Effect of an Endothelin B Receptor Agonist on the Tumor Accumulation of Nanocarriers

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Enhancing blood flow to tumors is a prominent strategy for improving the tumor accumulation of macromolecular drugs through the enhanced permeability and retention (EPR) effect. IRL-1620 is an agonist of the endothelin B receptor, and is a promising molecule to enhance tumor blood flow by activating endothelial nitric oxide synthase. However, contradictory effects on tumor blood flow modulation have been reported because the effects of IRL-1620 may differ in different animal models. Here, we examined for the first time the effect of IRL-1620 on the EPR effect for PEGylated liposomes in a CT-26 murine colon cancer model. Co-injection of IRL-1620 at an optimum dose (3 nmol/kg) nearly doubled the tumor accumulation of liposomes compared with controls, indicating that IRL-1620 enhanced the EPR effect in the present colon cancer model. Co-injection of IRL-1620 is a promising strategy to improve the therapeutic effects of macromolecular drugs while reducing their side effects.

Key words enhanced permeability and retention effect; endothelin B receptor; liposome; tumor accumulation

INTRODUCTION

Selective and substantial accumulation of anti-cancer drugs in tumors is crucial to maximize therapeutic efficacy and reduce adverse effects. Recently, vasodilators have been utilized to increase the tumor accumulation of anti-cancer drugs to improve the efficacy of chemotherapy. 1 This approach has been utilized for small molecular anti-cancer drugs 2 but also for macromolecular drugs. 3-5 Macromolecular drugs accumulated more specifically in tumors and showed extended retention because of the unique features of tumor tissues, such as the insufficient formation of tight junctions by the vascular endothelium and undeveloped lymphatic vessels. This is called the enhanced permeability and retention (EPR) effect. 4, 5

As vasodilators, nitric oxide (NO) donating molecules have been utilized by many research groups including us to enhance the EPR effect for macromolecular drugs. 6-9 However, most NO donors are unstable molecules and spontaneously release NO, which limits their practical application. In vivo, NO could also be produced by endothelial NO synthase (eNOS) in the endothelium. 10 Therefore, activation of eNOS is an alternative strategy to attain NO-induced vasodilation.

Several ligand/receptor combinations are known to directly activate eNOS, such as the vascular endothelial growth factor (VEGF)/VEGF receptor, the bradykinin/B2 receptor, and the endothelin-1/endothelin B (ETB) receptor. 11 Among these, a combination of the endothelin-1/ETB receptor has been utilized to enhance the tumor accumulation of small molecule anti-cancer drugs. IRL-1620, derived from endothelin-1, is a highly selective and high affinity peptide agonist of the ETB receptor. 12 Gulati and colleagues reported that systemic injection of IRL-1620 resulted in increased blood flow in inoculated tumor tissue in rats. This resulted in the enhancement of the tumor accumulation of small anti-cancer drugs and tumor regression. 13, 14 Based on these reports, a phase II clinical trial in which co-administration of IRL-1620 and a small molecule anti-cancer drug was performed in patients with biliary tract cancer was reported. 15 Unfortunately, the trial failed to meet the predetermined endpoint. Severe side effects, because of the non-specific delivery of the anti-cancer drug, were observed. Moreover, contradictory effects of IRL-1620 on the modification of tumor blood flow were reported by Tozer and colleagues. They found that tumor blood flow was reduced in response to a systemic injection of IRL-1620 in their rat models. 16 Because the ETB receptor is involved in both vasodilation and vasoconstriction in organs, 17-20 the discrepancy between these two results may result from differences between different animal models.

In the current study, we examined for the first-time the effect of IRL-1620 on the EPR effect for nanoparticles. Here we used a liposome modified with polyethylene glycol as a nanoparticle and applied it in a BALB/c mouse grafted with murine CT-26 colon cancer cells. We found an improvement in the tumor accumulation of the liposomes via the EPR effect, which is promising in terms of increasing the therapeutic effects and reducing the side effects of macromolecular anti-cancer drugs.

MATERIALS AND METHODS

Materials and Reagents Hydrogenated soybean phosphatidylcholine (HSPC) and N-(carboxy-methoxypolyethylene-glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG) were purchased from NOF Corporation (Tokyo, Japan). Cholesterol was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide (XenoLight DiR) was supplied by Caliper Life Sciences (Hopkinton, MA, U.S.A.). IRL-1620 was purchased from Torcis Bioscience (Ellristol, MO, U.S.A.).

Preparation of Liposomes (LPs) LPs were prepared via the lipid–thin film hydration method. In the first step, solutions of HSPC, cholesterol, and DSPE-PEG in chloroform were mixed together. The molar ratio of the lipid components was HSPC : cholesterol : DSPE-PEG = 55 : 40 : 5, including 1 mol% of Xenolight DiR (vs. total lipids) as a fluorophore. Next, the chloroform was removed to create a lipid film using a rotary evaporator and a 60°C water bath to maintain the
temperature above the gel–liquid crystal transition temperature of HSPC (52°C). The lipid film was then dried completely under reduced pressure in a vacuum desiccator overnight. Next, the dried lipid film was hydrated to produce 100 mM lipids with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4, 5% glucose) by intermittently heating at 60°C and vortexing for 30 min. LPs were subsequently extruded through 200 nm (21 times) and then 50 nm polycarbonate membranes (41 times). The obtained LPs were stored at 4°C. The average hydrodynamic diameter and ζ potential were determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Worcestershire, U.K.).

**Cell Line** CT-26 cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics containing 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, and 0.25 µg mL⁻¹ amphotericin B (all were purchased from Gibco Invitrogen Co., Grand Island, NY, U.S.A.). The cells were maintained in a humidified incubator containing 5% CO₂ in air at 37°C.

**Mice** Male 5-week-old BALB/c mice were purchased from Kyudo Co., Ltd. (Saga, Japan). The experimental handling of mice was performed with the approval of the Ethics Committee for Animal Experiments (Kyushu University, Japan) and in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan).

**Effect of IRL-1620 on the Accumulation of LPs in Tumors** The CT-26 colon carcinoma model was used to investigate the tumor accumulation of LPs. Mice were inoculated through subcutaneous injections of 2×10⁶ CT-26 cells suspended in 100 µL of Hank’s balanced salt solution (Gibco Invitrogen Co.) into the dorsum after their fur was removed. The mice were then closely monitored until their tumors reached standard volumes of approximately 150 mm³. Tumor volume \( V \) was measured using the following formula: \( V (\text{mm}^3) = (L \times W^2)/2 \), where \( L \) and \( W \) represent the long and short dimensions of the tumor tissue, respectively. In the next step, animals were divided into three treatment groups which were intravenously (i.v.) injected with LPs, LPs plus 3 nmol/kg IRL-1620, or LPs plus 9 nmol/kg IRL-1620. In vivo photos to evaluate the fluorescence of DiR dye were taken using an IVIS® Lumina instrument (Xenogen, Alameda, CA, U.S.A.) every 6 h post-injection. At 48 h after injection, mice were sacrificed under isoflurane anesthesia using a blood collection method in which blood was harvested from the heart through a heparinized syringe. The plasma was extracted from the whole blood solution through centrifugation (600 × g) for 15 min. Tumors and major organs (lungs, spleens, hearts, kidneys, and livers) were excised, rinsed with DPBS, weighed,
and each organ was imaged to determine its DiR dye fluorescence using the IVIS® Lumina instrument. To quantify LP accumulation, all tissue samples were subsequently homogenized in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% triton X-100). Next, the tissue extracts were clarified through centrifugation at 12000 × g for 15 min, and the fluorescence intensity of each supernatant sample was measured at an excitation wavelength of 730 nm and an emission wavelength of 790 nm using an Infinite M Plex microplate reader (TECAN, Switzerland). To exclude all possible interference caused by endogenous species, the LP content in each organ or tumor tissue sample was determined from a standard curve after subtracting the background fluorescence intensity of the respective control tissue obtained from mice receiving no treatment.

**Statistical Analysis** All data are expressed as mean ± standard deviation (S.D.). Data were evaluated using one-way ANOVA and statistical analysis was performed using GraphPad Prism software. A value of $p \leq 0.05$ was considered to be statistically significant.

**RESULTS**

PEGylated LPs containing a near-IR fluorescent probe were prepared through a standard hydration method. LPs had a diameter of 72 nm with a narrow size distribution (polydispersity index: 0.041), and their $\zeta$-potential was −21 mV. These LP characteristics were suitable for the EPR effect.

We examined the effect of IRL-1620 on LP tumor accumulation. LPs were intravenously co-injected with IRL-1620 at different doses (3 and 9 nmol/kg). Time-dependent accumulation of LPs in the tumor was monitored through in vivo imaging (Figs. 1A, B). Compared with the control group, IRL-1620 at a dose of 3 nmol/kg significantly boosted the tumor accumulation of LPs. This significant enhancement appeared from 6 h post-injection, reached a plateau at 24 h and persisted until the final time point of 48 h (Fig. 1B). In contrast, a significant difference was not observed for a group of mice with a higher dose of IRL-1620 (9 nmol/kg). We excised tumors and other tissues at 48 h post-injection and measured their fluorescence intensity (Figs. 1C, S1). In tumor, mice injected with IRL-1620 at 3 nmol/kg demonstrated a nearly two-fold increase in the fluorescence intensity, while such a remarkable augmentation was not observed with the 9 nmol/kg dose.

To quantify the accumulated LPs in each organ, organs were resected and homogenized to measure the fluorescence intensity of the homogenates (Fig. 2). It was shown that tumor accumulation of LPs in the mice treated with 3 nmol/kg IRL-1620 was 70% higher than in those not treated with IRL-1620. Such a difference was not observed for the mice treated with a high dose of IRL-1620. A slight change in the LP accumulation was also detected in the liver and spleen at a dose of 3 nmol/kg.

![Fig. 2. Biodistribution of LPs in BALB/c Mice Bearing CT-26 Tumors 48 h Post-injection](image)

The amount of LPs in each tissue is expressed as % injected dose (I.D.)/g-tissue (or mL-plasma). Results are expressed as mean ± S.D. (n = 5). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared with control (no injection of IRL-1620).

![Fig. 3. Expected Mechanism of Enhancement in LP Tumor Accumulation Produced by Co-injected IRL-1620](image)

(A) LPs demonstrated tumor accumulation because of the EPR effect. (B) Co-administered IRL-1620 at an optimum dose induces ET$_B$ receptor signaling to produce NO via eNOS activation, which induces vasodilation of the tumor vasculature and weakening of epithelial tight junctions, thereby enhancing the tumor accumulation of LPs. Such enhancements in LP tumor accumulation were not observed for a high dose of IRL-1620, which may be caused by a desensitization in ET$_B$ signaling. (Color figure can be accessed in the online version.)
DISCUSSION

The effect of IRL-1620 on LP tumor accumulation is illustrated in Fig. 3. We found that an optimum dose of IRL (3 nmol/kg) modestly enhanced the accumulation of co-administered LPs in several organs such as the liver and spleen (Fig. 2), and substantially enhanced LP accumulation in the tumor. A larger dose (9 nmol/kg) resulted in almost no effect on biodistribution. The higher LP accumulation in tumors compared with that in the organs may stem from the higher expression of the ETB receptor in tumors than in other tissues.21,22 The enhancement of LP tumor accumulation was also highest with NO-donating LPs.23

An optimum dose of IRL in enhancing tumor blood perfusion was also observed in rat models by Gulati and colleagues.15,16 A high dosage of IRL-1620 would result in desensitization of the ETB receptor-mediated activation of eNOS which induces vasodilation and increases vascular permeability. Desensitization of ETB receptor-mediated vasodilatation was also reported for another ETB agonist sarafotoxin S6c in resected rabbit veins.24 Generally, receptor desensitization is an important feature of G protein-coupled receptors which could not only overstimulate and potential damage to activated cells, but saturation of the cells by weak stimuli.25 The existence of ETB desensitization with ETB agonists for vasodilation seems to be an advantage in terms of avoiding unwanted side effects.

According to a previous report, the modulation of tumor blood flow produced by IRL-1620 is a transient phenomenon and lasts for 30 min.29 However, in the present time course of accumulation (Fig. 1B), the difference between the two groups (LPs alone vs. optimum dose of IRL-1620) increased with time until 24 h. At present we cannot explain the discrepancy between the previous results and ours, but a continuous enhancement in LP tumor accumulation created by IRL-1620 would be advantageous.

CONCLUSION

The effects of IRL-1620 on tumor blood flow have been controversial because the effects may differ in different tumor models. In the present murine colon cancer model, we found that co-administration of IRL-1620 at an optimum dose (3 nmol/kg) nearly doubled the accumulation of LPs in tumors via the EPR effect. The enhanced accumulation of LPs was largest in tumors compared with other organs. This is the first report to utilize IRL-1620 to enhance the EPR effect of nanoparticles. For tumors in which IRL-1620 works to increase blood flow, a combination of IRL-1620 with macromolecular anticancer agents holds promise to boost therapeutic effects, while reducing adverse effects.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Sonveaux P. Provascular strategy: targeting functional adaptations of mature blood vessels in tumors to selectively influence the tumor vascular reactivity and improve cancer treatment. *Radiother. Oncol.*, 86, 300–313 (2008).

2) Yasuda H. Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. *Nitr. Oxide*, 19, 205–216 (2008).

3) Ojha T, Pathak V, Shi Y, Hennink WE, Moonen CT, Storm G, Kiessling F, Lammers T. Pharmacological and physical vessel modulation strategies to improve EPR-mediated drug targeting to tumors. *Adv. Drug Deliv. Rev.*, 119, 44–60 (2017).

4) Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumor-tropic accumulation of proteins and the antitumor agent smanes. *Cancer Res.*, 46, 6387–6392 (1986).

5) Maeda H, Tsukigawa K, Fang J. A retrospective 30 years after discovery of the enhanced permeability and retention effect of solid tumors: next-generation chemotherapeutics and photodynamic therapy—problems, solutions, and prospects. *Microcirculation*, 23, 173–182 (2016).

6) Kinoshita R, Ishima Y, Chuang VT, Nakamura H, Fang J, Watanabe H, Shimizu T, Okihira K, Ishida T, Maeda H, Otogiri M, Maruyama T. Improved antitumor effects of albumin-bound paclitaxel nanoparticle via augmentation of EPR effect and albumin–protein interactions using S-nitrosated human serum albumin dimer. *Biomaterials*, 140, 162–169 (2017).

7) Seki T, Fang J, Maeda H. Enhanced delivery of macromolecular antitumor drugs to tumors by nitroglycerin application. *Cancer Sci.*, 100, 2426–2430 (2009).

8) Tahara Y, Yoshikawa T, Sato H, Mori Y, Zahangir MH, Kishimura A, Mori T, Katayama Y. Encapsulation of a nitric oxide donor into a liposome to boost the enhanced permeation and retention (EPR) effect. *MedChemComm.*, 8, 415–421 (2016).

9) Islam W, Fang J, Imamura T, Etrich T, Subr V, Ulbrich K, Maeda H. Augmentation of the enhanced permeability and retention effect with nitric oxide–generating agents improves the therapeutic effects of nanomedicines. *Mol. Cancer Ther.*, 17, 2643–2653 (2018).

10) Wang PG, Xian M, Fang X, Wu X, Wen Z, Cai T, Janzczuk AJ. Nitric oxide donors: chemical activities and biological applications. *Chem. Rev.*, 102, 1091–1134 (2002).

11) Zhao Y, Vanhoutte PM, Leung SW. Vascular nitric oxide: Beyond eNOS. *J. Pharmacol. Sci.*, 129, 83–94 (2015).

12) Fleming I. Molecular mechanisms underlying the activation of eNOS. *Pflugers Arch.*, 459, 793–806 (2010).

13) Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, 332, 411–415 (1988).

14) Watakabe U, Urade Y, Takai M, Umemura I, Okada T. A reversible radioligand specific for the ETB receptor,[125I] Tyr13-Suc-[Glu9, Ala11, 15]-endothelin-1 (8–21), [125I] IRL 1620. *Biochem. Biophys. Res. Commun.*, 185, 867–873 (1992).

15) Rajeshkumar NV, Rai A, Gulati A. Endothelin B receptor agonist, IRL 1620, enhances the anti-tumor efficacy of paclitaxel in breast tumor rats. *Breast Cancer Res. Treat.*, 94, 237–247 (2005).

16) Rajeshkumar NV, Matwyslyn G, Gulati A, IRL-1620, a tumor selective vasodilator, augments the uptake and efficacy of chemotherapeutic agents in prostate tumor rats. *Prostate*, 67, 701–713 (2007).

17) Kim R, Chioorean EG, Amin M, Rocha-Lima CMS, Gandhi J, Harris WP, Song T, Portnoy D. Phase 2 study of combination SPI-1620 with docetaxel as second-line advanced biliary tract cancer treatment. *Br. J. Cancer*, 117, 189–194 (2017).

18) Bell KM, Chaplin DJ, Poole BA, Prise V, Tozer GM. Modification of blood flow in the HSN tumour and normal tissues of the rat by
the endothelin ETB receptor agonist, IRL 1620. *Int. J. Cancer*, **80**, 295–302 (1999).

19) Cemazar M, Wilson I, Prise VE, Bell KM, Hill SA, Tozer GM. The endothelin B (ET B) receptor agonist IRL 1620 is highly vasoconstrictive in two syngeneic rat tumour lines: potential for selective tumour blood flow modification. *Br. J. Cancer*, **93**, 98–106 (2005).

20) Bell KM, Prise V, Chaplin D, Tozer G. Tumour blood flow modification by endothelin-related peptides in the rat HSN fibrosarcoma. *Br. J. Cancer Suppl.*, **27**, S161–S163 (1996).

21) Alanen K, Deng D, Chakrabarti S. Augmented expression of endothelin-1, endothelin-3 and the endothelin-B receptor in breast carcinoma. *Histopathology*, **36**, 161–167 (2000).

22) Égidy G, Juillerat-Jeanneret L, Jeannin JF, Korth P, Bosman FT, Pinet F. Modulation of human colon tumor-stromal interactions by the endothelin system. *Am. J. Pathol.*, **157**, 1863–1874 (2000).

23) Yoshikawa T, Mori Y, Feng HT, Phan KQ, Kishimura A, Kang JH, Mori T, Katayama Y. Rapid and continuous accumulation of nitric oxide-releasing liposomes in tumors to augment the enhanced permeability and retention (EPR) effect. *Int. J. Pharm.*, **565**, 481–487 (2019).

24) Sudjarwo SA, Hori M, Tanaka T, Matsuda Y, Okada T, Karaki H. Subtypes of endothelin ETA and ETB receptors mediating venous smooth muscle contraction. *Biochem. Biophys. Res. Commun.*, **200**, 627–633 (1994).

25) Cramer H, Müller-Esterl W, Schroeder C. Subtype-specific desensitization of human endothelin ETA and ETB receptors reflects differential receptor phosphorylation. *Biochemistry*, **36**, 13325–13332 (1997).