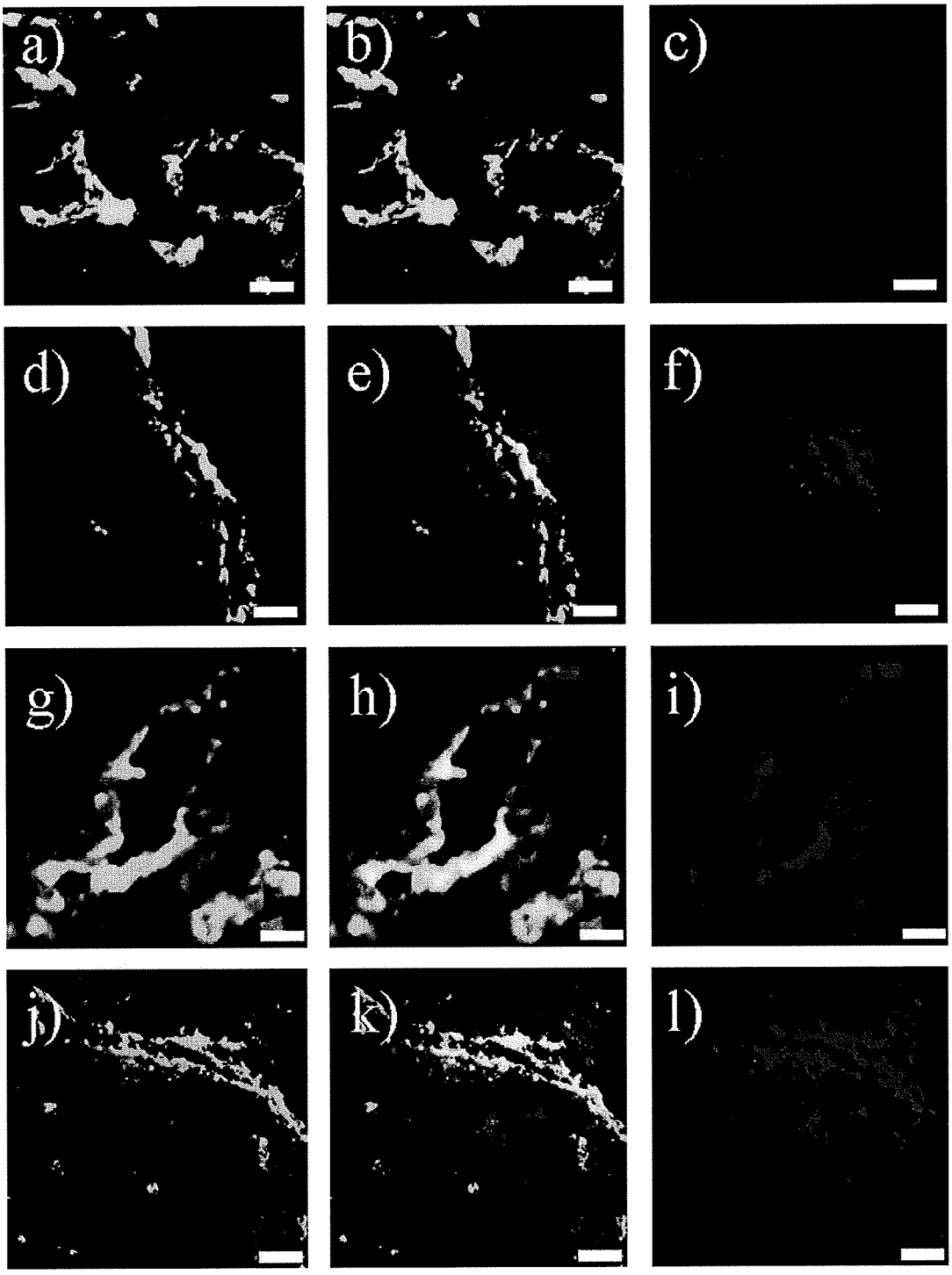


**Fig. 4.** Localization of Dual-PEG-Lip in the tumor. Colon26 NL-17-bearing mice were intravenously injected with PEG-Lip (a–c), PRP-PEG-Lip (d–f), NGR-PEG-Lip (g–i) or Dual-PEG-Lip (j–l) labeled with $DiIC_{18}$ at day 10 after tumor implantation. At 3 h after injection, the tumors were dissected, and then frozen-sections (10 μm) were prepared. Immunofluorescence staining for CD31 was performed to visualize endothelial cells. Green images indicate CD31-positive regions (a, d, g and j), and red images show liposomal distribution (c, f, i and l). Panels b, e, h and k represent the merged images of them. Yellow portions indicate the localization of liposomes at the site of vascular endothelial cells. Scale bars represent 20 μm.

of liposomes to tumor tissue. However, intratumoral distribution of Dual-PEG-Lip in Colon26 NL-17-bearing mice was obviously different. The results showed that targeting liposomes bound to angiogenic vessels whereas PEG-Lip did not. Moreover, Dual-PEG-Lip showed further enhanced targeting activity to angiogenic vessels in comparison to PRP-PEG-Lip or NGR-PEG-Lip along with the result from the *in vitro* association experiment. Thus, the association

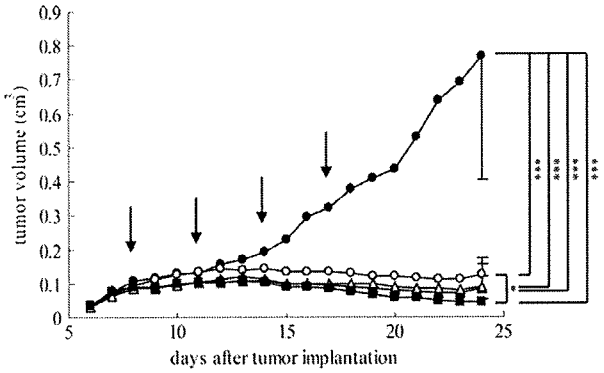


**Fig. 5.** Therapeutic effect of Dual-PEG-DOX in tumor-bearing mice. Colon26 NL-17-bearing mice ($n = 5$) were intravenously injected with PBS (closed circles), PEG-DOX (open circles), PRP-PEG-DOX (closed triangles), NGR-PEG-DOX (open triangles) or Dual-PEG-DOX (closed squares) at days 8, 11, 14 and 17 after tumor implantation. Injected dose of liposomal DOX were 3 mg/kg as DOX in each administration. Tumor volume and body weight (data not shown) of tumor-bearing mice were monitored. Data represent the mean tumor volume and SD, where the SD bars are shown only for the last points (day 24). Arrows show the days of injection. Asterisks show the significant differences: $^{*}P < 0.05$ and $^{***}P < 0.001$.

with angiogenic vessels *in vivo* was also improved effectively by use of dual-targeting liposomes. In our previous studies, we demonstrated that DOX encapsulated in angiogenic vessel-targeted liposomes was localized exclusively to angiogenic endothelial cells and damaged them [25]. Finally the therapeutic effect of Dual-PEG-DOX against the solid tumor was examined. Our data showed that Dual-PEG-DOX strongly suppressed the tumor growth compared with other formulations due to their potent targeting ability to angiogenic vessels. The therapeutic effects of Dual-PEG-DOX might be further enhanced by adjusting the amount of DOX in the liposomes. These findings give enough evidences for usefulness of dual-targeting liposomes in ANET.

From the result obtained in this study, it would be expected that "dual-targeting" is a useful targeting strategy for ANET. For example, combination of peptides used here and RGD would be promising approach. In addition, the investigation about the rate of peptides on dual-targeting liposomal surface would be interesting since we modified an equal amount of APRPG and GNGRG as a tentative rate. The most notable finding presented here is the fact that the targeting ability of liposomes was enhanced by dual-targeting. Dual-targeting would be available for a number of targeting therapies because most of target organs express multiple address molecules.

## Conflicts of interest

None declared.

## Acknowledgement

This research was supported by Grant-in-Aid for Scientific Research on Priority Areas.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.06.008.

## References

[1] T. Sakakibara, F.A. Chen, H. Kida, K. Kunieda, R.E. Cuenca, F.J. Martin, R.B. Bankert, Doxorubicin encapsulated in sterically stabilized liposomes is superior to free drug or drug-containing conventional liposomes at suppressing growth and metastases of human lung tumor xenografts, Cancer Res. 56 (1996) 3743–3746.
[2] D.D. Lasic, Doxorubicin in sterically stabilized liposomes, Nature 380 (1996) 561–562.
[3] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[4] F.M. Muggia, Doxorubicin-polymer conjugates: further demonstration of the concept of enhanced permeability and retention, Clin. Cancer Res. 5 (1999) 7–8.
[5] J.W. Park, K. Hong, D.B. Kirpotin, O. Meyer, D. Papahadjopoulos, C.C. Benz, Anti-HER2 immunoliposomes for targeted therapy of human tumors, Cancer Lett. 118 (1997) 153–160.
[6] X.B. Xiong, Y. Huang, W.L. Lu, X. Zhang, H. Zhang, T. Nagai, Q. Zhang, Intracellular delivery of doxorubicin with RGD-modified sterically stabilized liposomes for an improved antitumor efficacy: in vitro and in vivo, J. Pharm. Sci. 94 (2005) 1782–1793.
[7] C. Brignole, D. Marimpietri, C. Gambini, T.M. Allen, M. Ponzoni, F. Pastorino, Development of Fab' fragments of anti-GD(2) immunoliposomes entrapping doxorubicin for experimental therapy of human neuroblastoma, Cancer Lett. 197 (2003) 199–204.
[8] F. Pastorino, C. Brignole, D. Marimpietri, P. Sapra, E.H. Moase, T.M. Allen, M. Ponzoni, Doxorubicin-loaded Fab' fragments of anti-disialoganglioside immunoliposomes selectively inhibit the growth and dissemination of human neuroblastoma in nude mice, Cancer Res. 63 (2003) 86–92.
[9] O. Ishida, K. Maruyama, H. Tanahashi, M. Iwatsuru, K. Sasaki, M. Eriguchi, H. Yanagie, Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo, Pharm. Res. 18 (2001) 1042–1048.
[10] J. Folkman, P.A. D'Amore, Blood vessel formation: what is its molecular basis?, Cell 87 (1996) 1153–1155
[11] M.S. O'Reilly, L. Holmgren, C. Chen, J. Folkman, Angiostatin induces and sustains dormancy of human primary tumors in mice, Nat. Med. 2 (1996) 689–692.
[12] M. Skobe, P. Rockwell, N. Goldstein, S. Vosseler, N.E. Fusenig, Halting angiogenesis suppresses carcinoma cell invasion, Nat. Med. 3 (1997) 1222–1227.
[13] T. Browder, C.E. Butterfield, B.M. Kraling, B. Shi, B. Marshall, M.S. O'Reilly, J. Folkman, Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer, Cancer Res. 60 (2000) 1878–1886.
[14] N. Oku, T. Asai, K. Watanabe, K. Kuromi, M. Nagatsuka, K. Kurohane, H. Kikkawa, K. Ogino, M. Tanaka, D. Ishikawa, H. Tsukada, M. Momose, J. Nakayama, T. Taki, Anti-neovascular therapy using novel peptides homing to angiogenic vessels, Oncogene 21 (2002) 2662–2669.
[15] R.K. Jain, Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy, Nat. Med. 7 (2001) 987–989.
[16] T. Asai, K. Shimizu, M. Kondo, K. Kuromi, K. Watanabe, K. Ogino, T. Taki, S. Shuto, A. Matsuda, N. Oku, Anti-neovascular therapy by liposomal DPP-CNDAC targeted to angiogenic vessels, FEBS Lett. 520 (2002) 167–170.
[17] W. Arap, R. Pasqualini, E. Ruoslahti, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model, Science 279 (1998) 377–380.
[18] R. Pasqualini, E. Koivunen, R. Kain, J. Lahdenranta, M. Sakamoto, A. Stryhn, R.A. Ashmun, L.H. Shapiro, W. Arap, E. Ruoslahti, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis, Cancer Res. 60 (2000) 722–727.
[19] E. Ruoslahti, RGD and other recognition sequences for integrins, Annu. Rev. Cell Dev. Biol. 12 (1996) 697–715.

[20] E. Koivunen, B. Wang, E. Ruoslahti, Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins, Biotechnology (NY) 13 (1995) 265–270.
[21] N. Maeda, Y. Takeuchi, M. Takada, Y. Sadzuka, Y. Namba, N. Oku, Anti-neovascular therapy by use of tumor neovasculature-targeted long-circulating liposome, J. Control. Release 100 (2004) 41–52.
[22] F. Pastorino, C. Brignole, D. Marimpietri, M. Cilli, C. Gambini, D. Ribatti, R. Longhi, T.M. Allen, A. Corti, M. Ponzoni, Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy, Cancer Res. 63 (2003) 7400–7409.
[23] N. Oku, K. Doi, Y. Namba, S. Okada, Therapeutic effect of adriamycin encapsulated in long-circulating liposomes on Meth-A-sarcoma-bearing mice, Int. J. Cancer 58 (1994) 415–419.
[24] J.M. Saul, A.V. Annapragada, R.V. Bellamkonda, A dual-ligand approach for enhancing targeting selectivity of therapeutic nanocarriers, J. Control. Release 114 (2006) 277–287.
[25] K. Shimizu, T. Asai, C. Fuse, Y. Sadzuka, T. Sonobe, K. Ogino, T. Taki, T. Tanaka, N. Oku, Applicability of anti-neovascular therapy to drug-resistant tumor: Suppression of drug-resistant P388 tumor growth with neovessel-targeted liposomal adriamycin, Int. J. Pharm. 296 (2005) 133–141.