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Evaluation of Multi-Target and Single-Target Liposomal Drugs for the Treatment of Gastric Cancer

Chien-Ho CHEN,[1,*] Der-Zen LIU,[2,*] Hsu-Wei FANG,[3] Hong-Jen LIANG,[4] Tzu-Sheng YANG,[3] and Shyr-Yi LIN[5,†]

[1]*School of Medical Laboratory Science and Biotechnology, Taipei Medical University, Taipei, 110, Taiwan*
[2]*Graduate Institutes of Biomedical Materials, Taipei Medical University, Taipei, 110, Taiwan*
[3]*Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei, 106, Taiwan*
[4]*Department of Food Science, Yuanpei University, Hsinchu, 300, Taiwan*
[5]*Department of Internal Medicine and Primary Care Medicine, Taipei Medical University and Hospital, Taipei, 110, Taiwan*

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We studied the effects of multi- and single-target liposomal drugs on human gastric cancer cell AGS both *in vitro* and *in vivo*. The cytotoxic effect of dihydrotanshinone I was significantly enhanced by treatment with octreotide-polyethylene glycol(PEG)-liposome, Arg-Gly-Asp(RGD)-PEG-liposome, and RGD/octreotide-PEG-liposome encapsulated with 0.5 μg/ml of dihydrotanshinone I to AGS cell for 24 h, compared to control. Furthermore, the AGS cell survival rate for multi-target *versus* single target liposomal drugs was significantly suppressed. Microsocpic examination revealed that significant cell death occurred in the multi- and single-target liposomal encapsulated drug groups. Significant suppression of tumor growth in AGS cell xenograft nude mice given octreotide-PEG-liposome, RGD/octreotide-PEG-liposome encapsulated drug, *versus* those given a free drug was noted after 13 d of experimentation with the multi-targeted liposome: up to 60.75% and 41.2% reduction of tumor volume as compared to dimethylsulfoxide (DMSO) control and the free drug groups respectively. The treated animals showed no gross signs of toxicity. The results have potential clinical application.

Key words: liposome; octreotide; Arg-Gly-Asp (RGD); xenograft; gastric cancer

Dihydrotanshinone I is a lipid-soluable component obtained from Danshen, the root of a Chinese herb, *Salvia miltiorrhiza*, which has been suggested to exert various biological functions, including anti-inflammation,[1)] anti-platelet,[2)] liver protection,[3)] and anti-tumor effects.[4–6)] Recent reports have suggested that dihydrotanshinone I inhibited topoisomerase I derived from the calf thymus,[7)] and induction of growth arrest at the S phase and apoptosis of a multidrug resistance erythroleukemia cell line, K562/ADR.[8)] These findings indicate the potential anti-tumor effect of dihydrotanshinone I, but at relatively high dosage (10 μg/ml). Finding strategies to enhance dihydrotanshinone I and other potential anti-tumor agents' effects toward cancer cells without damaging normal cells is an important issue. One approach is through liposome-carriage of drugs.

Liposome is a single or multi-lamellar vesicle (MLV) with a self-closing feature.[9)] It has both a hydrophilic head region and a hydrophobic carbon chain for the carriage of various drugs.[10)] It is mainly composed of natural phospholipid, which makes it highly bio-compatible and bio-degradable. Conventional liposome has been used in the carriage of drugs in cancer treatment.[11,12)] Due to its low specificity and stability, clinical usage is limited. In recent years, liposome has been modified either with polyethylene glycol (PEG) to prolong its stability in circulation,[13)] or with targeting molecules to interact with specific cells.[14,15)] The development of covalent binding of targeting molecules with PEG on liposome makes improved stability and specificity possible at the same time.[16–18)]

Gastric cancer is the second leading cause of cancer-associated deaths worldwide.[19,20)] Importantly, its incidence is 6–8 times greater in Asian and Eastern European countries.[19,20)] Surgical treatment is effective

* Contributed equally
† To whom correspondence should be addressed. Tel: +886-2-27361661 ext.3010; Fax: +886-2-27393422; E-mail: sylin@tmu.edu.tw

Abbreviations: DMSO, dimethylsulfoxide; DSPE-PEG, distearoylphosphatidyl-ethanolamine-N-poly(ethyleneglycol); FCS, fetal calf serum; MLV, multi-lamellar vesicle; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PEG, polyethylene glycol; RGD, Arg-Gly-Asp; SANPAH, succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate

for patients with early-stage disease. However, for those patients with regional or distant metastasis at the time of diagnosis, only 20–40% respond to chemotherapy.[21] Therefore, new modalities are necessary for the treatment of patients with advanced gastric cancer.

Octreotide is a somatostatin analog composed of eight amino acids.[22] As compared to somatostatin, it has higher efficacy and a longer half-life in the circulatory system.[22,23] The gastrointestinal tract is a major source and target of somatostatin, and the receptor for somatostatin has been detected in gastric carcinoma.[24] It has been found that somatostatin subtype 2 and 5 receptors are expressed in human gastric cancer cell AGS.[25] The RGD sequence in the ligand is specifically recognized by integrin receptors. The $\alpha v\beta 3$ integrin is highly expressed in vessels surrounding the tumor.[26] Suppression of the expression of $\alpha v\beta 3$ integrin has been associated with apoptosis of endothelial cells, which inhibited tumor growth.[27,28] The purpose of this study, using RGD or/and octreotide in preparing single and multi-target covalent PEG binding liposome encapsulated drugs, was to compare their cytotoxic effects with free drugs on AGS cells both *in vitro* and *in vivo*.

Materials and Methods

Distearoylphosphatidyl-ethanolamine-N-poly(ethyleneglycol) (DSPE-PEG) conjugation, and analysis and preparation of liposome. Liposome compositions DSPE-PEG-Oct and DSPE-PEG-RGD were kindly provided by Professor Shui-Tein Chen, Academia Sinica, Taiwan. The method of DSPE-PEG-Oct and DSPE-PEG-RGD has been described elsewhere in detail.[29] The lipid mixtures and hydrophobic dihydrotanshinone I were dissolved in chloroform. The organic solvents were then removed with a rotary evaporator under reduced pressure. A thin film of lipid was observed after the solvents were removed. Normal saline was then added to hydrate the thin lipid film. Hydration was followed by sonication in a water bath for at least 10–15 min to form an MLV suspension. The MLV liposomal suspension was then moved into a probe sonicator for 10 min at 50 °C to form a small unilamellar vesicle suspension.

Cell line and cell culture. AGS, a cell line obtained from human gastric cancer, (American Type Culture Collection, Manassas, VA) was maintained in 1:1 DME/F-12 (v/v) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 0.3 mg/ml glutamine in a humidified incubator (37 °C, 5% CO_2). Dihydrotanshinone I (Xi'an Honson Biotechnology Co., Xi'an, China) was added at the indicated doses in 0.1% dimethylsulfoxide (DMSO). For control specimens, the same volume of DMSO was added to a final concentration of 0.1% (v/v) without dihydrotanshinone I.

Determination of cell viability. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, as described previously.[30] Briefly, cells were seeded in a 24-well plate at a density of 1×10^5 cells/well and allowed to adhere overnight. After removal of the medium, 900 µl of fresh medium per well, containing 10 mmol/l Hepes (pH 7.4), was added. Then 50 µl of MTT was added to the wells and the plate was incubated for 2 h at 37 °C in the dark. The medium was removed, and 1000 µl of DMSO and 125 µl of Sorensens's glycine buffer was added to the wells. Absorbance was measured using an ELISA plate reader at 570 nm.

Experimental animals. Male, 4–5 week old, BALB/cAnN.Cg-Foxn1^{nu}/CrlNarl mice (National Laboratory Animal Center, Taipei, Taiwan) were housed in plastic cages (5 mice/cage), with wire tops and wood-chip bedding and in a control room under a 12/12 (light/dark) cycle at a ventilation rate of 12 l/h, a humidity of $60 \pm 5\%$, and a temperature of 23 ± 2 °C. Food and water were available *ad lib*. All experimental animal treatment followed the guidelines set up by the Institutional Animal Care and Use Committee of Taipei Medical University.

Experiment on AGS cell xenograft. AGS cells were implanted by injection of a tumor suspension (5×10^6 cells in 0.1 ml of medium mixed with 0.1 ml of matrigel) subcutaneously in the backs of male BALB/cAnN.Cg-Foxn1^{nu}/CrlNarl mice. Each mouse was implanted with one bolus of tumor cells. After 6–7 weeks of treatment, when the tumor volume reached about 200 to 300 mm^3, dosing with vehicle or liposomal composition began intraperitoneally 3 times a week. The tumor volume in mm^3 was measured every 2 d according to the formula: volume = 1/2 xW^2 xL, where W = width and L = length of the tumor, as previously described.[31] Mice were weighed every day and sacrificed at day 13, and their general health was monitored during the assay.

Immunohistochemistry study. As described previously,[32] paraffin-embedded blocks were sectioned at 5–7 µm thickness. After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, slides were immersed in 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. After intensive washing with PBS, the slides were incubated overnight at 4 °C with anti-Ki67 or anti-CD34 (Dako, Carpinteria, CA) antibody at a dilution of 1:100. After incubation with a biotinylated secondary antibody (1:200), the slides were incubated with peroxidase-conjugated streptavidin (Dako LSAB+kit; Dako). Reaction products were visualized by immersing the slides in diaminobenzidine tetrachloride, and finally counterstained with hematoxylin.

Statistics. Results were expressed as mean ± S.E. for each study. Data were subjected to one-way analysis

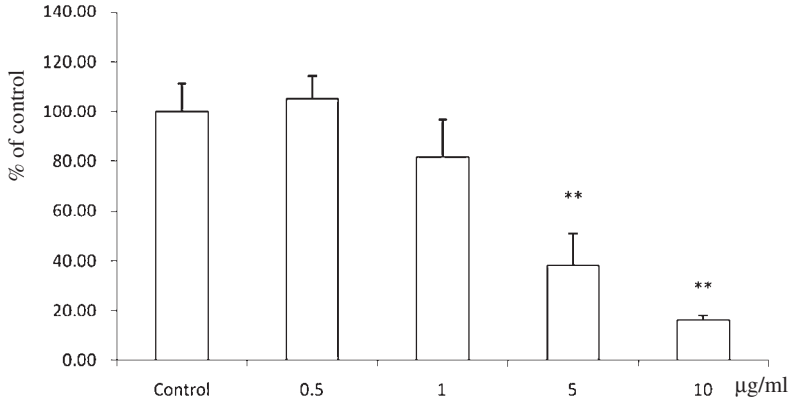

Fig. 1. Effects of Dihydrotanshinone I on the Proliferation of AGS Cells on MTT Assay.
Treatment of AGS cells with dihydrotanshinone I (0.5 to 10 μg/ml) for 24 h induced inhibiton of cell growth in a dose-dependent manner. **, $P < 0.01$ *versus* control. Data are the means ± SE for four wells in each group.

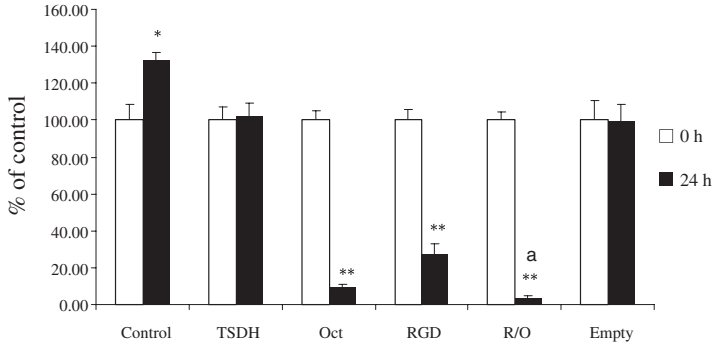

Fig. 2. Effects of Encapsulated Dihydrotanshinone I (TSDH) on the Proliferation of AGS Cells on MTT Assay.
Liposomes with compositions of octreotide-PEG-liposome (OCT), RGD-PEG-liposome (RGD), and RGD/octreotide-PEG-liposome (R/O) at a concentration of 5 mM were used to encapsulate 0.5 μg/ml of dihydrotanshinone I. AGS cells were treated for 24 h. Control, AGS tumor with DMSO treatment. Empty, liposome without TSDH encapsulation. *, $P < 0.05$ *versus* 0-h control; **, $P < 0.01$ *versus* TSDH 24 h treatment; a, $P < 0.05$ *versus* RGD, OCT-liposomes. Data are the means ± SE for four wells in each group.

of variance (ANOVA), followed by Student's t-test. A P value of 0.05 or less was considered statistically significant.

Results

First we used a spectrophotometer to obtain standard lines for RGD-SANPAH (succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate) and octreotide-SANPAH. The concentration of 260 μl of RGD-SANPAH was 9 mM, 0.0116 mM at a volume of 200 ml. The absorption intensity outside the dialysis membrane was 0.0115. Calculation was by the standard line formula for RGD-SANPAH: $y = 2.918x + 0.006$, where x is 0.0017 mM. Therefore, the graft density of RGD-SANPAH, was 1–0.0017 mM/0.0116 mM equal to 85.34%, which represents the conjugation percentage of DSPE-PEG-RGD. The concentration of 180 μl of octreotide-SANPAH was 5 mM, 0.0045 mM at a volume of 200 ml. The absorption intensity outside the dialysis membrane was 0.0262. Calculation was by the standard line formula for octreotide-SANPAH: $y = 19.78x - 0.018$, where x is 0.0014 mM. Therefore, the graft density of octreotide-SANPAH was 1–0.0014 mM/0.0045 mM equal to 69%, which represents the conjugation percentage of DSPE-PEG-octreotide.

MTT assay was performed to test the anti-proliferative effect of dihydrotanshinone I on AGS cells. As shown in Fig. 1, treatment of AGS cells with 1 to 10 μg/ml dihydrotanshinone I for 24 h dose-dependently suppressed AGS cell proliferation. Dihydrotanshinone I at conentrations 5 and 10 μg/ml significantly inhibited AGS cell proliferation, from 61.85% to 84.05% as compared to control respectively. There was no effect on AGS cell proliferation at a concentration of 0.5 μg/ml of dihydrotanshinone I.

Liposomes with compositions of octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome at a concentration of 5 mM were used to encapsulate 0.5 μg/ml of dihydrotanshinone I. As shown in Fig. 2, MTT assay, treatment with the dihydrotanshinone I free drug and empty liposome for 24 h had no effect on AGS viability as compared to the control, indicating that liposome at this concentration had no

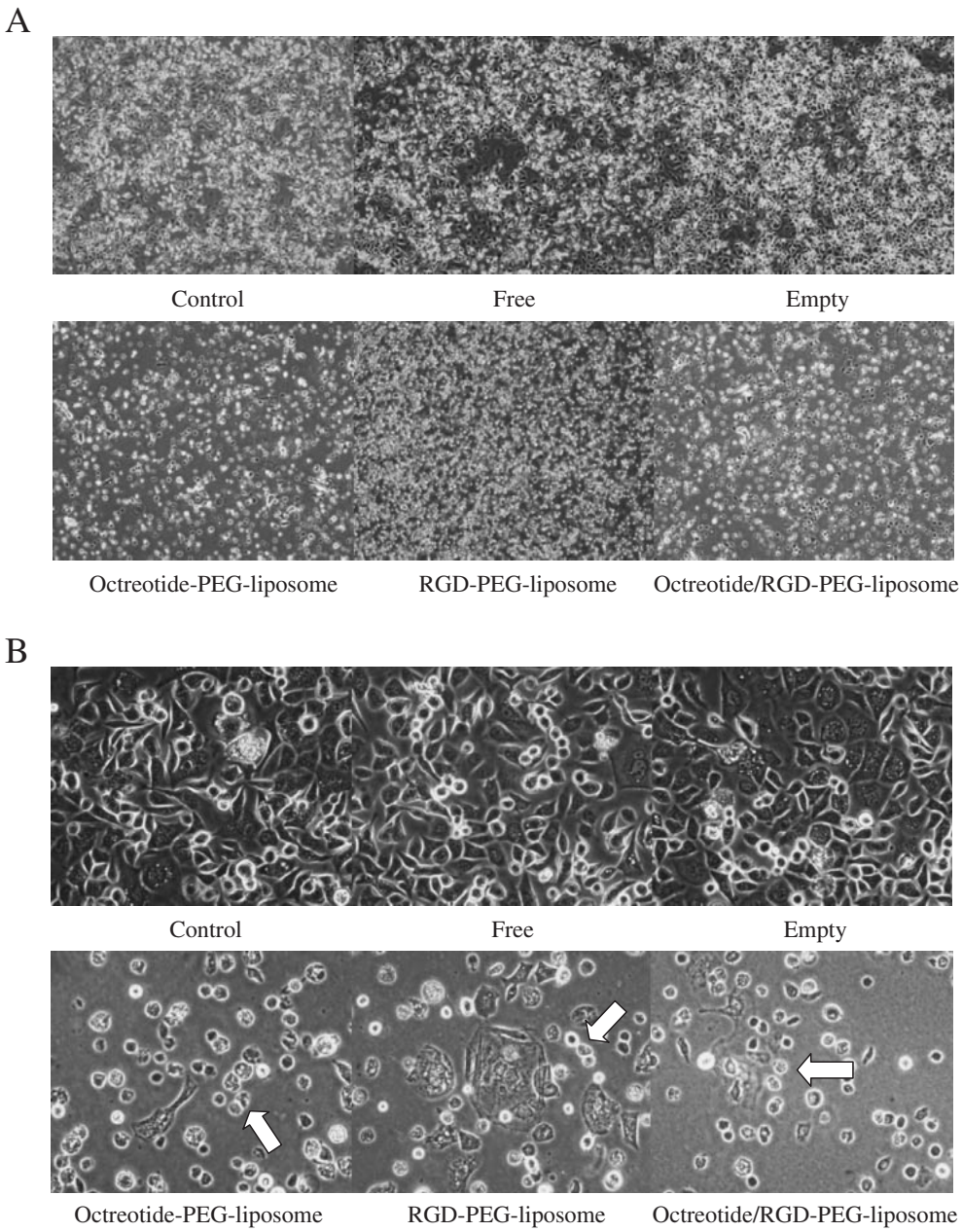

Fig. 3. Effects of Encapsulated Dihydrotanshinone I on Cell Morphologic Changes in AGS Cells by Microscopic Observation.

Liposomes with compositions of octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome at a concentration of 5 mM were used to encapsulate 0.5 μg/ml of dihydrotanshinone I. AGS cells were treated for 24 h. A, 40 X; B, 200 X. Control, AGS tumor with DMSO treatment. Empty, liposome without dihydrotanshinone I encapsulation. Free, 0.5 μg/ml of dihydrotanshinone I without encapsulation. Arrow indicates apoptotic nuclei.

toxicity on AGS cells. However, treatment with octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome encapsulated with 0.5 μg/ml dihydrotanshinone I for 24 h significantly suppressed AGS cell growth as compared to the control, suggesting that liposome encapsulation enhanced the cytotoxic effect of dihydrotanshinone I on AGS cells. Furthermore, the AGS cell survival rates for octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome were 7.41%, 19.7% and 2.66% respectively after 24 h of treatment. The multi-target liposomal drug showed significant enhancement of cytotoxicity on AGS cells, as compared to the single-target liposomal drug.

A similar experimental design was conducted in Fig. 2, but the cells were directly observed under a light microscope at 40× and 200× magnification. As shown in Fig. 3A, the low-power magnification, treatment with dihydrotanshinone I free drug and empty liposome for 24 h had no effect on AGS cell morphology as compared to the control, indicating that liposome under this concentration had no toxicity on AGS cells. However,

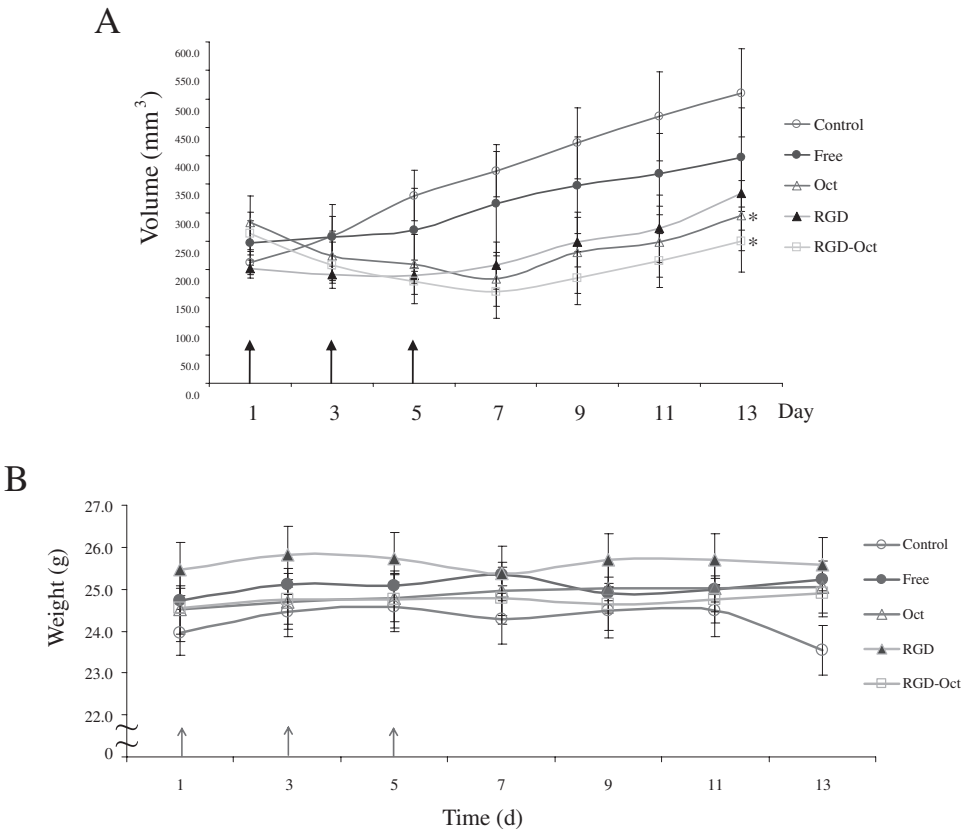

Fig. 4. Effects of Encapsulated Dihydrotanshinone I on (A) Tumor Growth of AGS Cell Xenograft and (B) Body Weight Changes in Nude Mice. Mice were injected subcutaneously with AGS cells in the back for 6–7 weeks until the tumor volume reached about 200 to 300 mm^3, and dosing with vehicle or liposomal composition began intraperitoneally 3 times a week (arrows). Control, AGS tumor with DMSO treatment. Free, 10 mg/kg of dihydrotanshinone I without encapsulation. The same dose of dihydrotanshinone I (10 mg/kg) encapsulated with octreotide-PEG-liposome (OCT), RGD-PEG-liposome (RGD), and RGD/octreotide-PEG-liposome (R/O). $*$, $P < 0.05$ *versus* DMSO control and free drug. Data are the means ± SE for five mice in each group.

treatment with octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome encapsulated with 0.5 μg/ml of dihydrotanshinone I for 24 h significantly suppressed AGS cell growth as compared to the control, suggesting that liposome encapsulation enhanced the cytotoxic effect of dihydrotanshinone I on AGS cells. Under the high-power magnification, Fig. 3B, significant numbers of AGS cells showed cell death (arrow), evidenced by cell shrinkage and pyknosis (chromatin condensation), suggesting the occurrence of apoptosis after they were treated with octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome individually for 24 h. The multi-target liposomal drug showed more induction of cell death on AGS cells than the single-target liposomal drug.

We next examined whether administration of various liposome compositions of encapsulated dihydrotanshinone I would affect the growth of tumors derived from human gastric cancer cells in an *in vivo* setting. As shown in Fig. 4A, reductions in tumor volume in mice given octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-PEG-liposome *versus* those given free drug were detected after 7 d of treatment. This difference became progressively more conspicuous, the average tumor volume of octreotide-PEG-liposome and RGD/octreotide-PEG-liposome-treated mice differing significantly from the free drug group after 13 d of experimentation with the multi-targeted liposome, up to 60.75% and 41.2% reductions in tumor volume as compared to the control and free drug groups respectively. However, as shown in Fig. 4B, there was no significant change in body weight among the treatment groups.

A previous report explained that monoclonal antibody Ki67, which recognizes a human nucleus antigen that is present in proliferating cells but absent in resting cells,[33] can be used as a marker of the tumor proliferative fraction. CD34 is an endothelial antigen that has been used to determine microvasculature vessel density as a direct marker of the degree of angiogenesis.[34] To test the *in vivo* effects of octreotide and of RGD-based liposome on cell proliferation and angiogenesis respectively, an immunohistochemistry study was performed using AGS tumor xenograft. As shown in Fig. 5A, brown staining by anti-Ki67 antibody in octreotide-PEG-liposome and RGD/octreotide-PEG-liposome decreased as compared to the control, suggesting inhibition of cell proliferation. Also shown in Fig. 5B, brown staining by

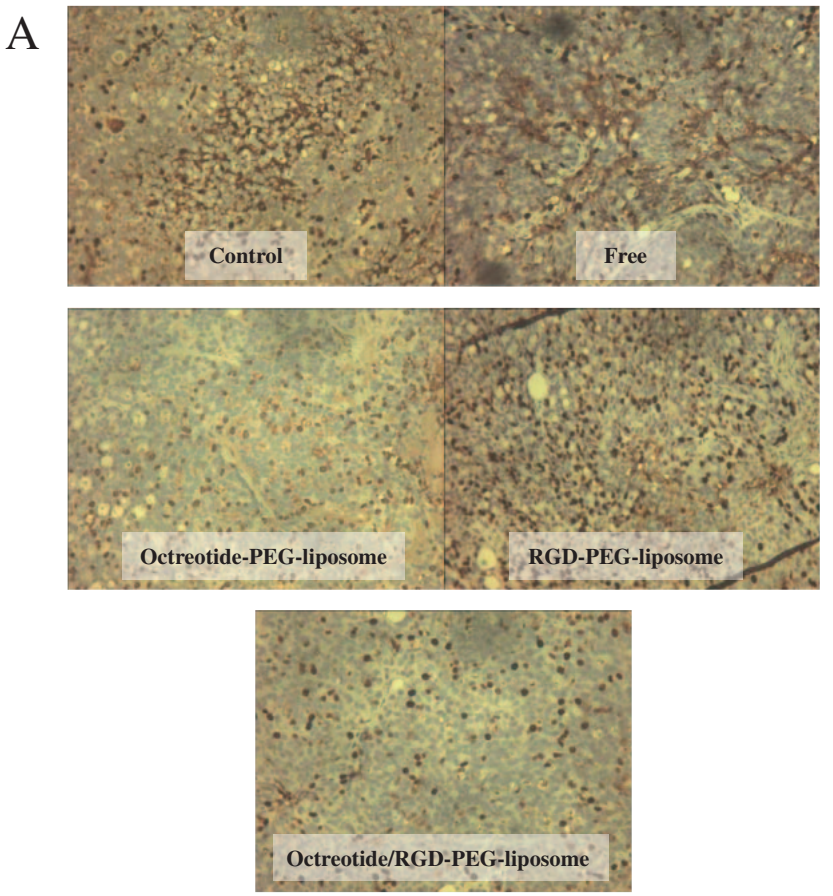

Fig. 5. Immunohistochemistry of AGS Tumor Xenografts Obtained after Completion of Treatments, as Shown in Fig. 4.

A, anti-Ki67; B, anti-CD34 staining, 200 X. Control, AGS tumor with DMSO treatment. Free, 10 mg/kg of dihydrotanshinone I without encapsulation. The same dose of dihydrotanshinone I (10 mg/kg) encapsulated with octreotide-PEG-liposome (OCT), RGD-PEG-liposome (RGD), and RGD/octreotide-PEG-liposome (R/O).

anti-CD34 antibody in RGD-PEG-liposome and RGD/octreotide-PEG-liposome decreased as compared to the control, suggesting inhibition of angiogenesis. These findings indicate that targeting of molecules on liposome can be specifically effective *in vivo*.

Discussion

In the present study, we found that RGD/octreotide covalent PEG binding liposome enhanced the anti-cancer effect of dihydrotanshinone I on AGS cells in cell culture and nude mice xenograft models. The treated nude mice showed no significant toxicity during the experiments. To our knowledge, this is the first demonstration that single or multi-target liposome inhibits the growth of gastric cancer cells both *in vitro* and *in vivo*.

Targeting of somatostatin receptor by octreotide has been found to provide cell selectivity with lower toxicity,[35] and to enhance antitumor action against gastic cancer cells.[25] Angiogenic vascular endothelial cells targeted by RGD-PEG-liposome has been found to enhance the treatment effect in a mouse experimental arthritis model by specific association with blood vessels at the site of inflammation.[36] Furthermore, RGD-modified sterically stabilized liposome has been found to enhance the antitumor effect of doxorubicin both *in vitro* and *in vivo*.[37] In Fig. 2, MTT assay of AGS cells with various non-encapsulated or encapsulated liposomes, we found that PEG-liposome (empty) control showed no significant cytotoxicity. We also tested the cytotoxic effect of non-encapsulated liposome controls, such as octreotide-PEG-liposome, RGD-PEG-liposome, and RGD/octreotide-liposome. These showed no significant toxicity as compared with PEG-liposome (unpublished data). These findings are supported by previous reports that RGD or somatostatin-targeting liposome or carrier showed no apparent toxicity.[35,37] Hence, we inferred that the *in vitro* anti-tumor effects of various liposomes of encapsulated TSDH is in part due to targeting mechanism. As Fig. 4B also shows, no significant difference in body weight change occurred between the target-liposome and control groups, suggesting no significant toxicity. One study found that the RGD-modified liposome loaded with doxorubicin caused no increase in doxorubicin accumulation in the liver, lung, kidney, or heart of mice as compared to non-targeting liposome.[38] Taken together, these findings suggest that a targeting mechanism is involved in the anti-tumor effect of various liposome of encapsulated TSDH *in vivo*. Our results indicate that multi-target RGD/octreotide-PEG-liposome further enhanced anti-tumor action as compared to single-target RGD-PEG-liposome and octreotide-PEG-liposome. An immunohistochemistry study with anti-Ki67 and anti-CD34 indicated the specific involvement of target molecules in anti-proliferation and antiangiogensis effects on AGS tumor xenograft, suggesting that a multi-target liposome can have additive effects through different signaling mechansisms.

The molecular mechanism of dihydrotanshinone I anti-cancer effects remains elusive. It was found that dihydrotanshinone I reduced topoisomerase I activity[7] and induced apoptosis in leukemia cells.[8] Previous reports have suggested that topoisomerase I inhibitors, such as camptothecin, CPT-11, and SN-38, induce apoptosis in human cancer cells,[39,40] involving stabilization of the topoisomerase I-DNA complex and generation of strand breaks and cell death.[41] In our study, treatment with single or multi-target covalent PEG binding liposome-encapsulated dihydrotanshinone I, but not liposome vehicle, significantly induced cell death as judged by microscopic examination of AGS cells, suggesting activation of apoptosis by dihydrotanshinone I.

In conclusion, RGD/octreotide-PEG-liposome enhanced the antitumor activity of encapsulated dihydrotanshinone I against gastric cancer cells without gross toxicity. The results from the present *in vitro* and *in vivo* studies suggest that multi-target liposomal drugs have potential applications in clinical usage.

Acknowledgments

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References

1) Chen, T. H., Hsu, Y. T., Chen, C. H., Kao, S. H., and Lee, H. M., Tanshinone IIA from *Salvia miltiorrhiza* induces heme oxygenase-1 expression and inhibits lipopolysaccharide-induced nitric oxide expression in RAW 264.7 cells. *Mitochondrion*, **7**, 101–105 (2007).
2) Wang, N., Luo, H. W., Niwa, M., and Ji, J., A new platelet aggregation inhibitor from *Salvia miltiorrhiza*. *Planta Med.*, **55**, 390–391 (1989).
3) Park, E. J., Zhao, Y. Z., Kim, Y. C., and Sohn, D. H., PF2401-SF, standardized fraction of *Salvia miltiorrhiza* and its constituents, tanshinone I, tanshinone IIA, and cryptotanshinone, protect primary cultured rat hepatocytes from bile acid-induced apoptosis by inhibiting JNK phosphorylation. *Food Chem. Toxicol.*, **45**, 1891–1898 (2007).
4) Wang, X., Bastow, K. F., Sun, C. M., Lin, Y. L., Yu, H. J., Don, M. J., Wu, T. S., Nakamura, S., and Lee, K. H., Antitumor agents. 239. Isolation, structure elucidation, total synthesis, and anti-breast cancer activity of neo-tanshinlactone from *Salvia miltiorrhiza*. *J. Med. Chem.*, **47**, 5816–5819 (2004).
5) Liu, J., Shen, H. M., and Ong, C. N., *Salvia miltiorrhiza* inhibits cell growth and induces apoptosis in human hepatoma HepG(2) cells. *Cancer Lett.*, **153**, 85–93 (2000).
6) Franek, K. J., Zhou, Z., Zhang, W. D., and Chen, W. Y., *In vitro* studies of baicalin alone or in combination with *Salvia miltiorrhiza* extract as a potential anti-cancer

agent. *Int. J. Oncol.*, **26**, 217–224 (2005).
7) Lee, D.-S., Lee, S.-H., Kwon, G.-S., Lee, H.-K., Woo, J.-H., Kim, J.-G., and Hong, S.-D., Inhibition of DNA topoisomerase I by dihydrotanshinone I, components of a medicinal herb *Salvia miltiorrhiza* Bunge. *Biosci. Biotechnol. Biochem.*, **63**, 1370–1373 (1999).
8) Lee, D. S., and Lee, S. H., Biological activity of dihydrotanshinone I: effect on apoptosis. *J. Biosci. Bioeng.*, **89**, 292–293 (2000).
9) Sessa, G., and Weissmann, G., Phospholipid spherules (liposomes) as a model for biological membranes. *J. Lipid Res.*, **9**, 310–318 (1968).
10) Weinstein, J. N., and Leserman, L. D., Liposomes as drug carriers in cancer chemotherapy. *Pharmacol. Ther.*, **24**, 207–233 (1984).
11) Swenson, C. E., Bolcsak, L. E., Batist, G., Guthrie, T. H., Jr., Tkaczuk, K. H., Boxenbaum, H., Welles, L., Chow, S. C., Bhamra, R., and Chaikin, P., Pharmacokinetics of doxorubicin administered i.v. as Myocet (TLC D-99; liposome-encapsulated doxorubicin citrate) compared with conventional doxorubicin when given in combination with cyclophosphamide in patients with metastatic breast cancer. *Anticancer Drugs*, **14**, 239–246 (2003).
12) Harris, L., Batist, G., Belt, R., Rovira, D., Navari, R., Azarnia, N., Welles, L., and Winer, E., Liposome-encapsulated doxorubicin compared with conventional doxorubicin in a randomized multicenter trial as first-line therapy of metastatic breast carcinoma. *Cancer*, **94**, 25–36 (2002).
13) Klibanov, A. L., Maruyama, K., Torchilin, V. P., and Huang, L., Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.*, **268**, 235–237 (1990).
14) Leserman, L. D., Barbet, J., Kourilsky, F., and Weinstein, J. N., Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature*, **288**, 602–604 (1980).
15) Leserman, L. D., Weinstein, J. N., Moore, J. J., and Terry, W. D., Specific interaction of myeloma tumor cells with hapten-bearing liposomes containing methotrexate and carboxyfluorescein. *Cancer Res.*, **40**, 4768–4774 (1980).
16) Torchilin, V. P., Klibanov, A. L., Huang, L., O'Donnell, S., Nossiff, N. D., and Khaw, B. A., Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. *FASEB J.*, **6**, 2716–2719 (1992).
17) Blume, G., Cevc, G., Crommelin, M. D., Bakker-Woudenberg, I. A., Kluft, C., and Storm, G., Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. *Biochim. Biophys. Acta*, **1149**, 180–184 (1993).
18) Torchilin, V. P., Levchenko, T. S., Lukyanov, A. N., Khaw, B. A., Klibanov, A. L., Rammohan, R., Samokhin, G. P., and Whiteman, K. R., p-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains *via* p-nitrophenylcarbonyl groups. *Biochim. Biophys. Acta*, **1511**, 397–411 (2001).
19) Murray, C. J., and Lopez, A. D., Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet*, **349**, 1498–1504 (1997).
20) Pisani, P., Parkin, D. M., Bray, F., and Ferlay, J., Estimates of the worldwide mortality from 25 cancers in 1990. *Int. J. Cancer*, **83**, 18–29 (1999).
21) Cli, F., Celik, I., Aykan, F., Uner, A., Demirkazik, A., Ozet, A., Ozguroglu, M., Tas, F., Akbulut, H., and Firat, D., A randomized phase III trial of etoposide, epirubicin, and cisplatin versus 5-fluorouracil, epirubicin, and cisplatin in the treatment of patients with advanced gastric carcinoma. *Cancer*, **83**, 2475–2480 (1998).
22) Bauer, W., Briner, U., Doepfner, W., Haller, R., Huguenin, R., Marbach, P., Petcher, T. J., and Pless, J., SMS 201-995: a very potent and selective octapeptide analogue of somatostatin with prolonged action. *Life Sci.*, **31**, 1133–1140 (1982).
23) Lamberts, S. W., Oosterom, R., Neufeld, M., and del Pozo, E., The somatostatin analog SMS 201-995 induces long-acting inhibition of growth hormone secretion without rebound hypersecretion in acromegalic patients. *J. Clin. Endocrinol. Metab.*, **60**, 1161–1165 (1985).
24) Miller, G. V., Farmery, S. M., Woodhouse, L. F., and Primrose, J. N., Somatostatin binding in normal and malignant human gastrointestinal mucosa. *Br. J. Cancer*, **66**, 391–395 (1992).
25) Szepeshazi, K., Schally, A. V., Nagy, A., Wagner, B. W., Bajo, A. M., and Halmos, G., Preclinical evaluation of therapeutic effects of targeted cytotoxic analogs of somatostatin and bombesin on human gastric carcinomas. *Cancer*, **98**, 1401–1410 (2003).
26) Brooks, P. C., Role of integrins in angiogenesis. *Eur. J. Cancer*, **32A**, 2423–2429 (1996).
27) Stromblad, S., Becker, J. C., Yebra, M., Brooks, P. C., and Cheresh, D. A., Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin alphaVbeta3 during angiogenesis. *J. Clin. Invest.*, **98**, 426–433 (1996).
28) Stromblad, S., and Cheresh, D. A., Integrins, angiogenesis and vascular cell survival. *Chem. Biol.*, **3**, 881–885 (1996).
29) Chung, T. W., Yang, M. G., Liu, D. Z., Chen, W. P., Pan, C. I., and Wang, S. S., Enhancing growth human endothelial cells on Arg-Gly-Asp (RGD) embedded poly (epsilon-caprolactone) (PCL) surface with nanometer scale of surface disturbance. *J. Biomed. Mater. Res. A*, **72**, 213–219 (2005).
30) Ho, Y. S., Wu, C. H., Chou, H. M., Wang, Y. J., Tseng, H., Chen, C. H., Chen, L. C., Lee, C. H., and Lin, S. Y., Molecular mechanisms of econazole-induced toxicity on human colon cancer cells: G0/G1 cell cycle arrest and caspase 8-independent apoptotic signaling pathways. *Food Chem. Toxicol.*, **43**, 1483–1495 (2005).
31) Osborne, C. K., Coronado, E. B., and Robinson, J. P., Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur. J. Cancer Clin. Oncol.*, **23**, 1189–1196 (1987).
32) Lee, W. S., Chen, R. J., Wang, Y. J., Tseng, H., Jeng, J. H., Lin, S. Y., Liang, Y. C., Chen, C. H., Lin, C. H., Lin, J. K., Ho, P. Y., Chu, J. S., Ho, W. L., Chen, L. C., and Ho, Y. S., *In vitro* and *in vivo* studies of the anticancer action of terbinafine in human cancer cell lines: G0/G1 p53-associated cell cycle arrest. *Int. J. Cancer*, **106**, 125–137 (2003).

33) Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U., and Stein, H., Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.*, **133**, 1710–1715 (1984).

34) Inda, A. M., Andrini, L. B., Garcia, M. N., Garcia, A. L., Fernandez Blanco, A., Furnus, C. C., Galletti, S. M., Prat, G. D., and Errecalde, A. L., Evaluation of angiogenesis with the expression of VEGF and CD34 in human non-small cell lung cancer. *J. Exp. Clin. Cancer Res.*, **26**, 375–378 (2007).

35) Huang, C. M., Wu, Y. T., and Chen, S. T., Targeting delivery of paclitaxel into tumor cells *via* somatostatin receptor endocytosis. *Chem. Biol.*, **7**, 453–461 (2000).

36) Koning, G. A., Schiffelers, R. M., Wauben, M. H., Kok, R. J., Mastrobattista, E., Molema, G., ten Hagen, T. L., and Storm, G., Targeting of angiogenic endothelial cells at sites of inflammation by dexamethasone phosphate-containing RGD peptide liposomes inhibits experimental arthritis. *Arthritis Rheum.*, **54**, 1198–1208 (2006).

37) Xiong, X. B., Huang, Y., Lu, W. L., Zhang, X., Zhang, H., Nagai, T., and Zhang, Q., Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for improved antitumor efficacy: *in vitro* and *in vivo*. *J. Pharm. Sci.*, **94**, 1782–1793 (2005).

38) Xiong, X. B., Huang, Y., Lu, W. L., Zhang, X., Zhang, H., Nagai, T., and Zhang, Q., Enhanced intracellular delivery and improved antitumor efficacy of doxorubicin by sterically stabilized liposomes modified with a synthetic RGD mimetic. *J. Control. Release*, **107**, 262–275 (2005).

39) Zhang, Z. W., Patchett, S. E., and Farthing, M. J., Topoisomerase I inhibitor (camptothecin)-induced apoptosis in human gastric cancer cells and the role of wild-type p53 in the enhancement of its cytotoxicity. *Anticancer Drugs*, **11**, 757–764 (2000).

40) Ikegami, T., Matsuzaki, Y., Al Rashid, M., Ceryak, S., Zhang, Y., and Bouscarel, B., Enhancement of DNA topoisomerase I inhibitor-induced apoptosis by ursodeoxycholic acid. *Mol. Cancer Ther.*, **5**, 68–79 (2006).

41) Morris, E. J., and Geller, H. M., Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *J. Cell Biol.*, **134**, 757–770 (1996).