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In vitro and *in vivo* evaluation of *N*,*N*,*N*-trimethylphytosphingosine-iodide (TMP) in liposomes for the treatment of angiogenesis and metastasis

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ABSTRACT

Phytosphingosine and methyl derivatives are important mediators on cellular processes, and are associated with cell growth and death. The antitumor activity of *N*,*N*,*N*-trimethylphytosphingosine-iodide (TMP) as a novel potent inhibitor of angiogenesis and metastasis was evaluated in B16F10 murine melanoma cells. The results indicated that TMP itself effectively inhibited *in vitro* cell migration, tube formation, and the expression of angiogenic factors as well as *in vivo* lung metastasis. However, TMP slightly suppressed *in vivo* experimental tumor metastasis in its free form and induced side effects including hemolysis and local side effects. Therefore, in an attempt to reduce the toxicity and the undesirable side effects of TMP, a liposomal formulation was prepared and tested for its effectiveness. TMP liposomes retained the effectiveness of TMP *in vitro* while side effects were reduced, and both *in vivo* experimental and spontaneous tumor metastasis were significantly suppressed. These results support the conclusion that TMP effectively inhibits *in vitro* angiogenesis as well as *in vivo* metastasis, and a liposomal formulation is more efficient delivery system for TMP treatment than solution.

1. Introduction

Angiogenesis is the process by which blood vessels are formed from pre-existing vessels for wound repair, the development of granulation tissue and tumors (Folkman, 1971; Ossowski and Reich, 1983). Various studies dealing with tumor angiogenesis have pointed out that tumor expansion with the formation of new blood vessels and tumor regression due to angiogenic inhibitors are correlated with metastasis (Holash et al., 1999; Hood et al., 2002; Weidner et al., 1991, 1993). During the process of tumor angiogenesis, highly permeable blood vessels are formed in and around solid tumors, thus providing an efficient translocation route for tumor cells to leave the primary tumor site, possibly inducing metastasis (Zetter, 1998). Therefore the use of angiogenic inhibitors for the suppression of metastasis has been an area of considerable interest (Ebos et al., 2009; Konno et al., 1995; O'Reilly et al., 1994; Pàez-Ribes et al., 2009). Sphingolipids and their metabolites, such as ceramide, sphingosine, sphingosine-1-phosphate (S1P) and phytosphingosine, have been identified as important mediators of cellular processes, in particular, cell proliferation, differentiation, senescence and apoptosis (Merrill, 2002; Spiegel and Merrill, 1996; Spiegel and Milstien, 2002; Woodcock, 2006). For instance, it has been reported that sphingosine (SPN) is a negative modulator of transmembrane signaling through protein kinase C (PKC) as well as an inhibitor of sphingosine kinase-1 (SK-1), which is implicated in cell growth and inhibitory apoptosis (Cuvillier and Levade, 2001; Hannun and Bell, 1989). In addition, the inhibitory effect of SPN derivatives on metastatic potential has also been reported (Okoshi et al., 1991). Interestingly, *N*,*N*,*N*-trimethylsphingosine (TMS) showed a much stronger inhibitory effect on PKC activity than *N*,*N*-dimethylsphingosine (DMS) and unsubstituted SPN (Endo et al., 1991). Phytosphingosine, which is similar in structure to sphingosine, is a major component of membranes produced by plants, fungi, mammalian tissue and some types of cancer cells (Jo et al., 2003). The inhibitory effects of synthetic phytosphingosine derivatives (*N*-monomehtylphytosphingosine and *N*,*N*-dimehtylphytosphingosine) on SK-1 activity were recently reported to be stronger than that of DMS (Park et al., 2010). Moreover phytosphingosine and its methyl derivatives induce the apoptotic cell death of cancer cells through ROS generation, caspase activation and Bax translocation (Kim et al., 2009; Park

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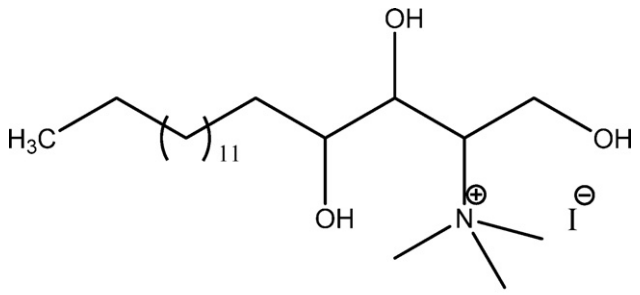

Fig. 1. Chemical structure of *N,N,N*-trimethylphytosphingosine-iodide (TMP).

et al., 2003, 2010). However, the anti-angiogenic and metastatic roles of phytosphingosine and its methyl derivatives are largely unknown in spite of their potential as anticancer cellular modulators.

Clinical studies of SPN derivatives indicate that they have a number of undesirable side effects such as hemolysis, hemoglobinuria, and inflammation at the injection sites, but that they also have potent inhibitory effects on tumor growth and metastasis (Endo et al., 1991). To reduce magnitude of the side effects and enhance the activities of SPN derivatives in *in vivo* systems, the use of liposomes that contain SPN derivatives as drug delivery carriers have been proposed (Park et al., 1994). It is well known that, when liposomes are used as carriers, the systemic circulation time is prolonged and drug accumulation in cancer tissue is increased, either through surface modification or lipid composition of liposomes, and that the side effects of a drug are reduced (Gabizon et al., 1982; Gabizon, 1995; Matsuo et al., 2001; Song et al., 2009).

N,N,N-Trimethylphytosphingosine-iodide (TMP, Fig. 1) was recently prepared for use as an antitumor agent (Namgoong and Park, 2003). In the present study, we evaluated the *in vitro* anti-angiogenic and *in vivo* anti-metastatic activities of TMP. In addition, the mechanism of its action was investigated. However, when TMP was used in *in vivo* systems in preliminary experiments, it was observed to cause side effects similar to TMS (data not shown). Therefore, a liposomal formulation was prepared, in an attempt to reduce the toxicity and undesirable side effects of TMP. The findings herein indicate that TMP is a potent inhibitor of angiogenesis and metastasis, and that liposomes that contain TMP retain these properties while reducing side effects during *in vivo* treatment.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N,N,N*-Trimethylphytosphingosine-iodide (TMP) was synthesized and donated by the Phytos Co. (Suwon, South Korea) (Fig. 1) (Namgoong and Park, 2003). For cell culture, Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM) and RPMI Medium 1640 (RPMI1640) and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). All other materials were used without further purification.

2.2. Synthesis of N,N,N-trimethylphytosphingosine-iodide (TMP)

Phytosphingosine (300 mg, 0.946 mmol), iodomethane (0.298 ml, 4.73 mmol) and K_2CO_3 (523 mg, 3.79 mmol) as a protector of hydroxyl groups were dissolved in 3 ml of methanol, and the mixture was stirred for 4 h at 50 °C. The solvent was evaporated under reduced pressure and 4 ml of distilled water was added to the resulting mixture. The solution was extracted with 8 ml of ethyl acetate, dried over Na_2SO_4 and filtered. The ethyl acetate was evaporated to give 260 mg of a white solid. The chemical structure and mass of TMP were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR and MS (FAB, Glycerol, *m/z*) spectra. The yield of product was 76% and the material was stored at −20 °C until used. IR (KBr) υ_{max}: 3009 (OH), 2918, 2850 (C–H) cm^{-1}. ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.95 (dd, 1H, CH_2O, J = 14.4 Hz), 3.89 (dd, 1H, CH_2O, J = 14.4 Hz), 3.76 (d, 1H, J = 8.7 Hz), 3.6 (dd, 1H), 3.11 (s, 9H, N^+CH_3), 1.68 (m, 1H, CH_2), 1.48 (m, 1H, CH_2), 1.23 (s, 24H, CH_2), 0.84 (t, 3H, CH_3) ppm. ^{13}C NMR (600 MHz, DMSO-d_6): δ 76.80, 71.01, 55.69, 52.18, 33.21, 31.21, 30.60, 29.15, 29.03, 28.99, 28.93, 28.62, 24.87, 22.00, 13.83 ppm. MS (FAB) calcd for TMP: 361 [M^+]) (Namgoong and Park, 2003).

2.3. Preparation and characterization of liposomes

Unless otherwise stated, the liposomes were composed of DPPC, CHOL (1:1 molar ratio) and various concentrations of TMP (from 0 to 400 μM). The liposomes were prepared by the thin film hydration method. Briefly, the lipids were dissolved in chloroform, and the solvent subsequently removed under reduced pressure at 50 °C, using a rotary evaporator (Buchi Rotavapor R-200, Switzerland). The resulting lipid film was hydrated with phosphate-buffered saline (pH 7.4) and then gently mixed. Each liposomal solution was extruded 5 times through a 200 nm polycarbonate filter, followed by five extrusions through a 100 nm polycarbonate filter (Whatman, USA) using an extruder (Northern Lipids Inc., USA). The particle size and zeta potential of the liposomes were determined by ELS-Z Electrophoretic light scattering particle size and zeta potential analyzer (ELS-Z, OTSUKA Electronics Co. Ltd., Japan) at room temperature. The particle size, poly dispersity and zeta potential of the TMP liposomes are shown in Table 1.

2.4. In vitro cytotoxicity studies

Cell viability, after the addition of TMP and TMP liposomes, was determined by using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Mosmann, 1983). Briefly, 5×10^3 cancer cells were cultured in 96-well flat-bottomed microtiter plates for 24 h at 37 °C in order to evaluate the cytotoxicity of TMP or TMP liposomes. Human cancer cell lines (HepG2, MCF7, A549 and A375P) and murine cancer cell line (B16F10) were used in this experiment. The medium was replaced with serum-free medium containing various concentrations of TMP or TMP liposomes, and subsequently cultured for 24 h. And then, MTT (5 mg/ml, 20 μl) was added to each well and the cells were incubated for another 4 h at 37 °C in a humidified 5% CO_2 atmosphere. A 190 μl aliquot was removed from each well, and then 150 μl of DMSO was added to solubilize the cells. Cell viabilities were then determined using a microplate reader (Emax, Molecular devices, Sunnyvale, CA) at 560 nm.

Table 1
Physical properties of TMP liposomes.

Concentration of TMP (μM)	Size (nm)	Poly dispersity	Zeta potential (mV)
0	155.16 ± 22.45	0.17 ± 0.07	−2.97 ± 5.77
400	152.23 ± 7.27	0.12 ± 0.05	−0.27 ± 4.84

Table 2
IC_{50} of TMP and TMP liposomes.

Name	Origin	Species	TMP IC_{50} (μM)	TMP Liposomes IC_{50} (μM)
HepG2	Liver	Human	26.61 ± 1.93	>400
MCF7	Breast	Human	14.42 ± 0.58	>400
A549	Lung	Human	15.28 ± 0.50	>400
A375P	Melanoma	Human	12.97 ± 0.49	372.46 ± 42.51
B16F10	Melanoma	Murine	33.34 ± 1.03	>400

2.5. Hemolytic effect of TMP or TMP liposomes

The hemolytic effect of TMP or TMP liposomes in rat blood was determined based on the increased absorbance due to the release of hemoglobin from lysed erythrocytes (Park et al., 1994; Yamaji et al., 1998). Briefly, 500 μl of fresh rat whole blood treated with heparin was incubated with various concentrations of TMP (from 0 to 2 mM) or TMP liposomal solutions at 37 °C for 30 min. After incubation, the mixture was centrifuged at 500 × *g* for 5 min to remove erythrocytes. An aliquot (200 μl) of supernatant was collected, and the absorbance due to the hemoglobin released from lysed erythrocytes was measured using a microplate reader (Emax, Molecular devices, Sunnyvale, CA) at 405 nm. Total hemoglobin concentration as reference was determined after freezing and thawing a sample of whole rat blood.

2.6. In vitro cell migration studies

The inhibition of cell migration was measured by using similar methods previously (Decaestecker et al., 2007; Liang et al., 2007). Briefly, B16F10 cells (1×10^5 cells/well) were cultured on 24 well plates at 37 °C in a humidified 5% CO_2 atmosphere until 90% confluency was reached. The cells were washed with PBS and a scratch was made on the cell monolayer by scrapping with a sterile pipette tip. Cells were washed with PBS again, followed by treatment with various concentrations of TMP (2 and 10 μM) or TMP (0, 100, 200 and 400 μM) liposomes in 1 ml of DMEM (1% serum) medium and then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. After 24 h, the medium was replaced by fresh conditioning medium, followed by 24 h incubation. After this treatment, unattached cells were washed out by rinsing with PBS and the attached cells were fixed in 2% paraformaldehyde. The cell migration from the wound edge was visualized under a reverse phase microscope and area of cell migration was analyzed using the Image Pro Plus (Ver 6.0) software. Experiments were done in triplicates, and the representative images were chosen.

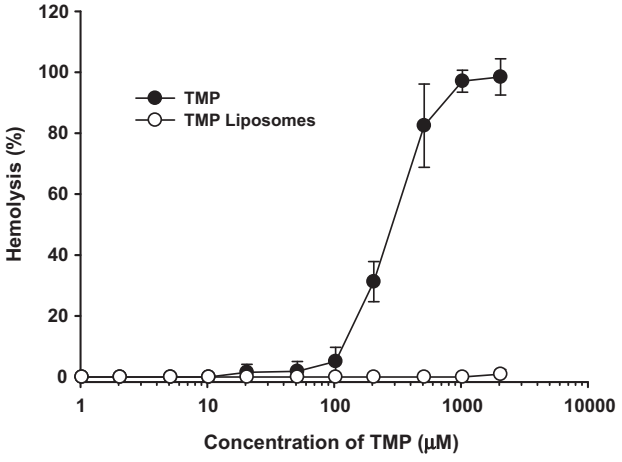

Fig. 2. TMP-induced hemolysis. Heparin-treated rat blood was incubated with various concentrations of TMP (●) or TMP liposomes (○) at 37 °C for 30 min. The hemolysis percentage was determined by measuring the amount of hemoglobin released at 405 nm.

2.7. Tube formation assay

The tubular structure of human umbilical vein endothelial cells (HUVECs) was evaluated by means of a tube formation assay (Abdollahi et al., 2003). Matrigel (250 μl) (10 mg/ml) (BD Biosciences, New Jersey) was polymerized for 30 min at 37 °C. HUVECs were suspended in M199 (5% FBS) medium (Sigma Chemical Co., St. Louis, MO, USA) at a density of 2.5×10^5 cells/ml, and 0.2 ml of cell suspension was added to each well that had been coated with Matrigel, together with the indicated concentrations of TMP or TMP liposomes, followed by incubation at 37 °C in a humidified 5% CO_2 atmosphere for 14 h. Morphological changes of the cells and HUVEC tubes formation were observed under a phase-contrast microscope and photographed at 40× magnification.

2.8. In vivo anti-metastatic activity studies

To evaluate the anti-metastatic effect of TMP and TMP liposomes, two lung metastasis models, *i.e.*, experimental metastasis model (intravenous injection of tumor cells) and spontaneous metastasis model (subcutaneous injection of tumor cells), were used following a previous report (Park et al., 1994). The experimental metastasis model was produced by injection of B16F10 murine melanoma cells (5×10^4) in 100 μl serum-free media into the lateral tail veins of C57BL/6 mice. They were administrated with various doses of TMP (0.4 and 2 mM) or TMP (0.4 and 2 mM) liposomes in 200 μl of serum-free media at 15 min, 5 and 10 days after tumor inoculation, respectively. On day 18, the lungs were harvested and fixed in 2% paraformaldehyde solutions after the animals had been sacrificed with carbon dioxide. The colony numbers in the lungs were compared after dissection to facilitate the measurements. The spontaneous metastasis model was produced by injection of B16F10 cells (2×10^5) in 40 μl of serum-free media into the footpad (subcutaneous injection) of C57BL/6 mice, and TMP (2 mM) or TMP (2 mM) liposomes were administrated on days 5, 10, 15, 20 and 25 after tumor inoculation, respectively. The primary tumors in feet of mice were excised on day 21. After the animals were sacrificed with carbon dioxide on day 38, the lungs were harvested and fixed in 2% paraformaldehyde solutions. The numbers of colonies in lungs were compared after dissection to facilitate the measurement. These experiments were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University.

2.9. Western blot analysis

Whole-cell extracts of B16F10 cells, which were incubated with various concentrations of TMP (2, 10 and 25 μM) or TMP (100, 200 and 400 μM) liposomes for 24 h, were prepared in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Thermo Scientific, Rockford, IL, USA). Protein concentrations were then determined using a bicinchoninic acid (BCA) protein assay kit following the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO, USA). Samples (50 μg of total protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., MA, USA). Protein transfer was checked by Ponceau S solution staining (Sigma Chemical Co., St. Louis, MO, USA). After blocking with 5% skim milk in phosphate buffered saline, the blots were incubated with specific antibodies including VEGF (Novus Biologicals, Littleton, CO, USA), MMP-2 (Santa Cruz Biotechnology, Inc., CA, USA), and β-actin (Abcam, Cambridge, UK), followed by the secondary antibody conjugated to horseradish peroxidase and detected by an enhanced chemiluminescence (ECL) reagent. The secondary antibodies and

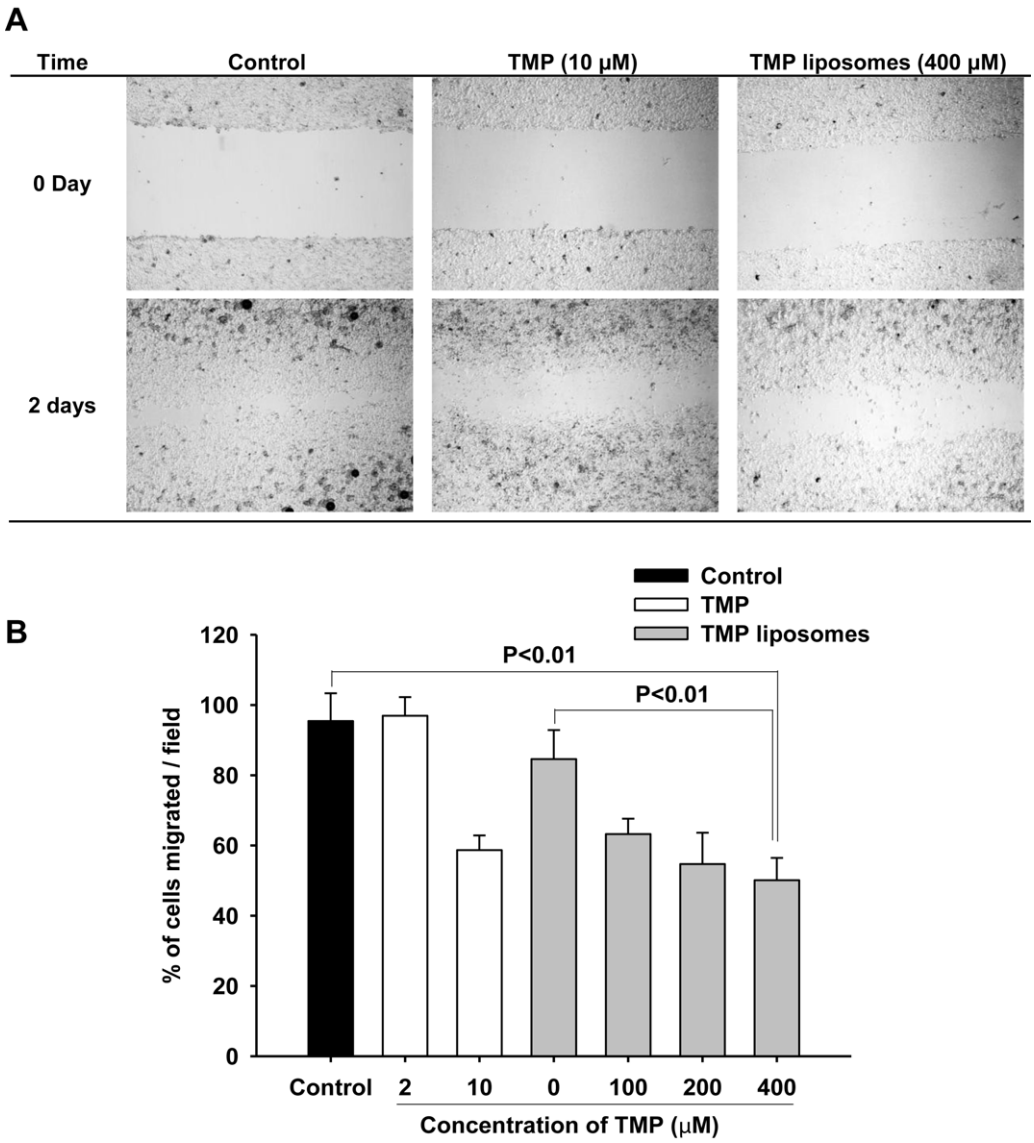

Fig. 3. *In vitro* cell migration studies. B16F10 cells (1×10^5 cells/well) were cultured reaching 90% confluence. The cells were incubated with various concentrations of TMP (TMP or TMP liposomes) for 2 days after creating a scratch on the cell monolayer by scraping with a sterile pipette tip. (A) Wound edge morphology was measured using reverse-phase microscopy (100× magnification). (B) The percent cell migration/field was evaluated from the cell migration area after 2 days.

ECL reagent were purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.10. Statistics

Values were presented as the mean ± standard deviation (S.D.). The Student's *t*-test was used to determine statistical significance between pairs of samples. A *p*-value below 0.05 was considered to be significant.

3. Results

3.1. Effects of TMP and TMP liposomes on cytotoxicity and hemolysis

The IC_{50} values for TMP or TMP liposomes on five-cancer cell lines are summarized in Table 2. We observed significantly lower cell cytotoxicity for TMP liposomes than for TMP on all five-cancer cell lines. Additionally, the sensitivity of B16F10 murine melanoma cells was lower compared to the other cell lines. These results indicate that, although TMP itself exhibits a severe cytotoxicity, liposomal formulations effectively reduce the cytotoxicity on both human and murine cancer cell lines. B16F10 cell line was used to evaluate the anti-metastatic effect of TMP and TMP liposomes due to their highly invasive malignant properties as well as their lower cytotoxic sensitivity compared to other cell lines.

After inoculating mice with high concentrations of TMP solutions (2 mM) through the tail vein, TMP induced hemoglobinuria and a local inflammation response (data not shown). As shown in Fig. 2, TMP did not induce hemolysis at concentrations of up to 100 μM, but erythrocytes were completely lysed in concentrations of 1 mM TMP and above. A hemolytic effect of TMP at concentrations of up to 2 mM was not observed in the case of treatment with TMP liposomes.

3.2. Inhibition of cell migration by TMP or TMP liposomes

Cell migration in metastasis, tissue invasion and cancer cell scattering are important markers for evaluating malignant tumors. We investigated the migration of B16F10 melanoma cells using the

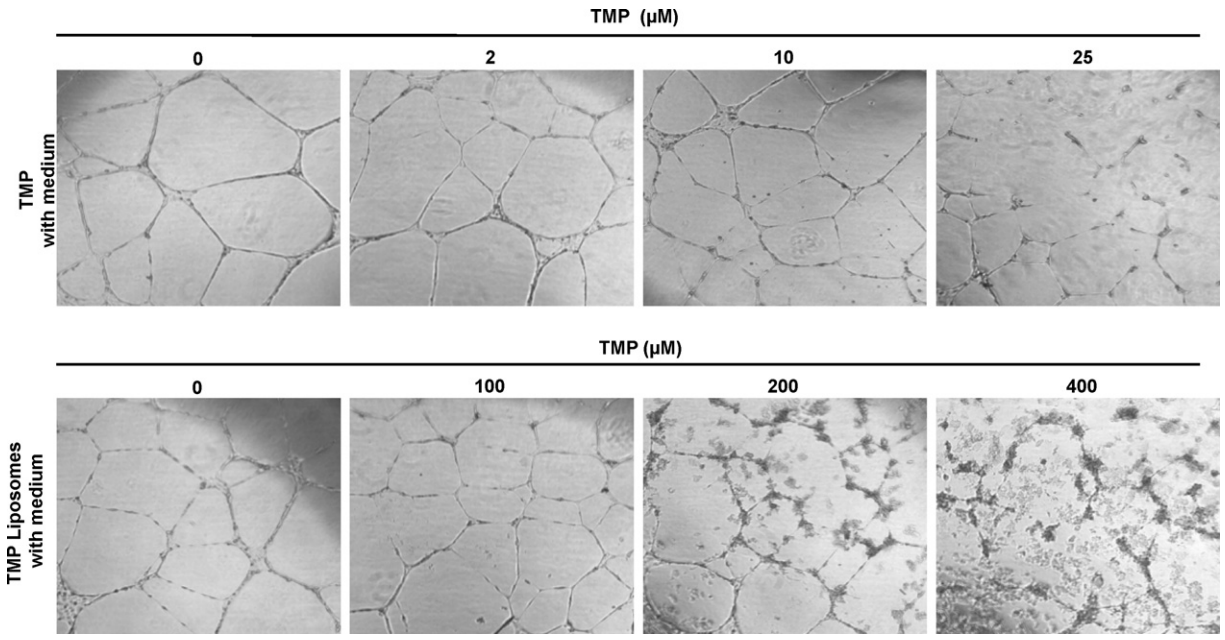

Fig. 4. Behavior of endothelial cells (HUVECs) after treatment with TMP (2, 5, 10 and 25 μM) or TMP liposomes (0, 100, 200 and 400 μM TMP).

scratch wound assay after treatment with TMP or TMP liposomes. Fig. 3A shows the morphology of the B16F10 cell monolayers after scraping with a sterile pipette (0 day), and those after 2 days of treatment with 10 μM TMP, liposomes containing 400 μM TMP in DMEM media and no treatment (control). After 48 h, B16F10 cells cultured in the absence of TMP (control and 0 μM TMP liposomes) or in the presence of 2 μM TMP solution had recolonized nearly 100% of the wound. However, B16F10 cells cultured in the presence of 10 μM TMP solution as well as 100, 200 and 400 μM TMP liposomes showed a significantly reduced wound healing of about 40–50% (Fig. 3B).

3.3. Reduction of tube formation

The production of new blood vessels is an essential process in angiogenesis. Therefore, we evaluated the inhibitory effects of TMP or TMP liposomes on HUVEC tube formation. As shown in Fig. 4, HUVECs that had been treated with the TMP solution formed tubular structures up to a concentration of 10 μM, although the formation of capillary networks was slightly reduced. When HUVECs were incubated with TMP solutions at concentrations above 25 μM, no capillary-like network could be detected. HUVECs treated with TMP liposomes containing levels of TMP up to 200 μM formed capillary-like networks. But the luminal structure of HUVECs treated with TMP (400 μM) liposomes was morphologically altered and formed fewer as well as weaker tubes when compared to that of HUVECs treated with TMP (0 μM) liposomes.

3.4. The effect of TMP and TMP liposomes on in vivo lung metastasis

As mentioned above, TMP inhibits tumor migration in B16F10 melanoma cells and tube formation in HUVECs, and the efficacy of the inhibition is dependent on the concentration of TMP. We also examined the issue of whether TMP inhibits tumor metastasis. To investigate the efficacy of TMP and TMP liposomes in an *in vivo* lung metastasis model, B16F10 cells were utilized. In experimental metastasis (Fig. 5), the median number of lung nodules after treatment with TMP (400 μM) or TMP (400 μM) liposomes was not significantly different from that of PBS. The number of lung

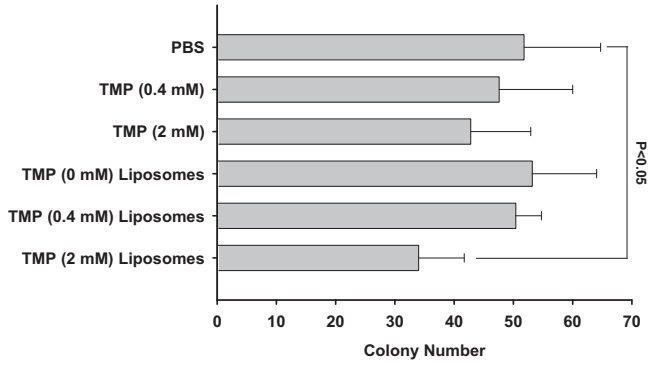

Fig. 5. Inhibitory effect of TMP and TMP liposomes in treating experimental metastasis. TMP or TMP liposomes were injected intravenously at 15 min, 5 and 10 days after inoculation of B16F10 cells *via* the tail vein in mice. Numbers of lung colonies were counted on day 18. The count of the number of metastatic colonies on the lung. The data represent the mean ± S.D ($n = 5$).

nodules after treatment with TMP (2 mM) liposomes was significantly reduced comparison with that of PBS, and treatment with TMP (2 mM) resulted in slight reduction in the number of nodules compared to that of PBS. For the treatment of spontaneous metastasis, TMP (2 mM) or TMP (2 mM) liposomes were injected through tail vein after inoculation of the footpad in mice with tumor cells. Both TMP (2 mM) and TMP (2 mM) liposomes significantly suppressed tumor metastasis compared to that of PBS and TMP (0 μM) liposomes (Fig. 6). In addition, no notable side effects were observed for the TMP (2 mM) liposomes, while severe local tissue damage was clearly observed when TMP (2 mM) solutions were used.

3.5. Cellular mechanism of TMP

The previous results showed that TMP and TMP liposomes inhibit not only tumor migration and the formation of tubular structures *in vitro*, but also *in vivo* lung metastasis. Vascular endothelial growth factor (VEGF) is a major signal protein that induces not only angiogenesis, but also vasculogenesis (Kim et al., 1993). MMP-2 is also associated with various cell behaviors, including cell migration and angiogenesis (Sang, 1998; Zheng et al., 2006). To better

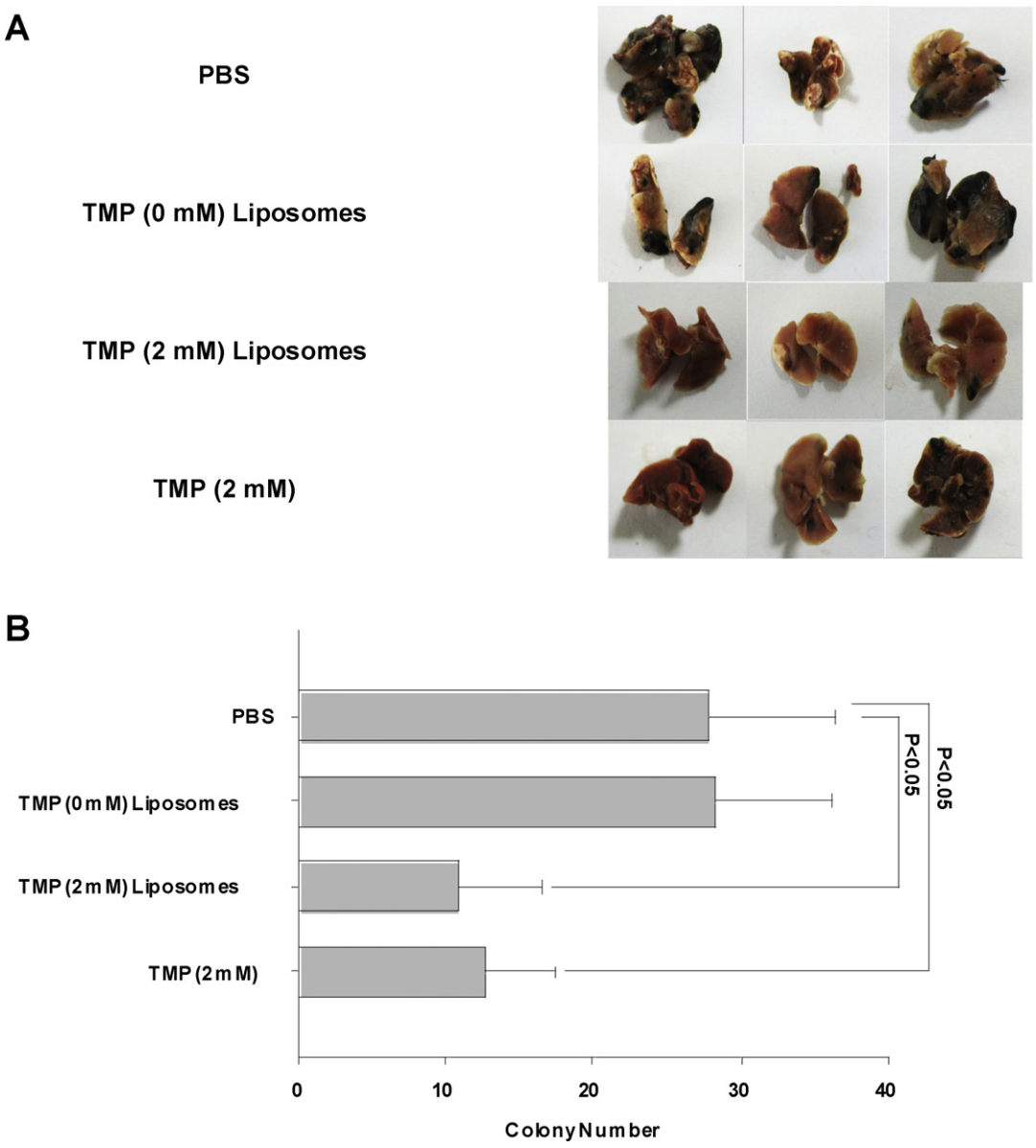

Fig. 6. Inhibitory effect of TMP and TMP liposomes in treating spontaneous metastasis. TMP or TMP liposomes were injected intravenously at 5, 10, 15, 20 and 25 days after inoculation of B16F10 cells *via* the footpad in mice. Lung colonies were counted on day 38. (A) Image for metastatic colonies on the lungs (representative images). (B) The number of metastatic colonies on the lung. The data represent the mean ± S.D ($n = 5$).

understand these relationships, we determined whether TMP or TMP liposomes inhibited the expression of VEGF and MMP-2 by western blotting. Western blot analysis showed that incubation with TMP and TMP liposomes inhibited the activity of VEGF and MMP-2 (Fig. 7). Moreover the expression of VEGF and MMP-2 was dependent on the concentration of TMP.

4. Discussion

Since the tumor metastasis is closely associated with angiogenesis, the focus of much of the current cancer research has been on anti-angiogenic compounds (Folkman, 1971; Rosen, 2000). Sphingolipids and their metabolites have been extensively studied as important mediators of cellular processes. Previous studies demonstrated that phytosphingosine derivatives induce apoptotic cell death through various cell signaling pathways (Kim et al., 2009; Park et al., 2003, 2010). Nevertheless, the physiological role of TMP, which is a phytosphingosine derivative, in tumor metastasis remains largely unknown. In this report, anti-angiogenic activities such as the inhibition of proliferation and cell migration and the suppression of tube formation by TMP were explored. Our results show that 10 μM TMP significantly suppresses the migration of B16F10 melanoma cells compared to the control (Fig. 3). The findings also show that TMP inhibits the formation of tubular structures at concentrations above 25 μM (Fig. 4). Although it is known that TMP plays an important role in tumor anti-angiogenic activity, its clinical application was difficult due to side effects including severe cell cytotoxicity, hemolysis and local inflammation in mice after intravenous injection (preliminary experiment, data not shown). Therefore, utilizing the well-known ability of liposomal formulations to enhance cellular uptake and reduce side effects (Gabizon et al., 1982; Gabizon, 1995; Matsuo et al., 2001), we used liposomes as a delivery carrier for clinical trials in an attempt to inhibit tumor metastasis by TMP.

We investigated the anti-angiogenic activities of TMP and TMP liposomes, and found evidence that they successfully inhibit

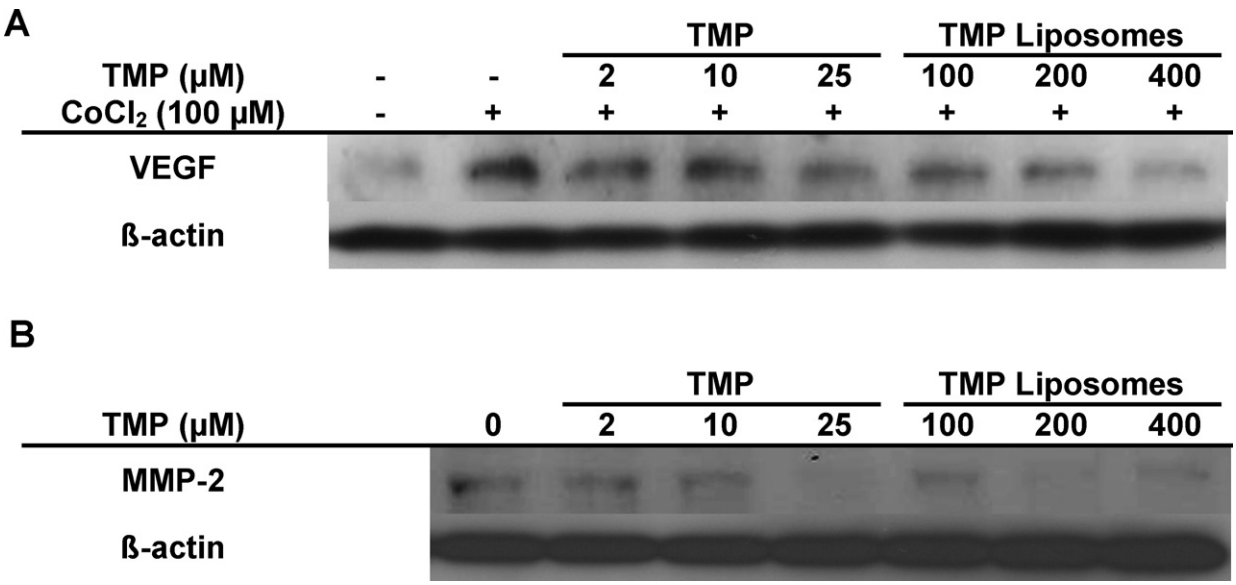

Fig. 7. Expression of VEGF (A) and MMP-2 (B) in B16F10 melanoma cells after treatment with TMP or TMP liposomes for 24 h, as determined by western blot.

cell migration and tube formation (Figs. 3 and 4). Additionally, TMP liposomes inhibited the migration of B16F10 cells in a concentration-dependent manner up to a TMP concentration of 400 μM, and no negative effects on culture growth were observed. Our results show that TMP and TMP liposomes effectively suppress angiogenic activities, and have inhibitory effects on *in vivo* tumor metastasis. In experimental tumor metastasis, the number of lung colonies in mice after intravenous administration of TMP (400 μM) or TMP (400 μM) liposomes was not significantly different compared to that of the control. However, TMP (2 mM) liposomes significantly suppressed the formation of lung colonies in mice when compared to the control, while TMP (2 mM) solution reduced lung colony formation, however not significantly (Fig. 5). This may be due to the fact that the suppression of metastasis by TMP or TMP liposomes was insufficient in experimental tumor metastasis model since tumor cells were directly introduced into the bloodstream *via* intravenous administration (Park et al., 1994). On the other hand, both TMP (2 mM) and TMP (2 mM) liposomes significantly reduced the number of lung colonies in mice in the spontaneous tumor metastasis model (Fig. 6). Since tumor cells were injected into the foodpads by subcutaneous injection, TMP could show the inhibition of metastatic effect more efficiently. Although it is also interesting to note that inhibitory effects on tumor metastasis by TMP and TMP liposomes were not significantly different at the 2 mM concentration. *In vivo* administration of TMP (2 mM) solution was not feasible due to undesirable side effects such as local thrombophlebitis and severe tissue damage as well as hemoglobinuria caused by hemolysis (data not shown), while TMP liposome did not show any notable side effects at the same concentration. Thus, a liposomal formulation could be a suitable system for delivering a high dosage of TMP and successful inhibit metastasis.

TMP might slightly offset the cell-signaling pathways of angiogenesis and inhibit metastasis in a highly metastatic B16F10 cell line. Previous studies suggested that various cancer cell lines are able to secrete pro-angiogenic factors such as VEGF and MMP-2. Thus, we investigated whether TMP and TMP liposomes could counteract the angiogenic factors of B16F10 cells. In the western blot assay, we found that TMP and TMP liposomes were able to slightly suppress the expression of VEGF and MMP-2 by B16F10 cells (Fig. 7). These results also indicate that the inhibitory effects of TMP on VEGF and MMP-2 expression are concentration-dependent.

5. Conclusion

The results of this study indicate that TMP exhibits significant anti-angiogenic and metastatic activities on B16F10 cells, and that this occurs by reducing the expression of angiogenic factors such as VEGF and MMP-2. However, TMP has undesirable side effects and low *in vivo* anti-metastatic activities. The use of liposomal carriers can deliver high dosage of TMP (up to 2 mM) with reduced cytotoxicity and side effects of TMP while at the same time these delivery systems can enhance the anti-angiogenic and metastatic activities of TMP both *in vitro* and *in vivo*. The findings reported here suggest that TMP is a potential inhibitor of tumor angiogenesis as well as metastasis, and that liposomal formulation of TMP can be an effective delivery system for this compound.

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