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Systemically Administered RNAi Molecule Sensitizes Malignant Pleural Mesothelioma Cells to Pemetrexed Therapy

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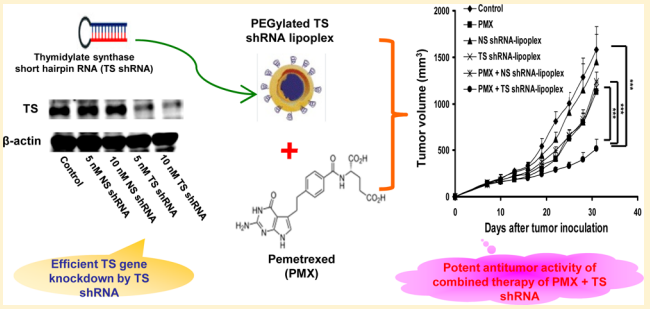
Supporting Information

ABSTRACT: Pemetrexed (PMX) is a key drug for the management of malignant pleural mesothelioma (MPM). However, its therapeutic efficacy is cruelly restricted in many clinical settings by the overexpression of thymidylate synthase (TS) gene. Recently, we emphasized the efficacy of locally administered shRNA designed against TS gene in enhancing the cytotoxic effect of PMX against orthotopically implanted MPM cells in tumor xenograft tumor model. Herein, we explored the efficiency of systemic, rather than local, delivery of TS RNAi molecule in sensitizing MPM cells to the cytotoxic effect of PMX. We here designed a PEG-coated TS shRNA-lipoplex (PEG-coated TS shRNA-lipoplex) for systemic injection. PEG modification efficiently delivered TS shRNA in the lipoplex to tumor tissue following intravenous administration as indicated by a significant suppression of TS expression level in tumor tissue. In addition, the combined treatment of PMX with systemic injection of PEG-coated TS shRNA-lipoplex exerted a potent antitumor activity in a s.c. xenograft tumor model, compared to a single treatment with either PMX or PEG-coated TS shRNA-lipoplex. Metastasis, or the spread, of mesothelioma substantially dedicates the effectiveness of treatment options. The systemic, in addition to local, delivery of tumor targeted anti-TS RNAi system we propose in this study might be an effective option to extend the clinical utility of PMX in treating malignant mesothelioma.



KEYWORDS: *malignant pleural mesothelioma, PEG-coated shRNA-lipoplex, pemetrexed, short hairpin RNA, thymidylate synthase*

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a locally invasive tumor mostly associated with past exposure to asbestos.[1−3] Treatment strategies of MPM range from traditional chemotherapy to radical procedures to remove the cancerous tissue. However, these therapeutic strategies showed poor prognosis due to either the development of drug resistance or the dissemination of the tumor to neighboring areas leading to "cancer metastasis".[4,5]

Pemetrexed (PMX), a relatively novel multitargeted antifolate, is able to simultaneously inhibit multiple folate-dependent enzymes involved in the synthesis of purines and pyrimidines. PMX, as a single gent, has shown modest activity in a phase II trial of patients with MPM,[6] and more recently, it has been approved in combination with cisplatin as first-line treatment of MPM.[7] However, the overall therapeutic outcome of patients with MPM remains very poor with response rates of approximately 40%. Many reports have emphasized a correlation between high expression levels of thymidylate synthase (TS), an essential precursor for DNA synthesis,[8,9] and reduced sensitivity to PMX in different types of tumors including colon, lung, and advanced breast cancer.[10−13]

Recently, we investigated the efficacy of chemically synthesized short hairpin RNA targeting TS (TS shRNA) in suppressing the TS expression and thereby modulating the cytotoxic effect of PMX against human mesotheliomal cell line, MSTO-211H. In that study, we showed that intrapleural "local" injection of non-PEGylated TS shRNA-lipoplexes repressed the expression of TS mRNA in human malignant mesotheliomal MSTO-211H cells and suppressed tumor progression. In addition, the combined therapy of TS shRNA-lipoplex and PMX significantly suppressed the progression of orthotopic thoracic tumors, in tandem with prolonging the survival of mice

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inoculated orthotopically with MSTO-211H cells, compared to a single treatment with either PMX or TS shRNA-lipoplex.[14]

It is worth noting that MPM cells are not confined in the thoracic cavity but that they have the potential to disseminate not only to neighboring tissues/organs such as regional lymph nodes, pericardium, peritoneum, and chest wall but much farther to reach the brain.[15,16] In addition, the therapeutic efficacy of intrapleurally administered therapeutic agents might be compromised, on the one hand, by the widespread distribution of thoracic tumors throughout the whole pleura and nearby tissues/organs and, on the other, by the excessive pleural effusion associated with MPM.[17] In this study, therefore, we focused on evaluating the effectiveness of systemic administration of TS shRNA-lipoplex in sensitizing the mesothelioma cells to the cytotoxic effect of PMX, using a subcutaneous xenograft mouse model. For systemic administration purpose, the composition of liposome of TS shRNA-lipoplex was manipulated to obtain PEG-coated TS shRNA-lipoplex. Inclusion of cholesterol, which has a membrane stabilizing effect, within liposomal membrane and grafting the hydrophilic polymer polyethylene glycol (PEG) onto the surface of liposomes were applied in order to enhance the *in vivo* stability/pharmacokinetics of administered TS shRNA-lipoplex following their systemic administration.[18−20] We here showed that combined treatment of PMX with systemic injection of PEG-coated TS shRNA-lipoplex significantly suppressed tumor growth in a subcutaneous xenograft mouse model. It appears that systemic delivery of tumor targeted anti-TS RNAi molecule along with PMX treatment might be an effective therapeutic option, in addition to local delivery, in treating mesothelioma showing either localized or distant "metastasis".

MATERIALS AND METHODS

Materials. Pemetrexed disodium (PMX) was purchased from Eli Lilly (IN, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanol-amine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and dioleoylphosphatidylethanolamine (DOPE) were kindly provided by NOF (Tokyo, Japan). A cationic lipid, *O*,*O*′-ditetradecanoyl-*N*-(alpha-trimethyl ammonio acetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical (Osaka, Japan). Opti-MEM I and Lipofectamine RNAiMAX were purchased from Invitrogen (CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

Animals and Tumor Cell Line. BALB/c nu/nu mice (male, 5-week-old) were purchased from Japan SLC (Shizuoka, Japan). Animals were allowed access to mouse chow and water and were handled under aseptic conditions. All animal experiments were conducted in alignment with the guidelines of Animal and Ethics Review Committee of Tokushima University. Among different human malignant pleural mesothelioma (MPM) cell lines, MSTO-211H cell line was chosen for this study based on the pronounced expression-level of TS gene.[21] A human MPM cell line, MSTO-211H, was purchased from the American Type Culture Collection (VA, USA) and was cultured in RPMI-1640 medium (Wako Pure Chemical) supplemented with 10% fetal bovine serum FBS (Japan Bioserum, Hiroshima, Japan) and maintained at 37 °C in a 5% CO_2/air incubator.

Chemically Synthesized shRNAs. Purified chemically synthesized shRNAs were purchased from Hokkaido System Science (Sapporo, Japan). Two shRNAs were designed; one to target TS (TS shRNA) and the other was nonspecific (NS shRNA, not to target any gene in human and mouse genome). The sequence of shRNA against TS was 5′-GUAACA-CCAUCGAUCAUGAUAGUGCUCCUGGUUGUCA-UGAUCGAUGGUGUUACUU-3′ and for a nonspecific shRNA was 5′-UCUUAAUCGCGUAUAAGGCUAGU-GCUCCUGGUUGGCCUUAUACGCGAUUAAGAUU-3′. shRNAs were reconstituted in RNase free TE buffer at a final concentration of 100 μmol/L.

Preparation of shRNA-Lipoplex for *in Vitro* Transfection. MSTO-211H cells were transfected with shRNA using Lipofectamine RNAiMAX (LfRNAiMAX, Invitogen, CA, USA) according to the manufacturer's instructions. In brief, 300 pmol of shRNA and 15 μL of LfRNAiMAX were diluted in Opti-MEM I (Invitrogen, CA, USA) to a total volume of 500 μL, respectively. The diluted shRNA and LfRNAiMAX were mixed and incubated at room temperature for 20 min to form shRNA/LfRNAiMAX complex.

***In Vitro* Gene Knockdown by Chemically Synthesized shRNA Transfection.** *In vitro* gene knockdown by chemically synthesized shRNA was examined with Western blotting as previously described.[22] Briefly, cells (5×10^5 cells/10 cm dish) were seeded 24 h before shRNA transfection. The cells were transfected with 5 or 10 nM of either TS shRNA or NS shRNA in the shRNA/LfRNAiMAX complex. Medium treated cells were served as control for the experiment. At 72 h post-transfection, the cells were harvested, washed with chilled PBS, and resuspended in ice-cold NP-40 lysis buffer (1% NP-40, 50 mM Tris HCl, 150 mM NaCl, 0.25% sodium deoxycholate) containing protease inhibitor (Sigma-Aldrich, St Louis, Mo, USA). The protein extract (20 μg per lane) was resolved on 12% SDS-PAGE gel (ATTO Corp., Tokyo, Japan) and transferred from gel to Hybond-ECL nitrocellulose membrane (GE Healthcare, CA, USA) and then blocked with Tris-buffered saline (pH 7.4) containing 5% BSA and 0.05% Tween 20 for 1 h at 25 °C. Membranes were then incubated overnight at 4 °C with primary antibody [(1:500 for mouse monoclonal anti-TS antibody (AnaSpec, CA, USA) or 1:5000 for mouse monoclonal anti-β-actin antibody (Abcam, Tokyo, Japan)]. Then membranes were further incubated with horseradish peroxidase-conjugated goat antimouse secondary antibody (1:2000, ICN Biomedical, CA, USA) at 25 °C for 1 h. Finally, membranes were visualized using the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, UK), and the obtained images were then analyzed using Multi Gauge v.3.2 (FujiFilm, Tokyo, Japan).

Cell Viability Assay. MSTO-211H cells (2×10^3) were seeded in 96-well plates in 200 μL of RPMI-1640 medium. At 24 h post-incubation, the cells were transfected with 5 or 10 nM of either TS shRNA or NS shRNA in the shRNA/LfRNAiMAX complex and further incubated for 24 h. Then, a fresh medium containing either 0.01 or 10 μg/mL PMX was added. After different incubation time intervals (24, 48, 72, and 96 h), MTT assay was conducted as described previously.[23]

Apoptosis Analysis. Cell apoptosis assay was done using Annexin-V-FITC Apoptosis Detection Kit (Roche) according to the manufacturer's recommended protocol. Briefly, cells (40 000 cells/well) were seeded in six-well plates 24 h before

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chemically synthesized shRNA transfection. The cells were transfected with 5 or 10 nM of either TS shRNA or NS shRNA in the shRNA/LfRNAiMAX complex for 24 h. After the transfection, fresh medium with or without PMX (0.01 μg/mL) was added, and the cells were further incubated for different time intervals (24, 48, 72, and 96 h) at 37 °C. Then, the cells were harvested, washed, and centrifuged for 5 min at 4 °C. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution and incubated in dark for 10−15 min at room temperature. Apoptotic cells were analyzed using flow cytometer, Guava EasyCyte Mini System (Guava Technologies, CA, USA), and the sample data were analyzed using CytoSoft software (Guava Technologies). All experiments were performed in triplicates.

Preparation of PEG-Coated shRNA-Lipoplexes. Cationic liposome composed of DOPE/CHOL/POPC/DC-6-14 (3:3:2:2, molar ratio) was prepared as described previously.[24] The mean diameter and zeta potential for cationic liposomes were 113.2 ± 10.2 nm and 25.3 ± 0.9 mV (n = 3), respectively, as determined with Zetasizer Nano ZS (Malvern Instruments Ltd., UK). For the determination of liposomal phospholipids concentration, a colorimetric assay was applied.[25]

For the preparation of shRNA-lipoplexes, shRNA and cationic liposome were mixed at a molar ratio of 2000:1 (lipid/shRNA) under heavy vortex mixing conditions. The zeta potential and mean diameter of shRNA-lipoplex were 18.6 ± 1.3 mV and 392.1 ± 37.0 nm (n = 3), respectively. For systemic application, shRNA-lipoplex was surface-decorated by polyethylene glycol (PEG)-conjugated lipid using a post-insertion technique as described previously.[26] The mean diameter and zeta potential of PEG-coated shRNA-lipoplex were 403.4 ± 26.2 nm and 16.1 ± 1.8 mV (n = 3), respectively. To identify the presence of free-shRNA in the prepared PEG-coated shRNA-lipoplex, electrophoresis was conducted on 2% agarose gel in 40 mM Tris−acetate buffer containing 1 mM EDTA and shRNA detection was performed using a UV transilluminator. The absence of bands related to free shRNA verified that practically 100% of the shRNA was complexed with PEG-coated lipoplex under our preparation condition (Figure S1). The *in vitro* stability of prepared shRNA-lipoplex was also investigated in the presence of mouse serum to verify the stability of our formulated lipoplex (Figure S2).

***In Vivo* Antitumor Activity by the Combined Treatment with Chemically Synthesized TS shRNA and PMX.** To establish the subcutaneous xenograft model, human MSTO-211H cells (5 × 10^6/100 μL PBS) were subcutaneously inoculated into the back region of 5-week-old male BALB/c nu/nu mice. The animals were then randomly grouped and treated with 9% sucrose (control), PEG-coated NS shRNA-lipoplex, PEG-coated TS shRNA-lipoplex, or PMX as single agents, or a combination of PMX with either PEG-coated NS shRNA-lipoplex or PEG-coated TS shRNA-lipoplex. The tumor size was measured every three days by a caliper, and tumor volume was estimated using the formula: tumor volume (mm^3) = (length) × $(\text{width})^2$ × 0.5.[27] Treated versus control percentage [T/C (%)] was calculated using the following formula:

$$T/C(\%) = [(\text{relative tumor volumes in the treated group} / \text{the control group}) \times 100]$$

where the relative tumor volume = final tumor volume/initial tumor volume.[28] PEG-coated lipoplex containing TS shRNA or NS shRNA (40 μg/mouse) was intravenously injected every other day (on day 7, 9, 11, 13, 15, and 17), and PMX (25 or 100 mg/kg) was intraperitoneally (i.p.) administered days 7−11 and 14−18, after the tumor cell inoculation. The PMX doses were selected based on the doses reported in many preclinical studies[12,29] and on our preliminarily experiments (data not shown). At the end of therapeutic experiments (i.e., when tumor size exceeded 1500 mm^3 in volume), mice were euthanized, tumors were dissected, and tumor specimens were analyzed for both the *in vivo* gene knockdown efficiency (suppression of TS protein expression) and the induction of apoptosis within tumor tissue. For the *in vivo* gene knock down efficiency of different treatments, Western blotting was performed as aforementioned. For detection of apoptotic cells in the tumor tissue, TUNEL assay using In Situ Cell Death Detection Kit, Texas red (Roche, Indianapolis, IN, USA), was carried out as described previously.[19] Induction of apoptosis in tumor sections was observed by using a fluorescence microscope (Axiovert 200M; Zeiss, Oberkochen, Germany), and the numbers of apoptotic cells in each tumor section were estimated in ten different microscopic fields using analyze software (AxioVision; Zeiss).

Biodistribution and Tumor Accumulation of PEG-Coated shRNA-Lipoplexes. To assess the tissue distribution of PEG-coated TS shRNA-lipoplex, MSTO 211H tumor-bearing mice were intravenously injected with ^{3}H-CHE-labeled TS shRNA-lipoplexes (25 mg phospholipid/kg body weight). Twenty-four hours post-test-lipoplex injection, blood samples (100 μL) were collected from the retro-orbital sinus, and the livers, spleens, and tumors were dissected. Radioactivity in blood and collected tissues was then assayed, as described previously.[30]

Statistical Analysis. The data were expressed as mean ± SD and analyzed using GraphPad InStat View software (GraphPad Software, CA, USA). A two-tailed unpaired Student's t test was performed to determine the significance of the differences between groups. The level of significance was set at $p < 0.05$.

RESULTS

Effect of shRNA Transfection on TS Expression in Human Mesothelioma Cell Line (MSTO-211H). The gene knockdown efficiency of chemically synthesized TS shRNA on TS expression was estimated in MSTO-211H cells by using Western blotting. As shown in Figure 1A, TS shRNA strongly suppressed TS expression at both shRNA concentrations used (5 nM and 10 nM), whereas the nonspecific shRNA (NS shRNA) had no effect on the TS expression at the same condition. Under this experimental condition, the knockdown efficacy of TS shRNA was approximately 75%, compared with the NS shRNA (Figure 1B).

Effect of TS Knockdown on Chemosensitivity of Mesothelioma MSTO-211H Cell Line to PMX *in Vitro*. At first, the cytotoxicity of PMX against MSTO-211H cells was investigated. As shown in Figure 2, PMX significantly reduced cell survival in a dose- and time-dependent manner with a maximum cellular growth inhibition observed at 96 h postincubation; IC_{50} at 96 h was 24.64 ± 0.001 ng/mL versus 828.6 ± 0.012 or 38.82 ± 0.001 ng/mL at 48 or 72 h post-incubation, respectively.

Next, we studied the contribution of TS shRNA-mediated TS-protein suppression to the cytotoxicity of PMX. MSTO-211H cells were transfected with chemically synthesized TS

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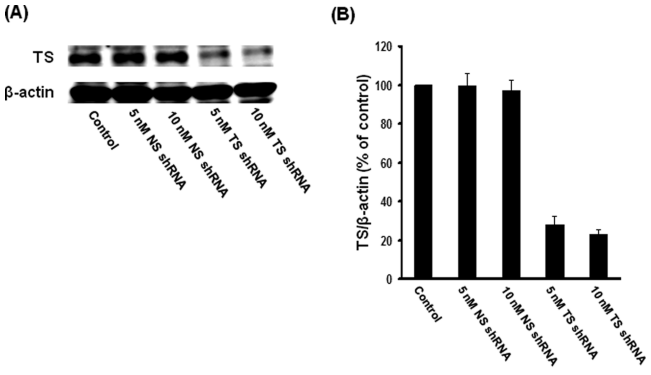

Figure 1. Suppression of TS protein expression by the transfection with chemically synthesized TS shRNA in human mesothelioma MSTO-211H cell line. (A) TS protein expression in control, nonspecific (NS) shRNA-, or TS shRNA-transfected MSTO-211H cells 72 h after transfection, as determined by Western blotting. β-actin was used for equal loading assessments. (B) Quantitative evaluation of the percent change in expression levels of TS protein against β-actin one. Data represents mean ± SD from three independent experiments.

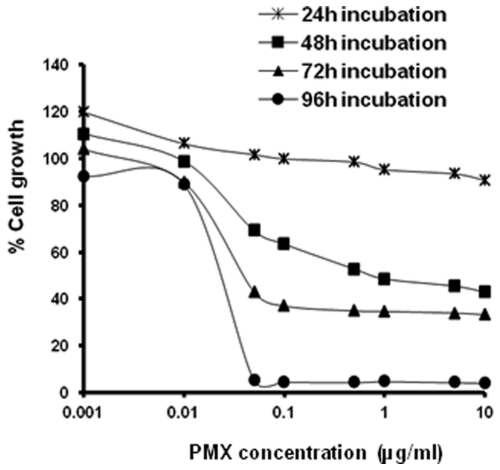

Figure 2. *In vitro* cytotoxicity of PMX on human mesothelioma MSTO-211H cells. MSTO-211H cells were incubated with media containing serial dilutions of PMX for different time intervals (24, 48, 72, or 96 h). After the specified incubation time, the medium was removed and cell survival was determined by the MTT assay. Data represent mean ± SD (n = 3).

shRNA, and then treated with PMX (0.01 or 1 μg/mL) for different times (24, 48, 72, and 96 h). As shown in Figure 3A,B, at low PMX concentration (0.01 μg/mL; lower than IC_{50} of PMX at 96 h), treatment with TS shRNA resulted in an increased sensitivity of MSTO-211H cells to PMX in a time-dependent manner, as compared with treatment with NS shRNA. A maximal cellular growth inhibition (more than 80%) was reached at 72 h post-transfection (Figure 3A,B). However, at higher PMX concentration (1 μg/mL) the effect of suppression of TS expression on the cytotoxicity of PMX was negligible, where a combination of TS shRNA and PMX did not show any superior cytotoxicity over PMX alone after any incubation time (Figure 3C,D). Taken together, the results suggest that down-regulation of TS protein expression significantly enhanced the chemosensitivity of mesothelioma MSTO-211H cells to PMX, raising the chance for reducing each dose and thereby the total dose of PMX when the combined therapy is carried out.

Induction of Apoptosis by a Combined Treatment with Chemically Synthesized TS shRNA and PMX *in Vitro*. To gain further insight into the effect of suppression of TS expression on the chemosensitivity of MSTO-211H cells to PMX, cellular apoptosis was determined using flow cytometric analysis of annexin V stained cells at different incubation time with PMX (24, 48, 72, and 96 h). As shown in Figure 4A,B, monotreatment with either shRNAs (TS shRNA or NS shRNA) or PMX did not induce significant apoptosis of MSTO-211H cells. In the NS shRNA-transfected cells, no significant change in the percent of apoptotic cells was induced by PMX treatment. However, in the TS shRNA-transfected cells, PMX treatment induced a 4.5 -fold increase in the percentage of apoptotic cells, compared with the cells treated with combination of NS shRNA and PMX, peaking at 72 h post-incubation.

***In Vivo* Antitumor Effect of the Combined Treatment with Chemically Synthesized TS shRNA and PMX in MSTO-211H Xenograft Model.** To scrutinize the effect of suppression of TS expression on tumor growth *in vivo*, the antitumor efficacy of the combined treatment of TS shRNA and PMX was evaluated in xenograft mouse model with MSTO-211H. Monotreatment with NS shRNA did not suppress tumor growth [T/C (%) = 98.64%] (Figure 5). Monotreatment with either TS shRNA or PMX or a combination treatment with NS shRNA and PMX slightly suppressed tumor growth [T/C (%) = 77.53%, 76.50%, and 81.28%, respectively]. Combination treatment of TS shRNA and PMX caused a potent tumor growth inhibition [T/C (%) = 37.14%], compared with other treatments ($p < 0.01$). Notably, all treatments were well tolerated as manifested by the absence of remarkable body weight loss among any of the treated animals (data not shown). These results suggest that combined treatment with TS shRNA and PMX exerts a potent antitumor efficacy without causing remarkable toxicity, relating to body weight loss, in a human mesothelioma xenograft mouse model.

Biodistribution and Tumor Accumulation of Test TS shRNA-Lipoplex. The biodistribution pattern of PEG-coated TS shRNA-lipoplex was evaluated following the intravenous injection of radio-labeled lipoplexes into MSTO-211 tumor-bearing mice. Test lipoplex distributed/accumulated substantially in the liver and spleen at 24 h post-test-lipoplex injection (Figure 6). Such preferential accumulation of test lipoplex in the major eliminating organs might explain the relatively low blood concentration of test dose. Nonetheless, no remarkable accumulation of test lipoplex was detected in either kidneys or lungs (Figure 6). The tumor accumulation of test lipoplex was also assessed at 24 h post-test-lipoplex injection. As depicted in Figure 6, test PEG-coated lipoplex accumulated to a certain extent in the tumor tissue. These results imply that the efficient antitumor efficacy of a combined treatment of PMX and PEG-coated TS shRNA is attributed, at least in part, to the efficient accumulation and/or delivery of TS shRNA within tumor tissue.

***In Vivo* Suppression of TS Expression and Induction of Apoptosis in Tumor Tissue Following Combined Treatment with TS shRNA and PMX.** TS expression levels in the treated tumors were determined by Western blotting (Figure 7A). Consistent with the *in vitro* TS suppression results, monotreatment with TS shRNA was efficient in suppressing TS expression in the tumor tissue, while monotreatment with NS shRNA showed no effect on the expression level of TS. In addition, a combined treatment with PMX and TS shRNA

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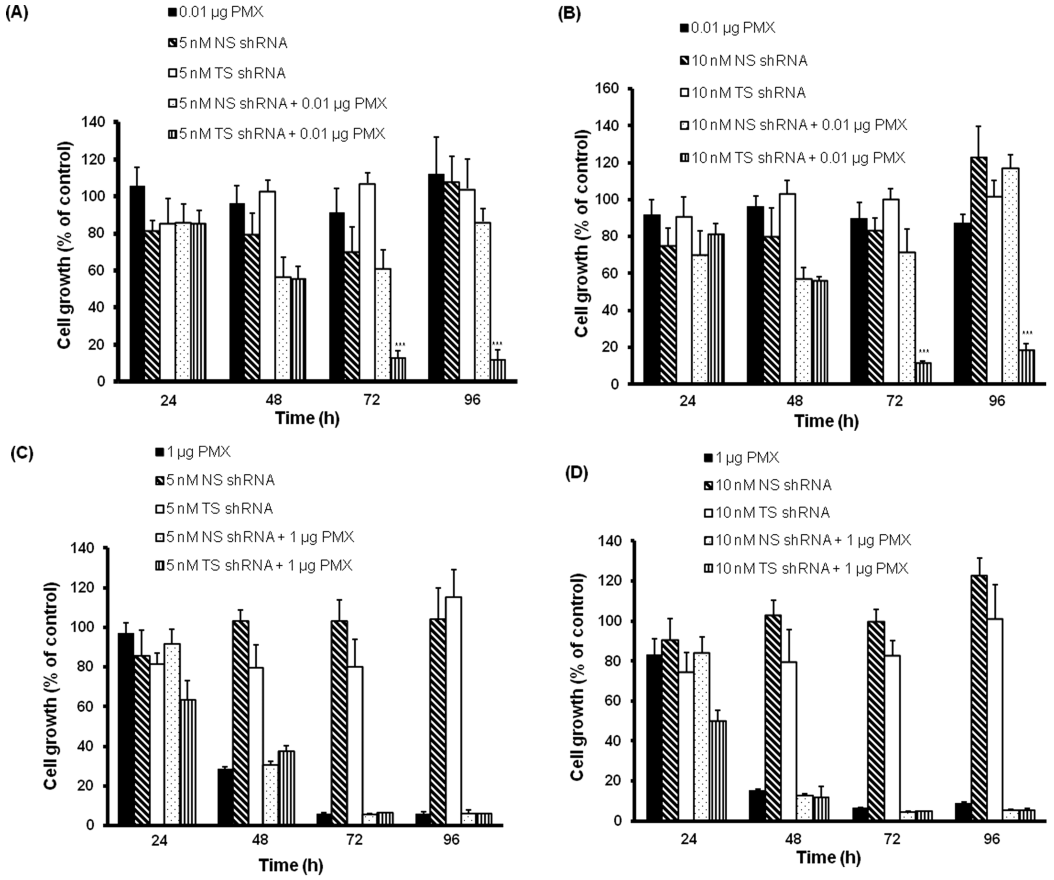

Figure 3. Effect of TS gene knockdown on the chemosensitivity of human mesothelioma MSTO-211H cells to PMX. MSTO-211H cells were transfected or not with either 5 or 10 nM of nonspecific (NS) shRNA or TS shRNA. At 24 h post-transfection, fresh media containing PMX (0.01 or 1 μg/mL) were added, and the cells were further incubated for different time intervals (24, 48, 72, or 96 h). Cell viability was then determined by MTT assay. Data were represented from three independent experiments. ***$p < 0.001$ versus TS shRNA- or PMX-treated cells.

significantly suppressed TS expression, compared to a combined treatment with PMX and NS shRNA (Figure 7A).

To elucidate partly the mechanism of the enhanced antitumor activity of combined treatment of PMX with systemic injection of PEG-coated TS shRNA-lipoplex (Figure 5), TUNEL assay was conducted (Figure 7B,C). The combined treatment of PMX with TS shRNA induced a potent apoptotic response within the tumor tissue (apoptotic index, 45.7 ± 5.3%), compared to the monotreatment with either PMX or TS shRNA (apoptotic indexes, 22.1 ± 2.9% and 17.4 ± 3.2%, respectively) or the combined treatment of NS shRNA with PMX (apoptotic index, 23.4 ± 4.1%). These results verify the contribution of apoptosis induction within the tumor tissue to the potent antitumor efficacy of the combined treatment against MPM.

DISCUSSION

Malignant pleural mesothelioma (MPM) represents an immense challenge because of its poor prognosis and remarkable resistance to current therapies.[31,32] Clinically, pemetrexed (PMX) represents a mainstay in most applied MPM therapeutic strategies. However, its antitumor efficacy is severely restrained by the development of drug resistance. Thymidylate synthase (TS), a folate-dependent enzyme, is considered the main target of PMX.[33,34] Many reports have indicated that TS expression in various tumors is prognostic for poor outcome in patients treated with PMX.[35,36] Recently, we emphasized that down-regulation of TS expression, using a chemically synthesized short hairpin RNA (shRNA), significantly increased the chemosensitivity of mesotheliomal MSTO-211H cells to PMX. In addition, we confirmed that the combined treatment of PMX and locally administered non-PEGylated TS shRNA-lipoplex substantially alleviated the progression of orthotopic thoracic tumors compared to each single treatment.[14]

Nevertheless, many reports have recently revealed the ability of MPM cells to metastasize not only to neighboring tissues/organs such as regional lymph nodes, pericardium, peritoneum, and chest wall but much farther to reach the brain.[15,16] In addition, the broad invasion of the whole pleura with thoracic tumors, in tandem with the mechanical stress imparted by pleural fluids associated with MPM could collectively compromise the efficacy of locally administered therapeutic agents. Instead, systemic administration of the therapeutic agent becomes a prerequisite. In the current study, therefore, we assessed the effectiveness of "systemically" administered PEG-coated TS shRNA-lipoplex in sensitizing the mesotheliomal cells to the cytotoxic effect of PMX using a subcutaneous xenograft mouse model. Combined therapy of PMX and "systemically" administered PEG-coated TS shRNA-lipoplex showed a superior antitumor efficacy compared with monotherapy with either agent alone or a combined therapy with PMX and NS shRNA in MSTO-211H tumor xenograft mouse model (Figure 5). This potent antitumor efficacy was achieved mainly via the induction of a potent apoptotic

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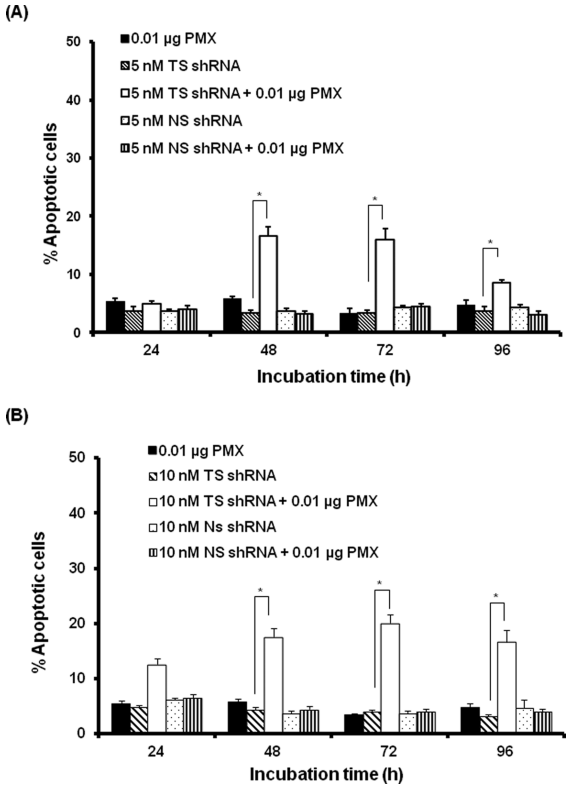

Figure 4. Induction of apoptosis in human mesothelioma MSTO-211H cells by a combined treatment with PMX and TS shRNA *in vitro*. MSTO-211H cells were transfected or not with either 5 or 10 nM of nonspecific (NS) shRNA or TS shRNA. At 24 h post-transfection, the cells were further treated with 0.01 μg/mL PMX. Apoptosis was determined by flow cytometry. Data represent mean ± SD (%) from three independent experiments. *$p < 0.05$.

response in MSTO-211H tumors (Figure 7B,C). Taken together, these findings may provide a proof-of-concept that the combined therapy of PMX and the tumor targeted anti-TS RNAi system (PEG-coated TS shRNA-lipoplex) represents a promising alternative therapy to the currently applied dismally effective therapeutic strategies for the treatment of human MPM.

Interestingly, we observed that a combined treatment of TS shRNA and high dose PMX (100 mg/kg) neither additively nor synergistically cause the tumor growth suppression (Figure S3). Several studies have revealed that after the exposure to fluoropyrimidines or antifolate TS inhibitors, the TS protein expression is induced in preclinical models, both cell lines and animal models.[11,37,38] Thus, small-molecule TS inhibitors can actually antagonize their therapy by promoting overexpression of TS. Based on these reports, we assume that treatment with a high dose of PMX (100 mg/kg) might result in an acute elevation in TS protein expression level. Thus, TS shRNA might not be efficient to completely suppress TS protein expression. However, treatment with a comparatively lower dose of PMX (25 mg/kg) might not trigger MSTO-211H cells to elevate TS protein expression level, leading to a superior tumor growth inhibition (Figure 5). These results obviously reveal that the therapeutic efficacy of PMX, in combination with TS shRNA, is mediated in a PMX dose-dependent manner. However, further studies are urged to precisely determine the optimal therapeutic window of PMX required to obtain the maximal antitumor efficacy.

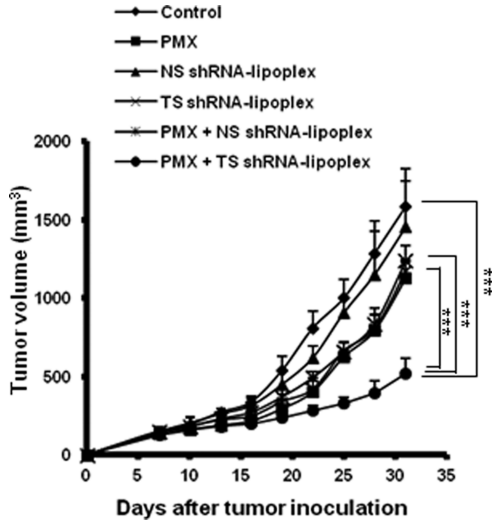

Figure 5. Tumor growth suppression by combination therapy of PMX plus PEG-coated TS shRNA-lipoplexes in human mesothelioma (MSTO-211H) tumor-bearing mice. Tumor xenograft animal model was established by subcutaneous inoculation of MSTO-211H cells in nude mice. PMX (25 mg/kg) was intraperitoneally administered daily for 5 consecutive days/week for 2 weeks. PEG-coated shRNA-lipoplex containing either nonspecific (NS) shRNA or TS shRNA (40 μg shRNA/mouse) was intravenously injected every 2 days (on day 7, 9, 11, 13, 15, and 17 after tumor cell inoculation). For the control group, sucrose was administered instead of PMX or PEG-coated shRNA-lipoplexes. Data represent mean ± SD ($n = 6$). ***$p < 0.001$.

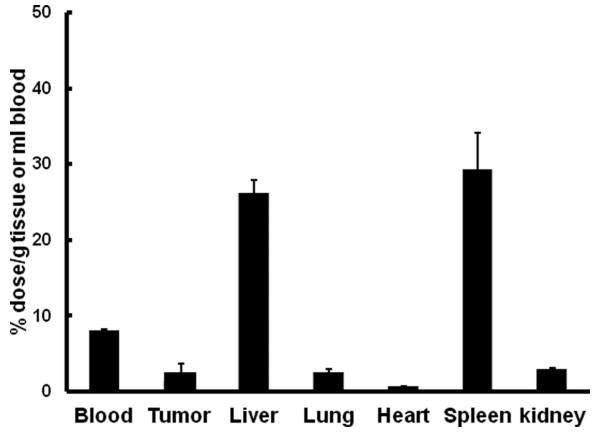

Figure 6. Biodistribution and intratumoral accumulation of test TS shRNA-lipoplex. MSTO211H-bearing mice were intravenously injected with radiolabeled TS shRNA-lipoplex (25 mg phospholipid/kg body weight). At 24 h after injection, blood and major organs were collected, and their level of radioactivity was determined. Data represent mean ± SD ($n = 3$).

For advanced clinical application of the RNAi technique, specific and effective delivery of chemically synthesized siRNA or shRNA to target tumor tissue should be granted. Recently, it was revealed that nanocarrier systems, such as nanomicelles and liposomes, containing siRNAs efficiently suppressed tumor growth in animal models.[39−41] In this study, we also confirmed that PEGylated cationic liposome was efficient in the systemic delivery of chemically synthesized shRNA to the target site as reflected by the superior antitumor efficacy (Figure 5) and the higher apoptotic potential (Figure 7A,B) induced by the combined therapy of TS shRNA-lipoplex and PMX without exerting severe side effects. To the best of our knowledge, this

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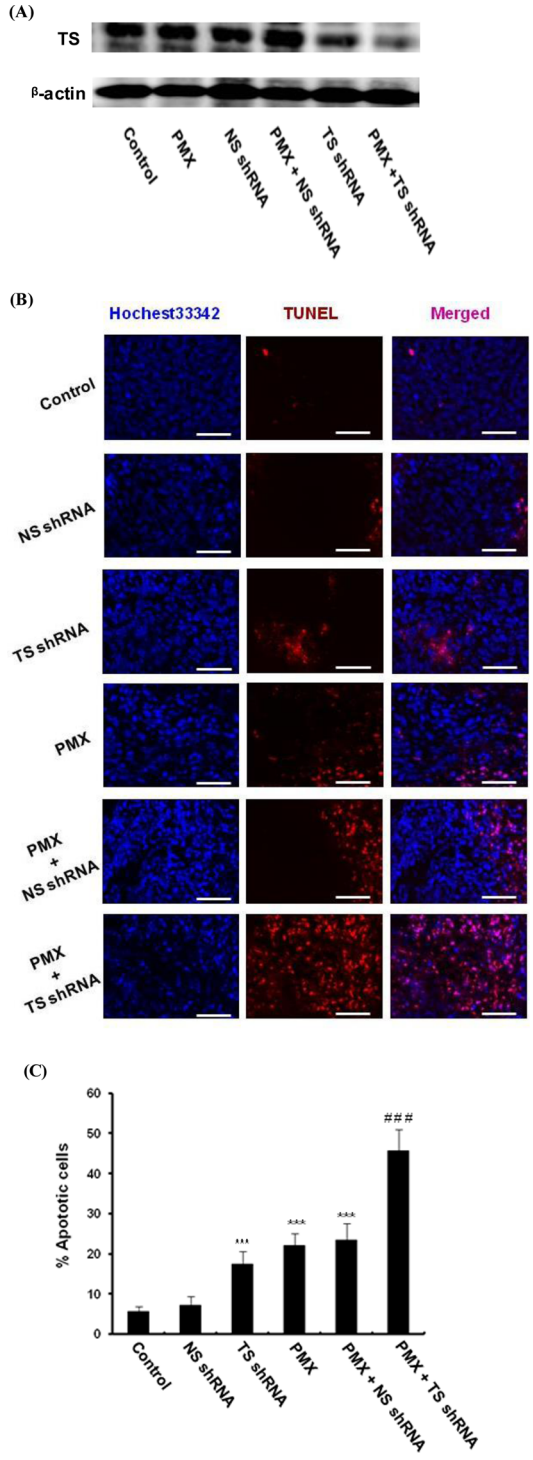

Figure 7. Suppression of TS protein and apoptosis induction in the human mesothelioma cell (MSTO-211H) tumor tissue by a combined treatment with PMX and PEG-coated TS shRNA-lipoplex. MSTO-211H tumor-bearing mice were treated in the same way as described above. When tumor xenograft exceeded 1500 mm^3 in volume, mice were euthanized and tumors were excised. Specimens of tumors were weighed and analyzed with Western blotting and TUNEL assay. (A) TS protein expression was determined by Western blot analysis. (B) Representative immunofluorescent tumor sections (TUNEL, red; Hoechst 33342, blue). The bar indicates 50 μm. Original magnification, ×400. (C) Percent of TUNEL-positive cells in the section. Data represent the mean ± SD. $^{***}p < 0.001$ versus sucrose. $^{###}p < 0.01$ versus PMX or PEG-coated TS shRNA-lipoplex.

is the first observation showing the efficacy of PEGylated cationic liposomes in delivering chemically synthesized shRNA to tumor tissues following their intravenous administration.

Currently, many therapeutic strategies are available for the treatment of localized primary tumors including surgical intervention, radio-/chemotherapy, or a combined approach. However, effective treatment of primary tumors along with local or distant metastasis, particularly micrometastases, is generally limited. Metastases, resulted from local and/or distant dissemination of malignant cells to the neighboring and/or far tissues, usually cause tumor recurrence and eventual treatment failure. An effective strategy to conquer both primary tumor and metastatic lesions are urgently needed for the complete cure of cancer. Systemic treatment strategy is one of the effective therapeutic approaches to treat both primary tumor and metastatic lesions. In the current study, we evidently demonstrated that systemically administered TS shRNA was efficient in the down-regulation of *in vivo* TS expression. In addition, the suppression of TS expression is associated with the increased *in vivo* antitumor efficacy of PMX in human MPM tumor-bearing mice model. These results imply that our tumor targeted anti-TS RNAi system could augment the therapeutic potential of PMX in many clinical settings. Consequently, the combined therapy of PMX and anti-TS RNAi system would represent a promising alternative therapeutic strategy to the currently applied therapeutic modalities for the treatment of human MPM especially those showing local and/or distance metastasis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00728.

(PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CHOL, cholesterol; DC-6-14, *O,O′*-ditetradecanoyl-*N*-(alpha-trimethyl ammonio acetyl) diethanolamine chloride; DOPE, dioleoylphosphatidylethanolamine; FBS, fetal bovine serum; FITC, flourescein isothiocyanate; HRP, horseradish peroxidase; $mPEG_{2000}$-DSPE, 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-*n*-[methoxy (poly ethylene glycol)-2000]; MPM, malignant pleural mesothelioma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer saline; PEG, polyethylene glycol; PMX, pemetrexed; POPC, 1-

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palmitoyl-2-oleoylphosphatidylcholine; TS, thymidylate synthase

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