Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1015–1017

Synthesis of angiogenesis-targeted peptide and hydrophobized polyethylene glycol conjugate

Noriyuki Maeda,[a,b] Yoshito Takeuchi,[a] Miki Takada,[a] Yukihiro Namba[b] and Naoto Oku[a,*]

[a]*Department of Medical Biochemistry and COE Program in the 21st Century, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan*
[b]*Nippon Fine Chemical Co., Ltd, Takasago, Hyogo, Japan*

Received 8 September 2003; accepted 20 November 2003

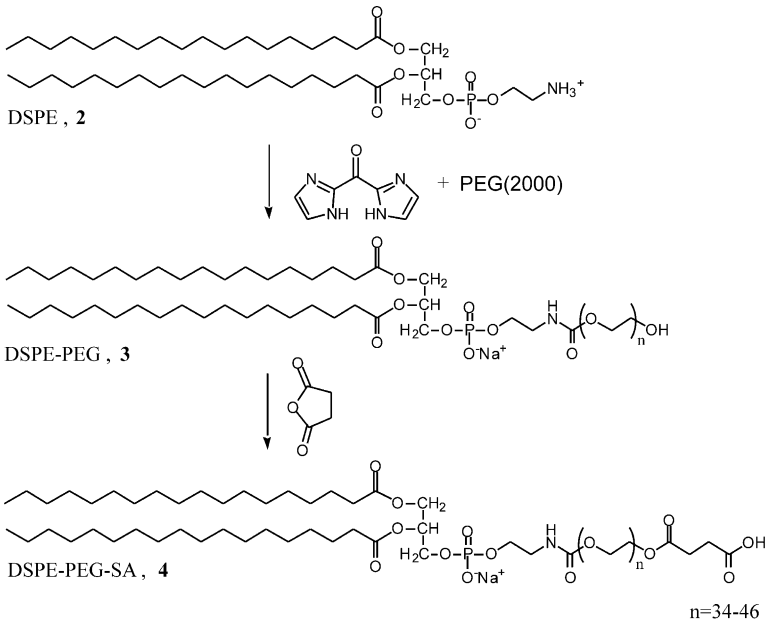
Abstract—For the purpose of cancer antineovascular therapy, a novel angiogenesis-targeted peptide, Ala-Pro-Arg-Pro-Gly, (APRPG) was attached to hydrophobized polyethylene glycol (distearoylphosphatidylethanolamine [DSPE]-PEG). DSPE-PEG and the 5-mer peptide were condensed with DCC-HOBt method. Liposome modified with this DSPE-PEG-APRPG conjugate highly accumulated in tumor of tumor-bearing mice.

Anigiogenesis is a critical event for maintenance, proliferation and metastasis of tumor,[1,2] and antineovascular therapy that causes tumor regression through damaging neovessel endothelial cells is expected to suppress both primary tumor and metastasis without aquiring drug resistance. The therapy is also expected for a broad spectrum of cancers. We previously isolated a peptide specifically bound to tumor angiogenic vasculature from a phage-displayed peptide library. The epitope sequence of the peptide was determined as APRPG.[3] In fact, APRPG-modified liposome highly accumulated in tumor tissue, and the liposome encapsulating anticancer drugs strongly suppressed tumor growth.[3,4] On the other hand, it is known that polyethylene glycol (PEG) conjugate liposome has long-circulating characteristics through avoidance of trapping by the reticuloendothelial system (RES) such as liver and spleen.[4,5] Furthermore, since liposomes have a tendency to passively accumulate in tumor tissues due to their enhanced permeability, long-circulating liposomes are more favorable for tumor targeting than conventional liposomes. Therefore, we aimed to endow angiogenesis-targeted liposome with long circulating character by the PEG conjugation. Conjugate **1** is the designed compound composed of phospholipid, polyethylene glycol and APRPG peptide. We synthesized DSPE-PEG-SA (**4**) and APRPG peptide separately, and then condensed them to obtain conjugate **1**. DSPE-PEG-SA was prepared as shown in Scheme 1. First, distearoylphosphatidylethanolamine (DSPE; **2**) 15.0 g and carbonyl diimidazole (CDI) 3.9 g were dissolved in 70 mL of toluene. Reaction was done at 100 °C for 1 h after addition of triethyl amine 2.0 g. Then polyethylene glycol (average molecular weight of 2000) 40.0 g dissolved in toluene was added dropwise to the solution. Solvent was evaporated in vacuo followed by the reaction, and the product was dissolved in acetone 500 mL and insoluble materials were filtrated and solvent was evaporated. The reaction mixture was exchanged into Na^+ salt with ion exchange resin. Purification by column chromatography on silica gave 11.4 g of the desired product **3** in a 26% yield. In order to use the PEG-end of obtained DSPE-PEG as a carboxylic group (referred to as **4**), it was allowed to react with succinic anhydride 2.1 g in the presence of pyridine 1.7 g in 100 mL of toluene. After powdering with ether, the yield of **4** was 80%.[6]

Preparation of APRPG peptide moiety was carried out by the liquid-phase method as shown in Scheme 2. *N*,*N*′-Dicyclohexylcarbodiimide (DCC, 1.1 equiv based on peptide) and 1-hydroxybenzotriazol (HOBt, 1.1 equiv based on peptide) were used for peptide coupling in DMF. HCl in 1,4-dioxane was used for deprotection of

* Corresponding author. Tel.: +81-54-264-5701; fax: +81-54-264-5705; e-mail: oku@u-shizuoka-ken.ac.jp

0960-894X/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.11.073

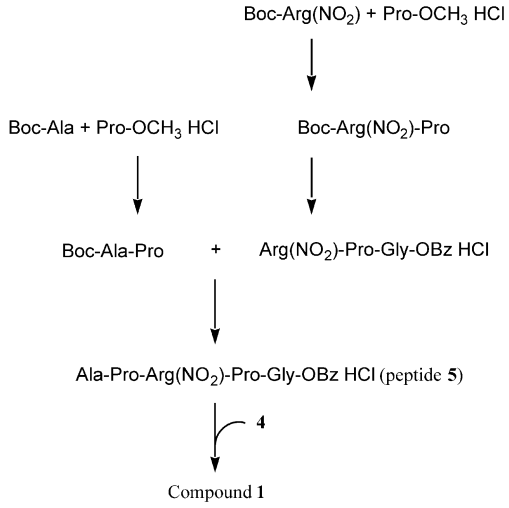


Scheme 1. Pathway for synthesis of DSPE-PEG-SA.

the Boc group of N-terminal and NaOH was used for deprotection of methyl ester group of C-terminal in water and methanol. In order to avoid racemization, segment condensation was proceeded between Boc-Ala-Pro and Arg(NO_2)-Pro-Gly-OBz to yield 78% of Boc-Ala-Pro-Arg(NO_2)-Pro-Gly-OBz. Next the Boc protecting group was deprotected by HCl in 1,4-dioxane to obtain peptide **5**.[7]

Peptide **5** was condensed with **4** (0.93 equiv based on **5**) in $CHCl_3$ by DCC (1 equiv based on **5**) and HOBt (1 equiv based on **5**). The progress of the reaction was monitored by TLC. The reaction was almost complete overnight without any serious side reactions. It was purified by column chromatography on silica. The yield was 83% based on **4**. Deprotections of NO_2 group of arginine side chain and benzyl ester group of glycine C-terminal were carried out by 10% palladium–carbon catalytic reduction under hydrogen atomosphere in methanol. It was purified by column chromatography on silica and ion exchange resin. This compound of single spot on TLC was in a 43% yield. This conjugate **1** was positive for Sakaguchi reagent, while negative for UV lamp on TLC. These showed that NO_2 protecting group and benzyl ester protecting group were deprotected simultaneously (Fig. 1).[8]

Thus obtained DSPE-PEG-APRPG was used for the modification of liposome, and we examined the biodistribution of PEG-APRPG conjugate liposome in



Scheme 2. Pathway for synthesis of conjugate **1**.

tumor-bearing model mice. Colon 26 NL-17 carcinoma cells (1.0×10^6 cells/mouse) were subcutaneously implanted in 5-week-old BALB/c male mice, and biodistribution study was performed when the tumor size had become about 10 mm in diameter. PEG-APRPG liposome was composed of distearoylphosphatidylcholine (DSPC), cholesterol and conjugate **1** (10:5:1 as a molar ratio) with trace of [^{3}H]cholesterol oleoyl ether

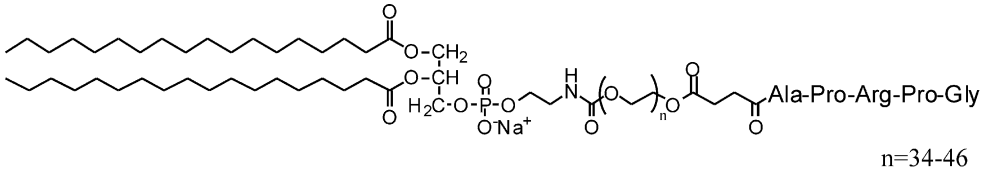


Figure 1. Structure of peptide-PEG-lipid conjugate **1**.

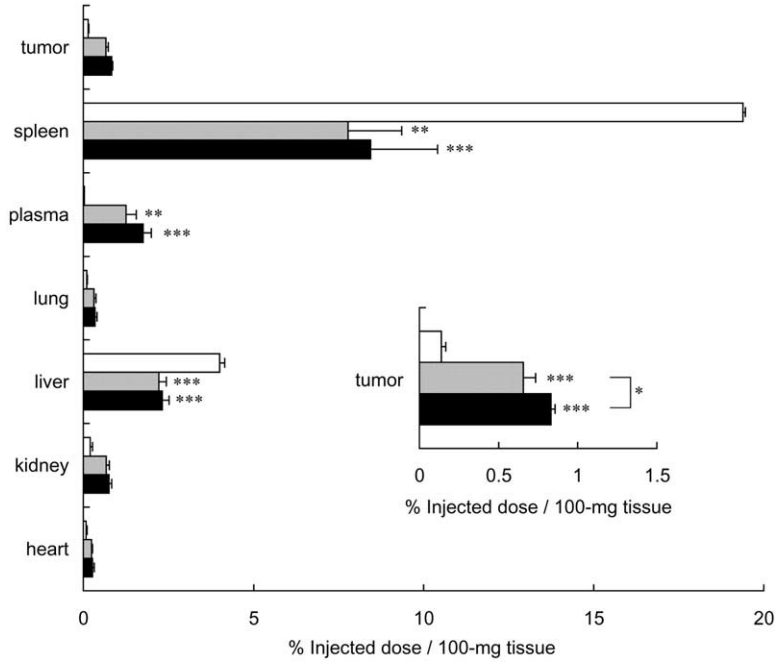


Figure 2. Biodistribution of PEG-APRPG modified liposome. Data are presented as the percentage of the injected dose per 100 mg tissue and S.D. in each tissue (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Open bars, non-modified liposome; shadow bars, PEG liposome; closed bars, PEG-APRPG liposome.

(74 kBq/mouse). PEG liposome composed of DSPC, cholesterol and DSPE-PEG; **3** (10:5:1) and non-modified liposome composed of DSPC, cholesterol (10:5) were also radiolabeled and used as control. Liposomes were hydrated with 0.3 M glucose solution and freeze-thawed for three cycles following the thrice extrusion through polycarbonate membrane filter with a 100 nm pore size. Size-matched Colon 26 NL-17 carcinoma-bearing mice were injected with the radiolabeled liposomes via the tail vein. At 24 h after the injection, the mice were sacrificed under diethyl ether anesthesia for obtaining the blood, heart, lung, liver, spleen, kidney, and tumor. After weighing each organ, the radioactivity was determined with a liquid scintillation counter. The results are shown in Figure 2.

The results indicate that PEG conjugate endowed liposomes with long-circulating character through avoidance of RES trapping. Furthermore, PEG-APRPG conjugate liposome accumulated in tumor more than PEG liposome at 24 h after the injection, suggesting that not only PEGylation enhanced passive targeting to tumor tissue through leaky endothelium of the tissue but also APRPG conjugation enhanced active targeting to the tumor angiogenic vasculature. Therefore, PEG-APRPG conjugate liposome developed in this study would be useful tools for anti-neovascular therapy.

References and notes

1. Folkman, J.; D'Amore, P. A. *Cell* **1996**, *87*, 1153.
2. O'Reilly, M. S.; Holmgren, L.; Chen, C.; Folkman, J. *Nat. Med.* **1996**, *2*, 689.
3. Oku, N.; Asai, T.; Watanabe, K.; Kuromi, K.; Nagatsuka, M.; Kurohane, K.; Kikkawa, H.; Ogino, K.; Tanaka, M.; Ishikawa, D.; Tsukada, H.; Momose, M.; Nakayama, J.; Taki, T. *Oncogene* **2002**, *21*, 2662.
4. Asai, T.; Shimizu, K.; Kondo, M.; Kuromi, K.; Watanabe, K.; Ogino, K.; Taki, T.; Shuto, S.; Matsuda, A.; Oku, N. *FEBS Lett.* **2002**, *520*, 167.
5. Woodle, M. C., Storm, G., Eds. *Long Circulating Liposomes: Old Drugs, New Therapeutics*; Springer-Verlag: Berlin, 1998.
6. Selected data for compound **4**: ^{1}H NMR (90 MHz, $CDCl_3$): δ 0.78–1.40 (66H, dialkyl H), 2.33 (4H, brt, CO–CH_2), 3.64 (160H, brs, PEG-H), 3.85–4.50 (9H, m, glycerol-H and O–CH_2–CH_2–N). FAB-MS m/z: 2314 + 44n (n = 0–13) $[M+H]^+$. R_f = 0.45 ($CHCl_3/CH_3OH$ = 4/1, v/v).
7. Selected data for compound **5**: ^{1}H NMR (600 MHz, CD_3OD): δ 1.52 (3H, d, J = 6.90, alanine CH_3), 1.75–2.27 (12H, m, proline C–CH_2–C and arginine C–CH_2–C), 3.22–3.34 (6H, m, proline N–CH_2–C and arginine N–CH_2–C), 3.59 (1H, m, arginine C*H*), 3.73 (2H, m, proline C*H*), 4.28 (1H, brq, alanine C*H*), 4.47 (2H, m, glycine CH_2), 5.15 (2H, dd, J = 12.3, 14.9, benzyl CH_2), 7.30–7.35 (5H, m, benzyl C_6H_5). FAB-MS m/z: 632.2 $[M+H]^+$.
8. Selected data for compound **1**, FAB-MS m/z: 2912 + 44n (n = 0–13) $[M+H]^+$. R_f = 0.74 ($CHCl_3/CH_3OH/H_2O$ = 60/30/5, v/v/v).