

Taspine derivative TAS9 regulates cell growth and metastasis of human hepatocellular carcinoma

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Abstract. Taspine has been indicated to be a potential anti-carcinogenic agent. The present study investigated the effects of TAS9, a modified taspine derivative, on the proliferation and migration of the SMMC-7721 human liver cancer cell line. First, the effects of TAS9 on SMMC-7721 cell growth were examined using MTT and colony formation assays. *In vivo* Transwell and wound healing assays were then performed to assess the inhibitory effects of TAS9 on cell invasion and migration, respectively. The expression of cell proliferation- and migration-associated signaling molecules was investigated by western blot analysis. The results indicated that TAS9 inhibited SMMC-7721 cell growth by downregulating the signaling molecules protein kinase C β (PKC β), Akt, mammalian target of rapamycin, mitogen-activated protein kinase kinase 2, RAF and c-Jun N-terminal kinase-1, and inhibiting SMMC-7721 cell migration by suppressing the expression of matrix metalloproteinase (MMP)-2, MMP-9, chemokine (C-X-C motif) receptor 4, nuclear factor κ B, p38 and p53. Small interfering RNA-mediated knockdown of PKC β in the SMMC-7721 cells significantly attenuated the tumor inhibitory effects of TAS9. In conclusion, the results of the present study suggested that TAS9 may have inhibitory effects on the proliferation and migration of SMMC-7721 cells, and may serve as a potential candidate for cancer treatment.

Introduction

The major cause of hepatocellular cancer-associated mortality is not only the growth of the primary tumor, but also the invasive spread of cancer cells to a secondary site (1,2). Tumor metastasis is a complex process involving numerous key steps, one of which is tumor cell migration, which is responsible for the entry of tumor cells into blood vessels and lymph nodes (3).

Various molecules and signaling cascades are involved in the proliferation- and metastasis-associated signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt, Ras/Raf/mitogen-activated protein kinase (MAPK) and phospholipase-C(PLC) γ /protein kinase C(PKC) signaling pathways, as well as matrix metalloproteinases (MMPs) and endogenous CXC chemokine receptor-4 (CXCR4) (4,5). The deterioration of the extracellular matrix (ECM) is an important step in tumor metastasis, in which MMPs, an important family of proteinases, have an active role. Among the MMPs that have been identified, increasing evidence showed that MMP2 and MMP9 are able to efficiently degrade native collagen types IV and V, fibronectin, entactin and elastin, and their overexpression is closely associated with poor prognosis in patients (6-8). MMP2 and -9 are therefore considered to be crucial for tumor cell migration and invasion, leading to metastasis.

Hepatocellular carcinoma (HCC), the fifth most common solid tumor type, is one of the leading causes for cancer-associated mortality worldwide (9). Despite significant advances in early detection and therapy, tumor recurrence in HCC patients can occur as metastases, whereas >90% of HCC-associated mortalities are the result of secondary local or distant disease. In the majority of patients diagnosed with HCC, the tumor can be surgically removed; however, the cancer is able to metastasize to distant organ sites. The best treatment option available for patients with HCC is systemic pharmacotherapy (9).

Taspine is a small molecular compound that exhibits various biological properties, including bacteriostatic, anti-biotic, antiviral, anti-inflammatory, anti-ulcer and anti-cancer effects (10-15). TAS9 (Fig. 1) is a modified taspine derivative with increased activity and solubility (16). In the present study, the effects of TAS9 on tumor growth and migration of SMMC-7721 human liver cancer cells as well as the associated signaling pathways were investigated to elucidate the mechanisms underlying the anti-tumorigenic effects of TAS9.

Materials and methods

Reagents. RPMI-1640, dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences (Logan, UT, USA). Trypsin was obtained from Amresco, LLC (Solon, OH, USA). Penicillin was purchased from the Harbin Pharmaceutical Group Co., Ltd. (Harbin, China) and

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streptomycin was purchased from North China Pharmaceutical Co., Ltd. (Shijiazhuang, China). The 96-, 12- and six-well plates were purchased from Corning Inc. (Corning, NY, USA). Crystal violet was purchased from Beijing Chemical Plant (Beijing, China). The 24-well polycarbonate millicell membranes were purchased from EMD Millipore (Billerica, MA, USA). The radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Shaanxi Pioneer Biotech Co., Ltd. (Xi'an, China). The bicinchoninic acid (BCA) Protein Assay Reagent kit and the enhanced Chemiluminescence (ECL) Plus Reagent kit were obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). The protease inhibitor and phosphatase inhibitor cocktail were purchased from Roche Diagnostics (Basel, Switzerland, USA). The polyvinylidene fluoride membranes were purchased from General Electric Company (Fairfield, CT, USA). The RNAfast200 kit was purchased from Shanghai Fastagen Biotechnology Co., Ltd. (Shanghai, China) and Lipofectamine[®] 2000 reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The PrimeScript RT Master mix Perfect Real Time kit and SYBR[®] Premix Ex Taq[™] II were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The non-fat milk was purchased from Wandashan (Heilongjiang, China). Tween-20 was purchased from Amresco, LLC. Monoclonal anti-rabbit Akt (cat. no. 4685), monoclonal anti-rabbit mammalian target of rapamycin (mTOR; cat. no. 2972), monoclonal anti-p53 (cat. no. 2527) rabbit, and monoclonal anti-rabbit p38 (cat. no. 8690) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Monoclonal anti-rabbit MMP-2 (cat. no. 1948-1), monoclonal anti-rabbit MMP-9 (cat. no. 2551-1) and monoclonal anti-rabbit RAF (cat. no. 1560-1) rabbit antibodies were purchased from Epitomics (Burlingame, CA, USA). Polyclonal anti-rabbit p65 (cat. no. 10745-1-AP), polyclonal anti-rabbit PKC β (cat. no. 12919-1-AP), monoclonal anti-mouse CXCR4 (cat. no. 60042-1-IG) and monoclonal horseradish peroxidase-conjugated GAPDH (cat. no. 60042-1-IG) antibodies were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). The corresponding secondary antibodies used were anti-mouse IgG (H+L; cat. no. 14709) and anti-rabbit IgG (cat. no. 14708) purchased from Cell Signaling Technology, Inc. The RNA oligo was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China).

Cell culture conditions. The SMMC-7721 human hepatocellular carcinoma cell line was obtained from Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and was cultured in RPMI-1640 medium supplemented with 10% FBS and 0.1% penicillin/streptomycin. The cultures were maintained at 37°C in a CO₂ incubator (Panasonic, Osaka, Japan) with a controlled humidified atmosphere containing 5% CO₂.

Colony formation assay. The SMMC-7721 cells were plated at a density of 5x10² cells/well in 12-well plates and then incubated for 24 h. The cells were subsequently treated with or without TAS9 at concentrations of 1.75, 3.5, 7 μ M for 48 h or without TAS9 as a control group. Colonies with cell numbers of >50 cells per colony were counted following staining with crystal violet solution. All the experiments were performed in triplicate wells in three independent experiments.

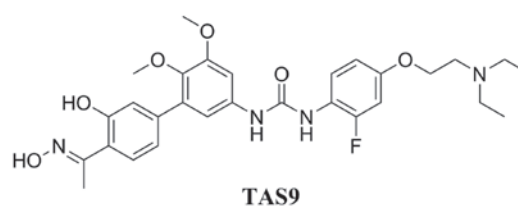


Figure 1. Chemical structure of TAS9.

Cell viability assay. The effects of TAS9 on cell viability were assessed using an MTT assay. Briefly, exponentially growing SMMC-7721 or small interfering (si)RNA-transfected cells were plated at a density of 5x10³ cells/well in 96-well plates and then cultured for 24 h. The cells were subsequently treated with TAS9 at 1.75, 3.5, 7 μ M for 10-15 days or without TAS9 as a control group. Cell proliferation reagent MTT was added to each well, and cells were incubated for a further 4 h at 37°C in an atmosphere containing 5% CO₂. The resulting formazan crystals were dissolved in DMSO (150 ml/well) with constant agitation for 15 min. Absorbance of the plates was read using a 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm.

Wound healing assay. The SMMC-7721 cells were seeded into 12-well plates (6x10⁵ cells/well) and cultured to 80% confluence overnight. Wounds were generated on the following day by performing line-shaped incisions of the monocell layers with pipette tips (100-200 μ l). The SMMC-7721 cells were then treated with or without TAS9 at concentrations of 1.75, 3.5 and 7 μ M, while cells were allowed to migrate into the scratched area. The migration of the cells was visualized at 0 h (immediately following wound scratching), 48 h and 72 h following treatment with TAS9 using a DM505 microscope (Nikon Corporation, Tokyo, Japan).

Migration assay. The cell migration assay was performed using a Transwell system, which allows the cells to migrate through an 8 mm-pore millicell polycarbonate membrane (Baihao, Tianjin, China). Briefly, the SMMC-7721 cells were serum-starved for 24 h and subsequently plated (1x10⁴ cells/well) in serum-free medium containing TAS9 at concentrations of 1.75, 3.5 and 7 μ M in the upper chamber of a 12-well plate. The lower chamber was filled with 1.5 ml medium supplemented with 10% FBS. After 48 h, the cells remaining on the upper surface of the membranes were gently removed using a cotton swab, and the cells on the lower surface of the membranes were fixed with cold methanol for 15 min and stained with 0.2% crystal violet. The cells that had migrated to the bottom of the membranes were visualized using a DM505 inverted microscope, and then counted in random fields. For each repetition, the cells in four randomly selected fields were counted and averaged. The data were expressed as a ratio of the untreated group.

Western blot analysis. The protein of the SMMC-7721 cells treated with or without TAS9 for 48 h was extracted using RIPA lysis buffer containing 10% protease inhibitor and phosphatase inhibitor cocktail (Roche Diagnostics) on ice for 30 min. The insoluble protein lysate was removed

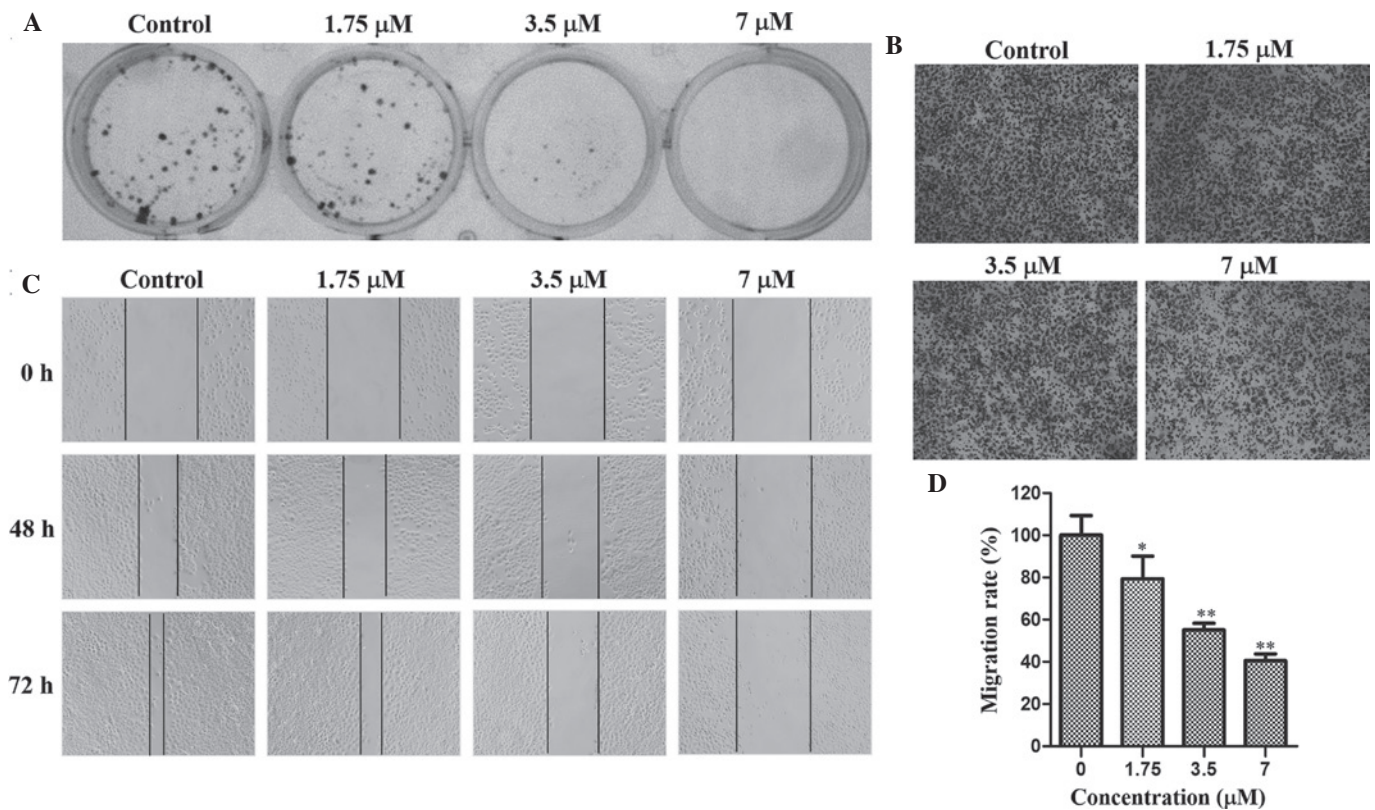


Figure 2. TAS9 suppresses SMMC-7721 human liver cancer cell colony formation and migration. (A) Representative images showing colony formation of SMMC-7721 cells. TAS9 inhibited colony formation of SMMC-7721 cells. (B) Images of cells migrated through polycarbonate membranes in Transwell assays stained with 0.2% crystal violet (magnification, x200). (C) Wound-healing assay of the cells treated with TAS9 (magnification, x200). (D) Quantification of the number of cells which migrated through the polycarbonate membranes in B. Values are expressed as the \pm standard deviation (n=3). *P<0.05 and **P<0.01, vs. the control group.

by centrifugation at 13,500 x g for 10 min at 4°C. The protein concentration was determined using a BCA Protein Quantification kit according to manufacturer's instructions. The cell lysates were denatured by boiling with a 5X reducing sample buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 5 min, and separated by 10% SDS-PAGE (Shaanxi Pioneer Biotech Co., Ltd.). Following electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Hangzhou Microna Membrane Technology Co., Ltd., Hangzhou, China) and blocked with 5% non-fat milk in tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd.) for 2 h at room temperature with continuous agitation. The membranes were then incubated with specific primary antibodies, including anti-MMP-2 (1:500), anti-MMP-9, anti-mTOR, anti-Akt, anti-PKC β , anti-mitogen-activated protein kinase kinase (MEK)-2, anti-RAF, anti-c-Jun N-terminal kinase-1 (JNK)-1, anti-CXCR4, anti-nuclear factor (NF)- κ B, anti-p38, anti-p53 (1:1,000) and anti-GAPDH (1:2,000) antibodies overnight at 4°C, followed by three washes with TBST every 10 min, and incubation with secondary antibodies at a dilution of 1:40,000 in TBST for 1 h at 37°C. The membranes were then washed three times with TBST for 10 min and developed using the ECL kit. A Lane 1D™ transilluminator (Beijing Creation Science Co., Ltd., Beijing, China) was used to capture images of the blots. Image-Pro Plus 5.1 (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

RNA interference. Specific knockdown was achieved using small interfering (si)RNAs targeting PKC β or a control siRNA. A smart pool of double-stranded siRNAs targeting PKC β as well as non-specific siRNAs were obtained from Shanghai GenePharma Co., Ltd. The siRNA sequences were as follows: Forward, 5'-GCGACCUC AUGUAUCACAUTT-3', and reverse, 5'-AUGUGAUACAUGAGGUCGCTT-3' for PKC β ; and forward, 5'-UUCUCCGAACGUGUCACGUTT-3', and reverse, 5'-ACGUGACACGUUCGGAGAATT-3' for the control. For transfection, siRNA was delivered at a final concentration of 80 nM using Lipofectamine® 2000 reagent according to the manufacturer's instructions. The cells were incubated for 24 h to allow knockdown of PKC β . These cells were then used for the proliferation assays.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the SMMC-7721 cells was isolated using a Total RNA Extraction kit (Takara Biotechnology Co.). Total RNA was then reverse-transcribed in 20 ml reaction solution (containing 4 μ l total RNA and 16 μ l PCR mixture) using a Revert AID™ First Strand cDNA Synthesis kit (Takara Biotechnology Co.). The cDNA was synthesized using Bio-Rad iScript Reverse Transcriptase (Bio-RAD Laboratories, Inc.) and the PCR reactions were performed using a Thermal Cycler Dice Real Time system (Takara Biotechnology Co.). The primer sequences were as follows: GAPDH forward, 5'-CACCCACTCCTCCACCTTTG-3' and reverse, 5'-CCACCACCCTGTTGCTGTAG-3'; PKC β

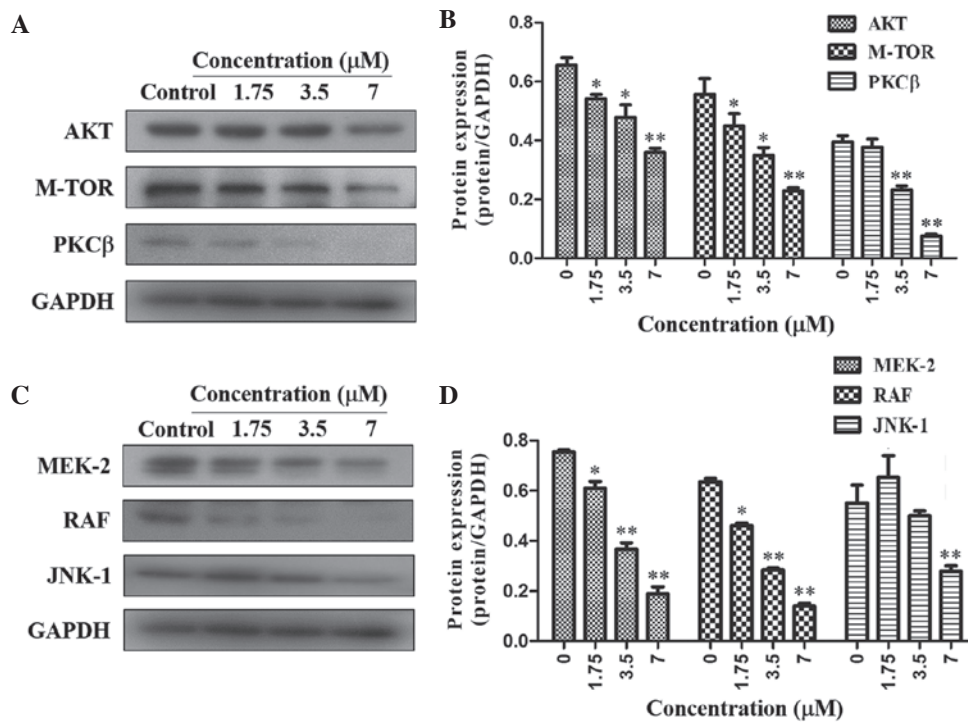


Figure 3. Effects of TAS9 on cell growth-associated signaling pathways. (A) Western blot analysis of the expression levels of Akt, m-TOR and PKC β in SMMC-7721 cells treated with TAS9. (B) The bands were quantified by densitometric analysis. (C) Western blot analysis of the expression levels of MEK-2, RAF and JNK-1 in SMMC-7721 cells treated with TAS9. (D) The bands were quantified by densitometric analysis. Values are expressed as the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01, vs. the control group. m-TOR, mammalian target of rapamycin, PKC β , protein kinase C β ; MEK-2, mitogen-activated protein kinase kinase 2; JNK-1, c-Jun N-terminal kinase-1.

forward, 5'-TTGGGATTTGACCAGCAGGAA-3' and reverse, 5'-GGTGGCACAGGCACATTGA-3', synthesised by Shanghai GenePharma Co., Ltd.. The thermocycling conditions were as follows: 95°C for 2 min and then 95°C for 10 s, and 45 cycles of 60°C for 20 sec. The relative levels of mRNA for each gene were normalized and represented as the ratio of the mRNA value of a target gene to that of the GAPDH gene.

Statistical analysis. Values are expressed as the mean \pm standard deviation of data from several repetitions. Statistical analyses of differences between groups were performed using GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and Student's *t*-test was used to analyze statistical differences between groups under various conditions. P<0.05 was considered to indicated a statistically significant difference.

Results

TAS9 inhibits SMMC-7721-cell proliferation. To assess the effects of TAS9 on cell proliferation, SMMC-7721 cells were treated with TAS9 at concentrations of 0, 0.4, 2, 10 and 50 μ M. The results showed that TAS9 inhibited the growth of SMMC-7721 cells in a dose-dependent manner and the IC₅₀ value of TAS9 on SMMC-7721 cells was 7.57 μ M. Furthermore, TAS9 suppressed colony formation of SMMC-7721 cells following 10-15 days of continuous culture. TAS9 evidently decreased the number of colonies formed by SMMC-7721 cells at concentrations of 1.75, 3.5 and 7 μ M (Fig. 2A). The MTT as well as the colony formation assay indicated

that TAS9 significantly inhibited the proliferation and clonogenicity of SMMC-7721 cells.

TAS9 inhibits SMMC-7721-cell metastasis. To investigate the effects of TAS9 on SMMC-7721-cell migration and invasion, wound healing and Transwell assays were performed. The Transwell assay indicated that after 48 h of incubation, cells migrated to the lower surface of the membrane, which was inhibited by TAS9 in a dose-dependent manner (Fig. 2B and D). In the wound healing assay, cells in the control group rapidly moved into the scratched area and almost covered the wounds, while cells treated with TAS9 had migrated to a lesser extent than those in control group after 72 h (Fig. 2C). TAS9 decreased the distance of cell migration in a dose-dependent manner, and fully inhibited cell migration at the highest concentration of 7 μ M. These results validated that TAS9 inhibited SMMC-7721-cell invasion and migration.

TAS9 inhibits cell growth via PI3K/Akt and MAPK signaling pathways. In order to elucidate the underlying mechanisms of the anti-proliferative effects of TAS9, the expression levels of PKC β , Akt and mTOR, which are representative molecules of the PI3K/Akt signaling pathways, were assessed. Of note, TAS9 significantly inhibited the expression of PKC β , Akt and mTOR in SMMC-7721 cells, suggesting that TAS9 may act via the PI3K/Akt signaling pathway to inhibit cell proliferation (Fig. 3A and B). In addition, the expression levels of MEK-2, RAF and JNK-1, which are representative molecules of the MAPK signaling pathway, were assessed. The results showed that the expression levels of MEK-2, RAF and JNK-1 were

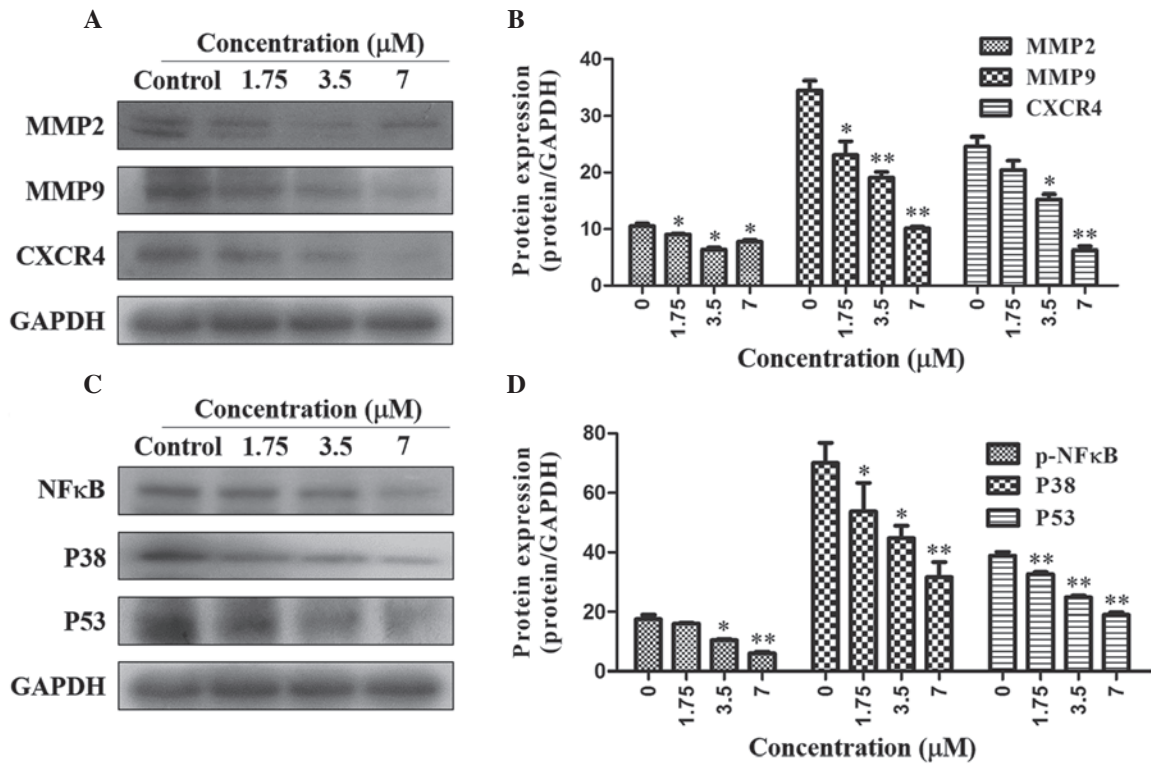


Figure 4. Effects of TAS9 on cell migration-associated signaling pathways. (A) Western blot analysis of the expression levels of MMP2, MMP9 and CXCR4 in SMMC-7721 cells treated with TAS9. (B) The bands were quantified by densitometric analysis. (C) Western blot analysis of the expression levels of NF- κ B, p38 and p53 in SMMC-7721 cells treated with TAS9. (D) The bands were quantified by densitometric analysis. Values are presented as the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01 vs. control group. MMP, matrix metalloproteinase; CXCR4, CXC chemokine receptor-4; p-NF- κ B, phosphorylated nuclear factor κ B.

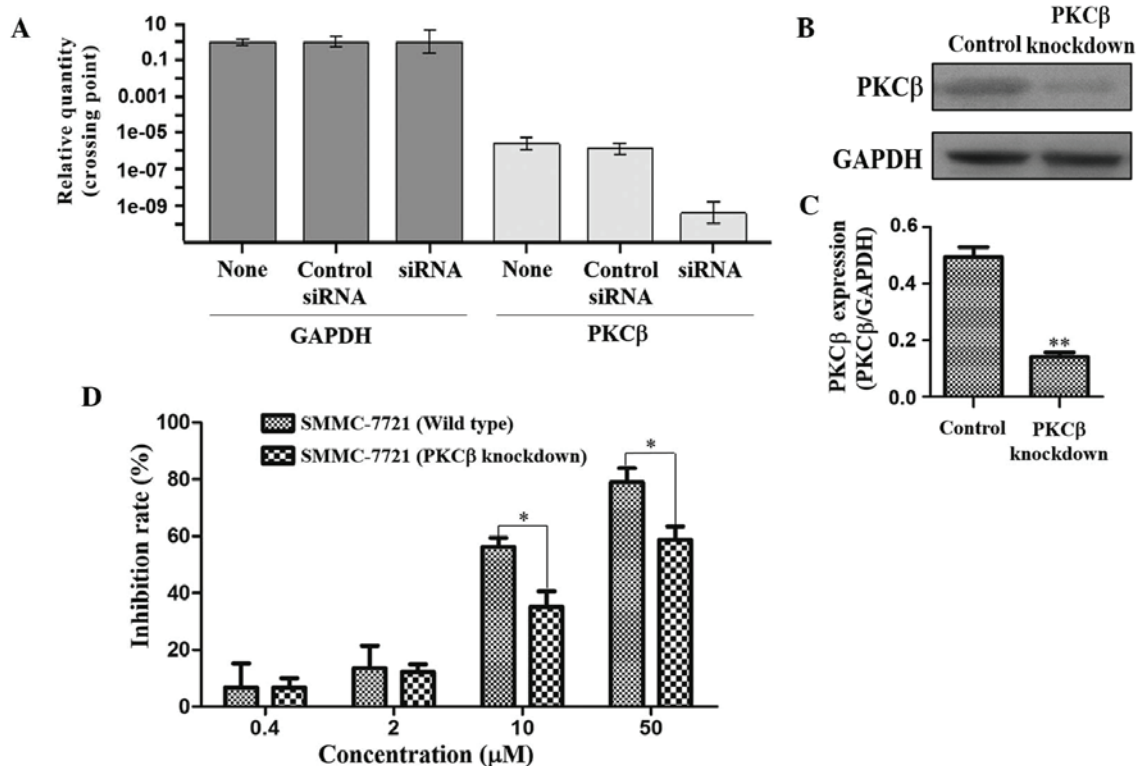


Figure 5. PKC β knockdown affects the anti-proliferative effects of TAS9 on SMMC-7721 cells. (A) mRNA expression levels of PKC β in SMMC-7721 cells transfected with 80 nM PKC β siRNA using Lipofectamine[®] 2000 and in SMMC-7721 control cells (wild-type) were determined by reverse transcription-quantitative polymerase chain reaction analysis. (B and C) Protein expression levels of PKC β in the SMMC-7721 cells transfected with 80 nM PKC β siRNA using Lipofectamine 2000. (D) Effects of TAS9 the proliferation of SMMC-7721 cells (wild-type) and knockdown cells. Values are expressed as the mean \pm standard deviation (n=3). *P<0.05 and **P<0.01, vs. the control group. siRNA, small interfering RNA; PKC, protein kinase C; None, untransfected cells.

significantly decreased in SMMC-7721 cells following treatment with TAS9 (Fig. 3C and D). Therefore, TAS-9 exerts its anti-proliferative effects by decreasing the expression of PKC β , Akt and mTOR in the PI3K/Akt signaling pathway as well as decreasing the expression of MEK-2, RAF and JNK-1 in the MAPK signaling pathway.

ETAS9 decreases cell migration-associated signaling. As shown in Fig. 4A and B, TAS9 was able to significantly inhibit MMP-2 expression; furthermore, MMP-9 expression was inhibited in a dose-dependent manner, as compared with that in the control. Furthermore, the expression of the cell migration-associated proteins CXCR4, NF- κ B, P38 and P53 was downregulated by treatment with TAS9 at the concentrations of 1.75, 3.5 and 7 μ M (Fig. 4C and D).

Knockdown of PKC β . As PKC β expression was most significantly downregulated by TAS9 amongst all proteins assessed in the present study (Fig. 3A and B), siRNA-mediated knockdown of PKC β was performed in SMMC-7721 cells in order to further elucidate its role in the mechanism of action of TAS9. The mRNA and protein expression levels of PKC β were quantified by RT-qPCR and western blot analyses in untreated, control-transfected and PKC β -knockdown SMMC-7721 cells. As shown in Fig. 5A-C, PKC β was selectively knocked down in the SMMC-7721 cells. In order to validate the roles of PKC β in SMMC-7721 cells treated with TAS9, wild-type and PKC β -knockdown SMMC-7721 cells were treated with TAS9 at various concentrations and subjected to an MTT assay. TAS9 inhibited the growth of native as well as PKC β -knockdown cells in a dose-dependent manner; however, the inhibitory effects on the SMMC-7721 PKC β -knockdown cells were markedly reduced as compared with those on the control cells (Fig. 5D). siRNA-mediated knockdown of PKC β in SMMC-7721 cells significantly attenuated the anti-proliferative effects of TAS9, suggesting that PKC β is involved in the mechanisms of action of TAS9.

Discussion

In the present study, the anti-tumorigenic effects of TAS9 on SMMC-7721 hepatocellular carcinoma cells were investigated. The anti-neoplastic effects of TAS9 may be attributed to the inhibition of cancer-associated biochemical mechanisms, including inhibition of cell proliferation, migration and invasion, as well as regulation of the corresponding signal transduction pathways. The results of the present study demonstrated that TAS9 exerts its anti-tumorigenic effects via inhibiting cell proliferation and migration in SMMC-7721 cells. TAS9 may have important roles in reducing cell survival and proliferation, as well as decreasing the migratory and invasive potential of SMMC-7721 cells.

The molecular signaling pathways associated with cellular growth are diverse and include the PI3K/AKT, MAPK and PLC γ /PKC signaling pathways (17-19). Upregulation of these pathways may cause aberrant cell proliferation. Upon activation by calcium and diacylglycerol, members of the PKC family can phosphorylate a diversity of protein targets at serine and threonine moieties to activate molecular signaling pathways (20,21). AKT regulates cell growth, proliferation,

migration and apoptosis by interacting with its numerous downstream protein targets (17). mTOR belongs to the PI3K protein family, and is an important kinase that can regulate cell growth and proliferation (18). The MAPK/ERK pathway is a junction point for numerous biochemical signaling pathways, which regulates cell proliferation, differentiation and development. JNK-1, MEK-2 and RAF are key checkpoints in these pathways (21). Furthermore, in the MAPK/ERK pathway, RAS binds to RAF and thereby activates to trigger a kinase cascade involving the phosphorylation of MRK, followed by phosphorylation of MAPK to affect cell growth and tumorigenesis (23,24). The results of the present study showed that TAS9 exerted its inhibitory effects by reducing the expression of PKC β , AKT, MEK-2, mTOR, JNK-1 and RAF, indicating TAS9 may target the PI3K/AKT, PLC γ /PKC and MAPK signaling pathways to suppress tumor progression.

The formation of metastasis mostly occurs at advanced tumor stages and requires a series of sequential events resulting in the translocation of cells from the primary tumor mass to distant sites of the body to form secondary tumors; these steps include the reduction of cell adhesion, as well as enhancement of the invasive, proliferative and vessel-forming potential of the cells (25,26). By contrast, interference with any of these steps may prevent tumor migration and metastasis. In the present study, TAS9 was demonstrated to exert inhibitory effects on cell migration by downregulating the expression of proteins associated with metastasis formation. MMP2 and MMP9 have key roles in cancer-cell invasion and metastasis; hence, inhibiting the expression of MMP2 and MMP9 is the most direct approach for anti-metastatic therapies (2,27). Furthermore, the expression of CXCR4 in cancer cells was linked to metastasis, and its inhibition may therefore block tumor migration (28). NF- κ B has important roles in numerous signaling pathways associated with cell proliferation and migration, as well as the promotion and progression of cancer (29,30). P53, which has anti-proliferative and tumor-suppressive effects, is a frequently mutated gene in human cancers; however, its overexpression may be correlated with tumor metastasis, recurrence and poor prognosis (31). Inhibition of P38 was shown to block the migration of epithelial cells (32). P53 as well as P38 are essential for cell migration, fusion and proliferation. In the present study, treatment with TAS9 reduced the protein expression levels of not only MMP-2 and MMP-9, but also CXCR4, NF- κ B, p53 and p38, indicating that TAS9 inhibited cell migration.

PKC β is important for cell survival and tumor development *in-vivo*, and inhibition of PKC β induces apoptosis and stimulates tumor necrosis factor and NF- κ B signaling (33). The results of the present study indicated a significant reduction in the protein expression levels of PKC β in SMMC-7721 cells following treatment with TAS9. To further investigate the role of PKC β , siRNA-mediated knockdown of PKC β in SMMC-7721 cells was performed, which significantly attenuated the inhibitory effects of TAS9. The inhibitory effects of TAS9 on the SMMC-7721 wild-type cells were markedly increased as compared with those on the SMMC-7721 PKC β -knockdown cells, which suggested that PKC β is an important target of TAS9.

In conclusion, the proliferative and migratory potential of SMMC-7721 hepatocellular carcinoma cells was suppressed by TAS9 via the MAPK and PI3K/AKT signaling pathways and by downregulating MMP2, MMP9, CXCR4, NF κ B, P38 and P53.

In addition, knocking down of PKC β significantly decreased the inhibitory effects of TAS9 on the proliferation of SMMC-7721 cells. These results suggested that TAS9 is a potential anti-cancer agent, which may be used for treating hepatocellular carcinoma.

Acknowledgements

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References

- Shen B, Chu ES, Zhao G, Man K, Wu CW, Cheng JT, Li G, Nie Y, Lo CM, Teoh N, *et al*: PPAR γ inhibits hepatocellular carcinoma metastases *in-vivo* and in mice. *Br J Cancer* 106: 1486-1494, 2012.
- Yodkeeree S, Chaiwangyen W, Garbisa S and Limtrakul P: Curcumin, demethoxycurcumin and bisdemethoxycurcumin differentially inhibit cancer cell invasion through the down-regulation of MMPs and uPA. *J Nutr Biochem* 20: 87-95, 2009.
- Zhen C, Chen L, Zhao Q, Liang B, Gu YX, Bai ZF, Wang K, Xu X, Han QY, Fang DF, *et al*: Gankyrin promotes breast cancer cell metastasis by regulating Rac1 activity. *Oncogene* 32: 3452-3460, 2013.
- Kim GD, Oh J, Park HJ, Bae K and Lee SK: Magnolol inhibits angiogenesis by regulating ROS-mediated apoptosis and the PI3K/AKT/mTOR signaling pathway in MES/EB-derived endothelial-like cells. *Int J Oncol* 43: 600-610, 2013.
- Song MK, Kim YJ, Song M, Choi HS, Park YK and Ryu JC: Polycyclic aromatic hydrocarbons induce migration in human hepatocellular carcinoma cells (HepG2) through reactive oxygen species-mediated p38 MAPK signal transduction. *Cancer Sci* 102: 1636-1644, 2011.
- Hidalgo M and Eckhardt SG: Development of matrix metalloproteinase inhibitors in cancer therapy. *J Natl Cancer Inst* 93: 178-193, 2001.
- Zhang XX, Fu ZY, Zhang Z, Miao C, Xu P, *et al*: Microcystin-LR promotes melanoma cell invasion and enhances matrix metalloproteinase-2/-9 expression mediated by NF κ B activation. *Environ Sci Technol* 46: 11319-11326, 2012.
- Chambers AF and Matrisian LM: Changing views of the role of matrix metalloproteinases in metastasis. *J Natl Cancer Inst* 89: 1260-1270, 1997.
- Wang C, Gao D, Guo K, Kang X, Jiang K, Sun C, Li Y, Sun L, Shu H, Jin G, *et al*: Novel synergistic antitumor effects of rapamycin with bortezomib on hepatocellular carcinoma cells and orthotopic tumor model. *BMC Cancer* 12: 166, 2012.
- Perdue G, Blomster RN, Blake DA and Farnsworth NR: South-American plants II: Taspine isolation and anti-inflammatory activity. *J Pharm Sci* 68: 124-126, 1979.
- Rollinger JM, Schuster D, Baier E, Ellmerer EP, Langer T and Stuppner H: Taspine: Bioactivity-guided isolation and molecular ligand-target insight of a potent acetylcholinesterase inhibitor from *Magnolia x soulangiana*. *J Nat Prod* 69: 1341-1346, 2006.
- Porrás-Reyes BH, Lewis WH, Roman J, Simchowicz L and Mustoe TA: Enhancement of wound healing by the alkaloid taspine defining mechanism of action. *Proc Soc Exp Biol Med* 203: 18-25, 1993.
- Itokawa H, Ichihara Y, Mochizuki M, Enomori T, Morita H, Shirota O, Inamatsu M, and Takeya K: A cytotoxic substance from *Sangre De Grado*. *Chem Pharm Bull* 39: 1041-1042, 1991.
- Zhang YM, He LC, Meng L, Luo WJ and Xu XM: Suppression of tumor-induced angiogenesis by taspine isolated from *Radix et Rhizoma Leonticis* and its mechanism of action *in vivo*. *Cancer Lett* 262: 103-113, 2008.
- Zhang YM, Jiang Q, Wang N, Dai B, Chen Y and He L: Effects of taspine on proliferation and apoptosis by regulating caspase-3 expression and the ratio of Bax/Bcl-2 in A431 Cells. *Phytother Res* 25: 357-364, 2011.
- Gao HP, Su P, Shi YL, Shen X, Zhang Y, Dong J and Zhang J: Discovery of novel VEGFR-2 inhibitors. Part II: Biphenyl urea incorporated with salicylaldehyde. *Eur J Med Chem* 90: 232-240, 2015.
- Zinda MJ, Johnson MA, Paul JD, Horn C, Konicek BW, Lu ZH, Sandusky G, Thomas JE, Neubauer BL, Lai MT, *et al*: AKT-1, -2, and -3 are expressed in both normal and tumor tissues of the lung, breast, prostate, and colon. *Clin Cancer Res* 7: 2475-2479, 2001.
- Hay N: The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* 8: 179-183, 2005.
- Faivre S, Kroemer G and Raymond E: Current development of mTOR inhibitors as anticancer agents. *Nat Rev Drug Discov* 5: 671-688, 2006.
- Philippi A, Roschmann E, Tores F, Lindenbaum P, Benajou A, Germain-Leclerc L, Marcaillou C, Fontaine K, Vanpeene M, Roy S, *et al*: Haplotypes in the gene encoding protein kinase c-beta (PRKCB1) on chromosome 16 are associated with autism. *Mol Psychiatr* 10: 950-960, 2005.
- Lintas C, Sacco R, Garbett K, Mirnics K, Militerni R, Bravaccio C, Curatolo P, Manzi B, Schneider C, Melmed R, *et al*: Involvement of the PRKCB1 gene in autistic disorder: Significant genetic association and reduced neocortical gene expression. *Mol Psychiatry* 14: 705-718, 2009.
- Chuang SM, Wang IC and Yang JL: Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. *Carcinogenesis* 21: 1423-1432, 2000.
- Luo W, Wang X, Zheng L, Zhan Y, Zhang D, Zhang J and Zhang Y: Brucine suppresses colon cancer cells growth via mediating KDR signalling pathway. *J Cell Mol Med* 17: 1316-1324, 2013.
- Balan V, Leicht DT, Zhu J, Balan K, Kaplun A, Singh-Gupta V, Qin J, Ruan H, Comb MJ and Tzivion G: Identification of novel *in vivo* Raf-1 phosphorylation sites mediating positive feedback Raf-1 regulation by extracellular signal-regulated kinase. *Mol Biol Cell* 17: 1141-1153, 2006.
- Lou L, Ye W, Chen Y, Wu S, Jin L, He J, Tao X, Zhu J, Chen X, Deng A, *et al*: Ardisin-like inhibits survival, invasion and metastasis of human hepatocellular carcinoma cells. *Phytomedicine* 19: 603-608, 2012.
- Adams LS, Phung S, Yee N, Seeram NP, Li L and Chen S: Blueberry phytochemicals inhibit growth and metastatic potential of MDA-MB-231 breast cancer cells through modulation of the phosphatidylinositol 3-kinase pathway. *Cancer Res* 70: 3594-3605, 2010.
- Chen QY, Zheng Y, Jiao DM, Chen FY, Hu HZ, Wu YQ, Song J, Yan J, Wu LJ and Lv GY: Curcumin inhibits lung cancer cell migration and invasion through Rac1-dependent signaling pathway. *J Nutr Biochem* 25: 177-185, 2014.
- Chen Y, Stamatoyannopoulos G and Song CZ: Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion *in vivo*. *Cancer Res* 63: 4801-4804, 2003.
- Shen HM and Tergaonkar V: NF kappaB signaling in carcinogenesis and as a potential molecular target for cancer therapy. *Apoptosis* 14: 348-363, 2009.
- Wang Z, Banerjee S, Li Y, Rahman KMW, Zhang Y and Sarkar FH: Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res* 66: 2778-2784, 2006.
- O'Connor PM, Jackman J, Jondle D, Bhatia K, Magrath I and Kohn KW: Role of the p53 tumor-suppressor gene in cell-cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. *Cancer Res* 53: 4776-4780, 1993.
- Sharma GD, He J and Bazan HE: p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: Evidence of cross-talk activation between MAP kinase cascades. *J Biol Chem* 278: 21989-21997, 2003.
- Surdez D, Benetkiewicz M, Perrin V, Han ZY, Pierron G, Ballet S, Lamoureux F, Rédini F, Decouvrelaere AV, Daudigeos-Dubus E, *et al*: Targeting the EWSR1-FLI1 oncogene-induced protein kinase PKC β abolishes ewing sarcoma growth. *Cancer Res* 72: 4494-4503, 2012.