongly than sugar-derived glucosidase inhibitors currently used for therapeutic purposes, such as voglibose (IC_{50} = 23.4 μM) and deoxynojirimycin (IC_{50} = 3.5 μM).[27,28] Glycosidases are elevated in the interstitial fluid of tumors and sera of tumor patients and correlate with increased metastatic potential. Thus, there was considerable interest in the development of glycosidase inhibitors.[29] Characteristic oligosaccharide structures and their arrangements on tumor-associated membrane glycoprotein receptors may lead to tumor vaccine developments.[30] The *p*-toluenesulfonylamido and 4-hydroxy groups of TSAHC may target a sequence and/or *N*-linked carbohydrates unique to the EC2 loop of TM4SF5 that is responsible for intramolecular and intermolecular interactions,[11] presumably causing abnormal reorganization of membrane receptors including integrins in TERMs. The potent inhibitory effects of TSAHC on TM4SF5-mediated cellular phenotypes and its apparent specificity thus suggest that this compound holds considerable promise for the treatment of TM4SF5-mediated tumors. An oral dose at 1 g/kg or multiple (14 times over 2 days) doses at 50 mg/kg of TSAHC did not cause any significant toxicity in rats (data not shown).

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