

Employing the Properties of Tumor Microenvironment to Boost the Drug Delivery and Effectiveness

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<https://hdl.handle.net/2324/4110485>

出版情報 : Kyushu University, 2020, 博士 (工学) , 課程博士
バージョン :
権利関係 :



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A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Graduate School of Engineering
Kyushu University

2020

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Abstract

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The microenvironment within tumor is structurally and functionally different from normal organs. Based on that, it is possible to utilize these special reactivities of vascular or released active molecules to enhance the therapeutic benefits. Comparing with small molecular chemotherapy drugs, nano-sized macromolecular particles showed more noticeable therapeutic efficacy and less systemic side effect. This phenomenon could attribute to the spontaneous and selective accumulation of macromolecules in the solid tumors by the enhanced permeability and retention (EPR) effect. However, due to the limited tumor blood flow, the drug accumulation through the EPR effect demand to be further improved. Therefore, the author utilizes the special properties of the tumor microenvironment for modifying blood flow to the tumor to enhance the EPR effect by using an endogenous nitric oxide (NO) to improve the chemotherapeutic effectiveness.

In chapter 2, the author employed IRL-1620, an agonist of the endothelin B

receptor, and examined for the first time of its effect on the EPR effect for PEGylated liposomes. In a CT-26 murine colon cancer model, the author found that 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 could be a promising strategy to improve the therapeutic effects of macromolecular drugs while reducing their side effects.

In chapter 3, the author examined the synergistic effect of mild NO released from the tumor associated macrophages (TAM) with chemotherapeutic agent Doxorubicin (Dox) in a mimetic tumor microenvironment. L-Arginine (Arg) which is a substrate of nitric oxide synthase (NOS) expressed in TAM was employed. By using a co-culture system of cancer cells with macrophages, the author found that mildly produced NO from Arg by co-cultured macrophages enhanced the cytotoxic effect of Dox to cancer cells. In addition, it was found the augmentation is affected by the order of the addition of Arg and Dox. Prior addition of Arg to Dox and simultaneous addition showed the same enhancement effect, but prior addition of Dox to Arg abolished the augmentation. This suggests that the co-administration of Arg with Dox may open new treatment modalities to improve chemo-therapies.

In chapter 4, based on the synergistic effect property observed above, the author developed an Arg-Dox co-encapsulating PEGylated liposome. Arg was encapsulated into the liposome during the extrusion procedure and Dox was incorporated through the remoted loading method. The author examined that the encapsulation efficiency (EE%) of Dox was comparable with the conventional Dox-LP. Moreover, the in vitro and in vivo stability of the developed liposome showed the same properties with Dox-LP. This will be a promising strategy to augment the chemotherapies without increasing the toxic effect.

In chapter 5, the author summarized the conclusion of this thesis.

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Chapter 1 Introduction and Background

1.1 Tumor microenvironment

Carcinomas are considered complex organs (Fig. 1-1) having a microenvironment consisting of tumor epithelial cells, fibroblasts, endothelial cells, and leukocytes. All of those cells interact either physically or via the secretion of paracrine signaling molecules. [1]

Until now, despite some remarkable achievements, cancer predominantly remains a seemingly intractable problem. Nevertheless, it has been growing recognized that targeting the tumor cell alone is not sufficient as there is an intricate mutually sustaining synergy between the tumor cells and their surrounding stroma. Thus, combination therapies have arisen attention that would not only improve the efficacy of standard care but also allow dose reduction to limit the toxicity to healthy tissues.

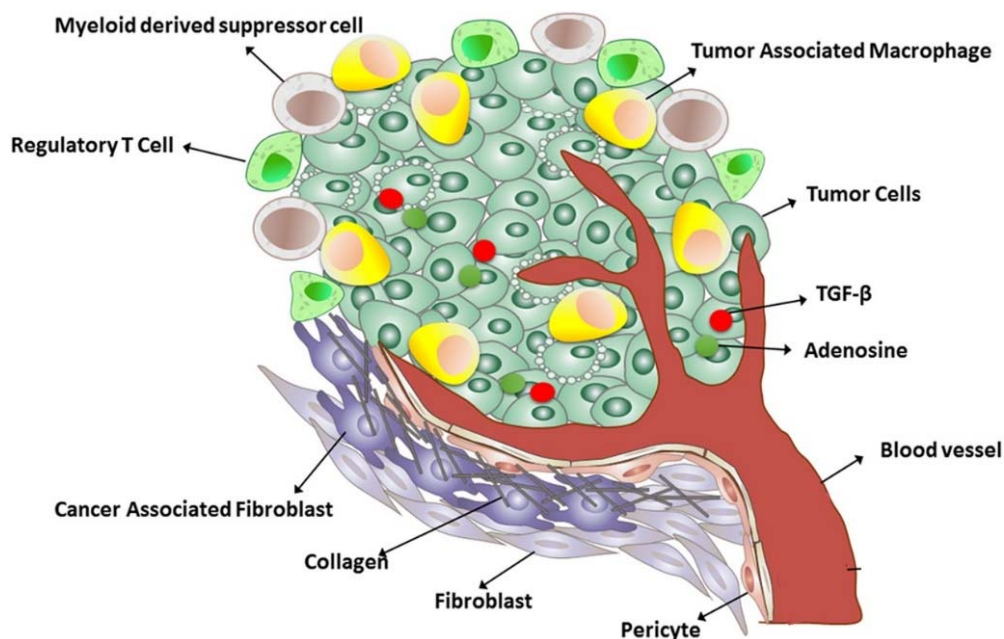


Figure 1-1. A schematic representation of the tumor microenvironment. [2]

1.2 Special properties of tumor blood vessel

In the tumor microenvironment, the vasculature is essential for keeping the tumor alive and facilitating its growth. In order to obtain sufficient nutrients to proliferate, tumor cells grow within a certain distance of a perfused blood vessel. Therefore, solid tumors must stimulate angiogenic and recruit their own vasculature to grow to a certain volume. [3]

Table 1-1. Characteristics of vasculature in solid tumors and normal tissues. [4]

Characteristics	Abnormal tumor vasculature	Normal vasculature
Organization	Normal vascular hierarchy absent Chaotic mixture of discontinuous, fenestrated capillaries Sac-like structures with dead ends	Normal hierarchy (Arterioles→Capillaries→Venules)
Pericytes	Absent or loosely associated with endothelial cells Fewer than in normal vasculature	Attached to endothelial cells of capillaries and venules Cover considerable surface of the vessel
Basement membrane	Either very thick or very thin or absent, which may lead to vessel leakiness	Present to closely interact with pericytes and Endothelial cells preventing vessel leakiness
Blood flow rate	Irregular or low Increased viscosity and geometric vessel resistance	Normal Normal viscosity and geometric vessel resistance

Compared with normal vascular, however, the tumor blood vessel is structurally different in many aspects (Table 1-1) which limited the nutrition supply and the efficacy of drug delivery. Studies of subcutaneous transplanted tumors in rats and mice reveal that under the imbalance of angiogenic regulators, the tumor vasculature develops inefficiently. They are unorganized and become increasingly tortuous (Fig. 1-2) and disfigured which hindering the vessel perfusion. Moreover, high interstitial fluid pressures will arise as tumors becoming larger and poor development of lymphatic vessels, which induces vessels in the tumor permanently shut, resulting in

blood stasis or even development of macroscopic necrosis. These insufficient perfusions observed in tumors set big obstacles for drug delivery since the rising dose is obviously limited by toxicity to normal tissues.

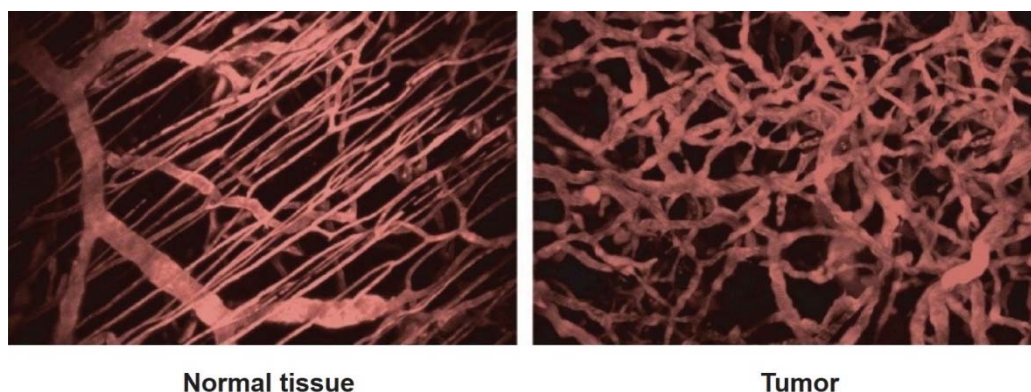


Figure 1-2. Organized vasculature in normal tissue contrasted with chaotic vasculature in tumors. [5] Copyright © 2017, Dove Medical Press Ltd.

Researchers in a study of surgical resections of human bronchial carcinoma also found that the tumor cells were grow in cords, where each cord only had a single blood vessel along its axis. [6] This can explain the availability of blood plasma components decreases with increasing distance from the blood vessels, and only tumor cells within the cords that close enough to a blood vessel could reach them.

In cancer therapy, techniques that targeting the tumor vasculature with different mechanisms continue to be used and developed to treat the solid tumors.

1.3 Exploiting tumor vasculature for improving drug delivery

Tumor vasculature plays direct or indirect role in the cancer treatment modalities. Various strategies of tumor vascular targeting are currently being explored, including vascular normalization that modulate the abnormal tumor microenvironment or modify the blood flow to the tumor that increases the tumor-specific drug delivery.

1.3.1 Vascular normalization to enhance drug delivery

As mentioned above, the abnormalities of tumor vasculature prevent sufficient and homogeneous distribution of anticancer drugs in tumors. [7, 8] Therefore, normalizing the vasculature to modulate the tumor microenvironment is a promising strategy. Through vascular normalization, the abnormal vasculature restored with the normal basement membrane and size of vessel pores, which result in improved perfusion and drug penetration. [9] Meanwhile, by correcting the rapid angiogenic signaling, vascular normalization repairs the abnormal structures and the function of tumor vasculature.

Anti-angiogenic treatment is an approach that enables the blood vessels to become more stable and uniform in structure and function which has made noticeable improvements in the delivery as well as the efficacy of anticancer therapeutics. [10-12] For example, heparanase act in angiogenesis [13], preclinical studies have obtained promising results through inhibition of this enzyme: the heparanase inhibitor SST0001 combined with conventional drugs (such as dexamethasone) or anti-VEGF agents showed synergistic effects on anti-angiogenesis and tumor growth inhibition.

[14] Moreover, another important angiogenic signaling factor VEGF has been observed overexpression in most tumors. [15] Thus, blocking the VEGF through a variety of monoclonal antibodies (mAbs) also has achieved promising results. Table 1-2 summarized some typical anti-angiogenic reagents that enhanced the tumor drug delivery.

Table 1-2. Vascular normalization agents for enhancement of drug delivery.

Priming agents	Priming mechanism	Drugs delivered	Tumor/tissue models	Ref.
Bevacizumab	Angiogenesis inhibition	Malphalan	Melanoma	11
A4.6.1 (anti-VEGF mAb)		CPT-11	Colon carcinoma	12
SST0001 (heparinase inhibitor)		Dexamethasone	Myeloma	14
DC101 (anti-VEGFR2 mAb)	IFP reduction	Albumin	Mammary carcinoma	45
PGE1		5-FU	Colon carcinoma	46
Inhibitory PDGF aptamer		Taxol	Thyroid carcinoma	47
Hyaluronidase		Liposomal doxorubicin	Osteosarcoma	48

IFP: interstitial fluid pressure

Although numerous success in improving cancer drug delivery have been achieved through vascular normalization, several factors still need to be considered before applying these approaches. Firstly, the dose- and time-dependent normalization window have to be optimized, since excessive vessel pruning might happen due to the high doses, which will result in poor perfusion. [16] In addition, the proposed normalization window may not work under all conditions. Such as NSCLC xenograft treatment with the anti-angiogenic agent pazopanib reduced interstitial fluid pressure but the improved oxygenation was short-lived and pazopanib could not promote liposomal penetration into these tumors. Moreover, normalized vessels with

insufficient fenestration won't be effective to the large NP's (>100 nm) leakage into the tumor tissue. Therefore, it has been reported that vascular normalization would improve tumor penetration only in the case of small NP (< 60 nm). [17]

Vascular normalization strategy has shown it is effective mainly against tumors with hyperpermeable and uncompressed vessels such as glioblastoma and ovarian carcinoma. [18] However, based on its low response rate in desmoplastic tumors which has compressed vasculature, it may not lead to desirable results. In such cases, combination strategies are needed.

In the chemotherapy domain, the tumor vasculature is relied upon to deliver the drugs to the tumor cells. Therefore, increasing the tumor blood perfusion is a promising strategy.

1.3.2 Modifying blood flow to tumors for specific drug delivery

For the chemotherapy, blood flow serves as a vehicle for the delivery of anticancer agents to tumor cells. As explained above, in growing tumors, due to the abnormal vascular structure and the changing microenvironment, blood flow is generally low. Thus, strategies have been developed to transiently increasing blood flow in tumors during the treatment to enhance the tumor drug delivery and sensitize tumors to the chemotherapy.

Numerous approaches and agents have been applied to modify the blood flow to tumors. Such as a known vasoconstrictor Angiotensin II (AT II). It has been reported that systemic infusion of AT II which elevated the arterial blood pressure induced a

strong and selective tumor blood flow increasing. [19, 20] But it was later confirmed that this effect varies depending on the tumor volume. [21] Moreover, Ca^{2+} antagonists which are inhibitors of vasoconstriction are also reported to increase the tumor blood flow [22], whereas its high heart toxicity limited its clinical application. [23]

Nitric oxide (NO), a radical gas, has been widely used to induce vasodilation and increase blood flow. However, the effect of systemic delivery of NO donors was controversial which reported to both increase [24, 25] or decrease the tumor blood flow. [26, 27] The uncertainty of systemic NO delivery to achieve predictable chemo-sensitization could be due to the failure to meet the tumor-specific blood flow increasing. Meanwhile, the responses of tumor vascular to NO donors are highly variable in function of time, dose, and tumor type. In mammals, NO is the most general endogenous vasodilator. It is produced by a family of enzymes termed nitric oxide synthases (NOS) including eNOS (endothelial NOS), nNOS (neuronal NOS), and iNOS (inducible NOS). Among these three isoforms, eNOS expressed by endothelial cells participates in the control of the vasodynamic balance. It catalyzes the production of NO that counteracts tonic vascular constriction of neuronal, endocrine, or local origin. It has reported that several ligand/receptor combinations are able to directly activate eNOS, such as the VEGF/VEGF receptor, the bradykinin/B2 receptor, and the endothelin-1/endothelin B (ET_B) receptor. [28]

As mentioned above, tumor-specific blood flow modifying is essential for the cancer therapy. Therefore, an active receptor Endothelin B (ET_B) seems to be a good target.

ET_B receptor present on both endothelial cells and vascular smooth muscle cells. On endothelial cells, ET_B receptors stimulate Ca²⁺ dependent eNOS activation to secrete NO, which results in vasodilation, whereas those on vascular smooth muscle cells produce vasoconstriction.[29] As for the special structure, tumor blood vessels often lack smooth muscle cells compared with normal blood vessels. In addition, numerous studies have confirmed that tumor vascular express more ET_B receptor than normal tissue. [30, 31] Therefore, employing a highly specific agonist of the ET_B receptor will lead to a selective tumor vasodilation and achieve the tumor blood flow modifying.

1.3.3 Enhanced permeability and retention effect for nanomedicine delivery

Selective improvement of drug effectiveness in tumor is always what our final purpose. Various strategies have been explored to utilize the special microscopic architecture of tumor blood vessel to enhance the chemotherapy efficiency. Among them, a well-known phenomenon in the tumor environment which is called enhanced permeability and retention effect (EPR effect) was discovered by Matsumura and Maeda about 30 years ago. It has been applied to increase drugs' tumor accumulation and accordingly decrease the dosage amount contributing the reduction of normal tissue's distribution. [32-34]

Blood vessels in tumors are irregular in shape and frequently absent the smooth-muscle layer, the endothelial cells are poorly aligned with gaps range from 100 nm to 800 nm between each other. Therefore, macromolecules of blood plasma

components could flow out to the tumor tissue through these gaps. Moreover, the poor lymphatic drainage in tumors results in the long-term retention of the accumulated macromolecules (Fig. 1-3). This phenomenon, namely the EPR effect, has become the basis for the selective targeting macromolecular drugs to the site of solid tumors. [32]

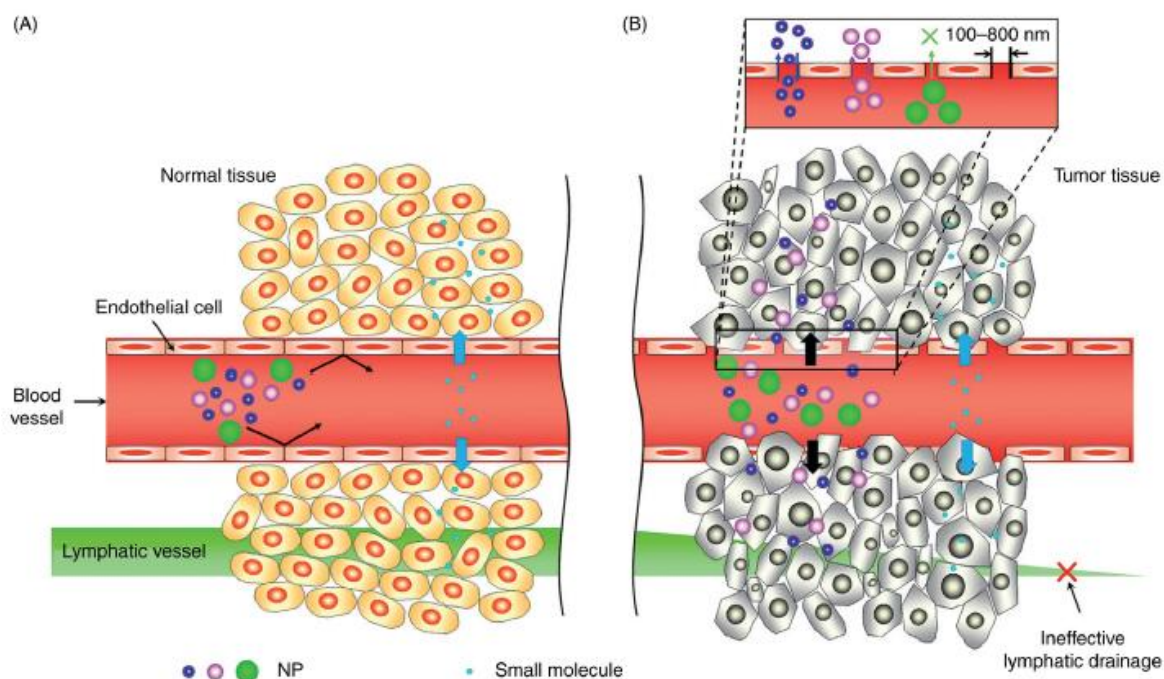


Figure 1-3. Schematic showing the leakage of different Nanoparticles (NP) and small molecules based on their size across (A) normal and (B) cancerous tissues. [35] Copyright © 2017 Elsevier Inc. All rights reserved.

1.4 Employing TAM to enhance the drug effectiveness

Widely distributed in all tissues, macrophages are a population of immune cells that can take various phenotypes, play a vital role in the host innate immune response against infections. [36] The relationship between macrophages and tumors has been the subject of numerous scientific reports over the last decade. Constituting an important group of immune cells in the tumor microenvironment (Fig. 1-4), macrophages have been found to be involved in tumor progression, which depends on the stage of tumor development and the tumor type. [37]

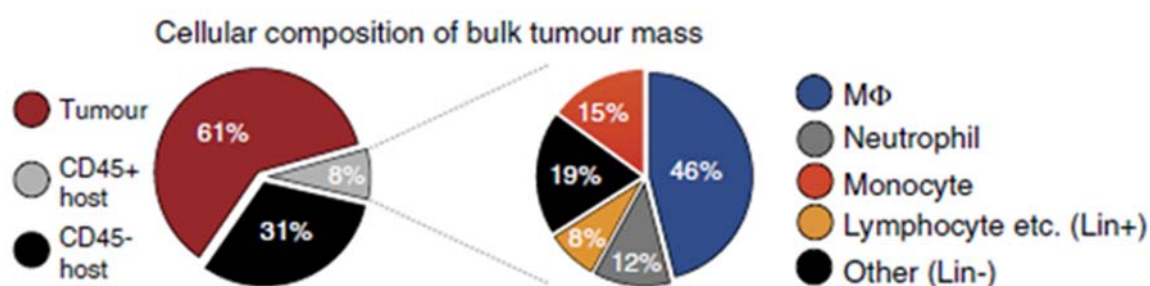


Figure 1-4. Cellular composition of bulk tumor mass. [38] Copyright © 2015 Macmillan Publishers Limited. All rights reserved.

At the tumor site, a significant portion of the immune cells are represented by tumor-associated macrophages (TAM), which be conventionally divided into two subgroups: M1 and M2 cells.[39] The functions and roles of these two subgroups have been sufficiently documented in different tumor conditions. Depending on the specific microenvironmental signals, due to the plasticity, macrophages can acquire different functional phenotypes: from initiation of proinflammatory reactions to inhibition of inflammation. Regarding the role played by macrophages in the

development of tumors, classically activated M1 macrophages are known to exert immunostimulatory and antitumor effects, whereas alternatively activated M2 macrophages can lead to immunosuppressive and protumoral responses. [40]

TAMs have captured the attention of scientists as a promising target for immunotherapy in cancer treatment. Early works on developing TAM-based immunotherapeutic approaches had focused on blocking the protumoral activity of M2. Subsequent research has been shifted to reprogramming the pro-tumorigenic phenotype (M2) to the anti-tumorigenic phenotype (M1). [41] Nonetheless, limited clinical success has been achieved to date because of low response rates observed in different types of cancers. Therefore, active molecules produced by M1 macrophages which show antitumorigenic effects have recently drawn attention.

Activating the Th1 immune response, M1 macrophages secrete high amounts of proinflammatory cytokines that induce killing of tumor cells, such as tumor necrosis factor (TNF), IL-12, and nitric oxide (NO). [42] The production of the inducible nitric oxide synthase (iNOS), a precursor for nitric oxide production, is indicative of an inflammatory response. NO produced following the activation of macrophages to an anti-tumorigenic state is treated as a hallmark of the M1 phenotype and can be a direct indication of response. [43, 44] It has been confirmed that NO produced by macrophages in the tumor microenvironment aids in arresting the cell cycle and inhibiting tumor progression.

1.5 Overview of this thesis

Improving chemotherapeutic drug delivery and effectiveness are always what we aim to. Making use of the characteristics of tumor microenvironment is a promising and effective strategy. To approach this purpose, two methods were developed in this thesis.

In the first strategy, the author sought to modify the blood flow to tumors to further enhance the EPR effect. The author utilized IRL-1620, an agonist of the endothelin B receptor, which is a promising molecule to enhance the blood flow to tumors by activating endothelial NO synthase. The enhancement of the PEGylated liposomes' tumor accumulation was examined by co-administration with IRL-1620. Results indicate that IRL-1620 enhanced the EPR effect in the murine colon cancer model.

In the second approach, the author utilized the NO produced from the TAM to augment the effect of chemotherapeutic agent. The enhanced sensitivity of cancer cells toward doxorubicin (Dox) was analyzed by employing the endogenous NO donor Arg. The author found that by co-culturing with macrophages, NO produced from Arg increased the cytotoxic effect of Dox to cancer cells. Moreover, it was confirmed that the sequence of adding Arg and Dox matter this augmentation effect. Furthermore, it is promising to co-encapsulate Arg and Dox into one nanocarrier to further enhance the drug efficacy.

Therefore, approaches that take advantage of the tumor microenvironment can be promising to realize more efficient and low toxic medications for cancer disease.

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Chapter 2 Effect of an endothelin B receptor agonist on the tumor accumulation of nanocarriers

2.1 Introduction

Selective and substantial accumulation of anti-cancer drugs in tumors is crucial to maximize therapeutic efficacy and reduce the 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] 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. [6-8] However, most NO donors are unstable molecules and spontaneously release NO,[9] 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 VEGF/VEGF receptor, the bradykinin/B2 receptor, and the endothelin-1/endothelin B (ET_B) receptor.[11] Among these, a combination of the

endothelin-1/ET_B receptor has been utilized to enhance the tumor accumulation of small molecular anti-cancer drugs. Endothelins (ET) are a family of small (21 amino acids) multifunctional peptides comprising of three isoforms in mammals: ET-1, ET-2, and ET-3 [25]. All of them exert their physiologic effects by binding to two high-affinity G-protein-coupled receptors, ET_A and ET_B [26]. ET-1 and the two ET receptors form the ET axis that plays an essential role in tumor and tumor vascular biology. ET_A and ET_B have opposite roles in vascular homeostasis [27] where ET_B mediates vasodilation and endothelial cell survival and proliferation [28]. In addition, ET_B receptors which are expressed on endothelial cells lining the interior surface of blood vessels are more abundant in tumor blood vessels compared with healthy ones [29, 30]. Therefore, utilizing selective ET_B receptor agonists can help to induce vasodilation and transiently increase tumor blood perfusion and thereby enhance the delivery of antineoplastic agents to solid tumors.

IRL-1620, derived from endothelin-1, is a highly selective and high affinity peptide agonist of the ET_B receptor. [12,13] Gulati et al. 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.[14,15] 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.[16] 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 et al. They found that tumor blood flow was reduced in response to a systemic injection of IRL-1620 in their rat models.[17,18] Because the ET_B receptor is involved in both vasodilation and vasoconstriction in organs (Fig. 2-1), the discrepancy between these two results may result from differences between different animal models. [17,19]

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.

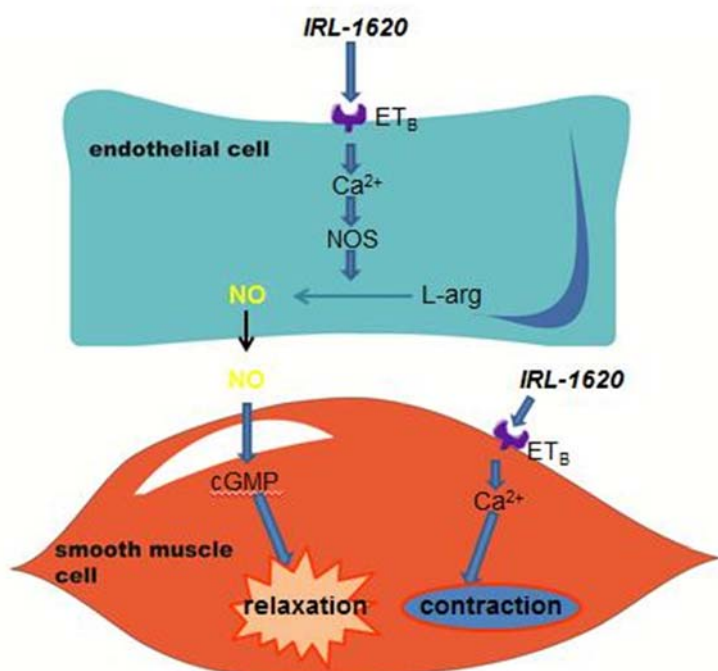


Figure 2-1. Mechanism of the IRL-1620 induces vasodilation.

2.2 Experimental section

2.2.1 Materials & reagents

Hydrogenated soybean phosphatidylcholine (HSPC) and N-(carbonyl-methoxypolyethyleneglycol2000)-1,2-distearoyl-snglycero-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, USA). IRL-1620 was purchased from Tocris Bioscience (Ellisville, MO, USA).

2.2.2 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. 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, UK).

2.2.3 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 μ g mL⁻¹ streptomycin, and 0.25 μ g mL⁻¹ amphotericin B (all were purchased from Gibco Invitrogen Co., Grand Island, NY, USA). The cells were maintained in a humidified incubator containing 5% CO₂ in air at 37°C.

2.2.4 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).

2.2.5 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 2×10^6 CT-26 cells suspended in 100 μ L 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: $V \text{ (mm}^3\text{)} = (L \times W^2)/2$, 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 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, USA) 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 $\times$ 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 12,000 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.

2.2.6 Statistical analysis

All data are expressed as mean $\pm$ SD. Data were evaluated using one-way analysis of variance and statistical analysis was performed using GraphPad Prism software. A value of $p \leq 0.05$ was considered to be statistically significant.

2.3 Results and discussion

2.3.1 Liposome preparation.

In order to improve the practical application of IRL-1620, the author sought to utilize the EPR effect that employed the representative drug carrier, liposomes, to combine with IRL-1620. PEGylated LPs containing a near-infrared fluorescent probe were prepared through a standard hydration method. Their physicochemical characteristics are summarized in Table 2.1. The diameter, polydispersity index (PDI), and ζ -potential of LP were suitable for the EPR effect.

Table 2.1 Characteristics of liposomes.

Sample	diameter (nm)	PDI	ζ -potential (mV)
LP	72	0.04	-21

2.3.2 In vivo tumor accumulation of LP.

The author examined the effect of IRL-1620 on LP accumulation in tumor of a mice colon cancer model. 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 (Fig. 2-2A, 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. 2-2B). In contrast, a significant difference was not observed for a group of mice with a higher dose of IRL-1620 (9 nmol/kg).

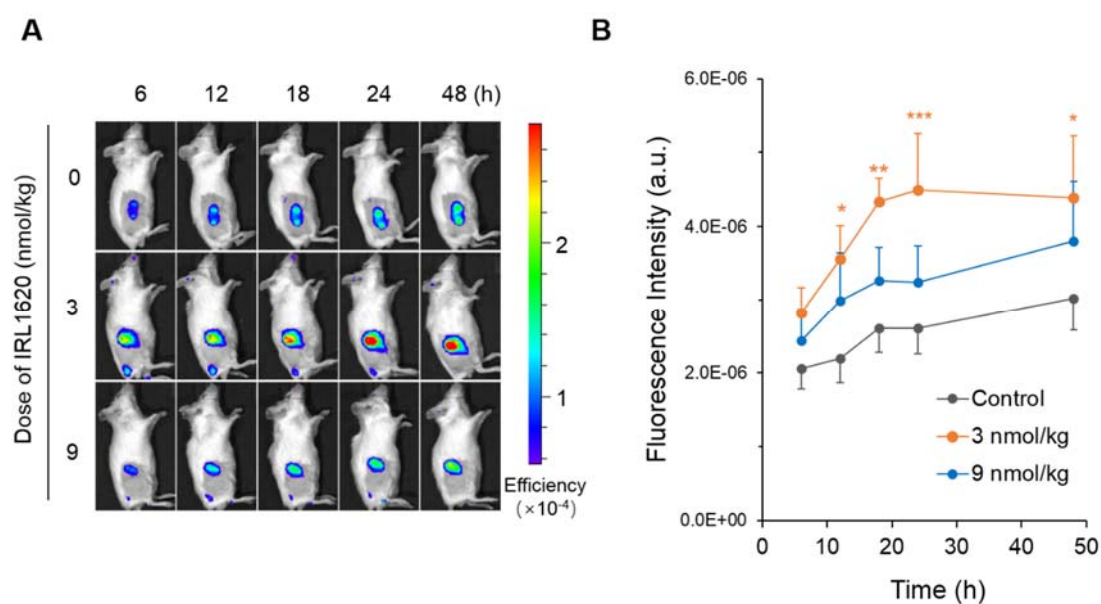


Figure 2-2. Effect of IRL-1620 on the tumor accumulation of LPs in CT-26 tumor bearing BALB/c mice. (A) Representative IVIS images of fluorescence resulting from LP at each time point. (B) Fluorescence intensity of the tumor region of accumulated LP at each time point. Results are expressed as means $\pm$ SD (n = 5). * P < 0.05, ** P < 0.01, *** P < 0.001 compared with control.

2.3.3 Ex vivo fluorescence assessment

Then the author excised tumors at 48 h post-injection and measured their fluorescence intensity (Fig. 2-3). Mice injected with IRL-1620 at 3 nmol/kg demonstrated a nearly two-fold increase in the fluorescence intensity in tumor, while such a remarkable augmentation was not observed with the 9 nmol/kg dose. It indicates that an optimum dose of IRL (3 nmol/kg) substantially enhanced LP accumulation in the tumor. While a larger dose (9 nmol/kg) resulted in almost no effect on the biodistribution. An optimum dose of IRL in enhancing tumor blood perfusion was also observed in rat models by Gulati et al. [14,15] And a high dosage of IRL-1620 would result in desensitization of the ET_B receptor-mediated activation of eNOS which induces vasodilation and increases vascular permeability. The desensitization of ET_B receptor-mediated vasodilation was also reported for another ET_B agonist sarafotoxin S6c in resected rabbit veins.[23] Desensitization of ET_B receptor signaling results from the internalization of the ET_B receptor.[24] The existence of ET_B desensitization from the higher dosage of ET_B agonists seems to be an advantage that could avoid uncontrollable blood pressure fluctuation.

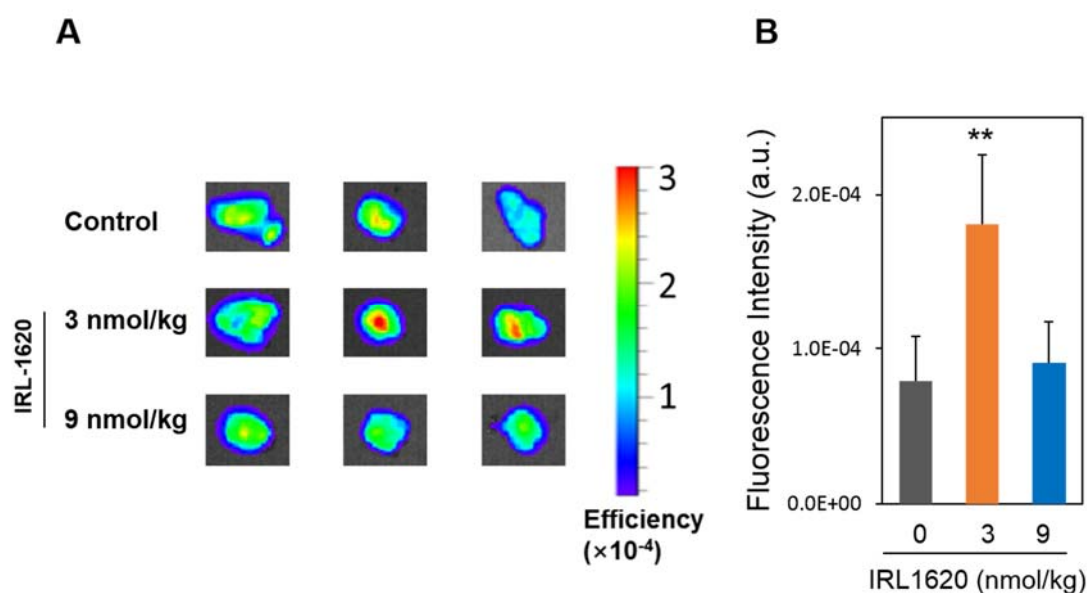


Figure 2-3. Representative ex vivo imagers of LP accumulation in CT-26 tumors. (A) IVIS images of fluorescence in excised tumor tissue. (B) Fluorescence intensity of tumor tissue from each group 48 h after injection. Results are expressed as means $\pm$ SD (n = 5). ** P < 0.01 compared with control.

2.3.4 The effect of IRL-1620 on the biodistribution of liposomes

To quantify the accumulated LPs in each organ, organs were resected and homogenized to measure the fluorescence intensity of the homogenates (Fig. 2-4). 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. These results consist with the ex-vivo imaging and revealed that IRL-1620

(3 nmol/kg) modestly enhanced the accumulation of co-administrated LPs in several organs such as the liver and spleen while greatly improved the tumor accumulation (Fig. 2-4). The larger increase of LP accumulation in tumors compared with that in other organs may stem from the higher expression of the ET_B receptor in tumors than in other tissues. [20,21] The enhancement of LP accumulation in tumor was also observed with NO-donating LPs. [22]

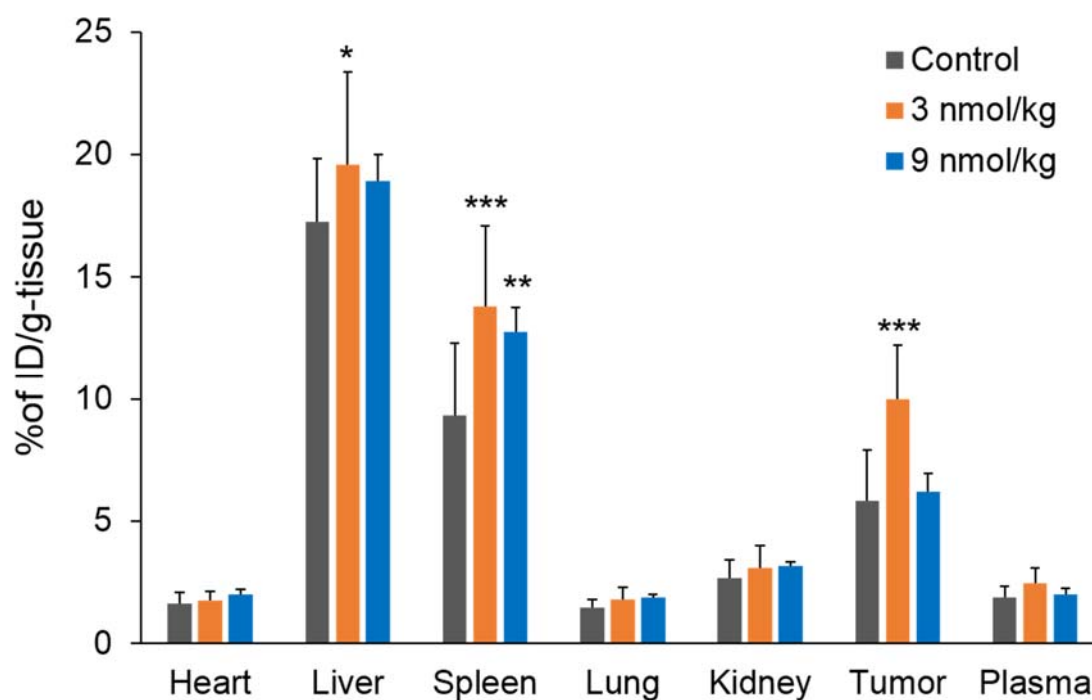


Figure 2-4. Biodistribution of LPs in BALB/c mice bearing CT-26 tumors 48 h post-injection.

The amount of LPs in each tissue is expressed as % injected dose (I.D.)/g-tissue (or mL-plasma).

Results are expressed as mean $\pm$ SD (n = 5). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared with control (no injection of IRL-1620).

According to a previous report, the modulation of tumor blood flow produced by IRL-1620 is a transient phenomenon and lasts for 30 min.[18] 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.

2.3.5 Mechanism of improved EPR effect by IRL-1620.

The expected mechanism of the enhanced tumor accumulation of LP through co-administration of IRL-1620 is shown in Fig. 2-5. (A) represent the LPs tumor accumulation via 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.

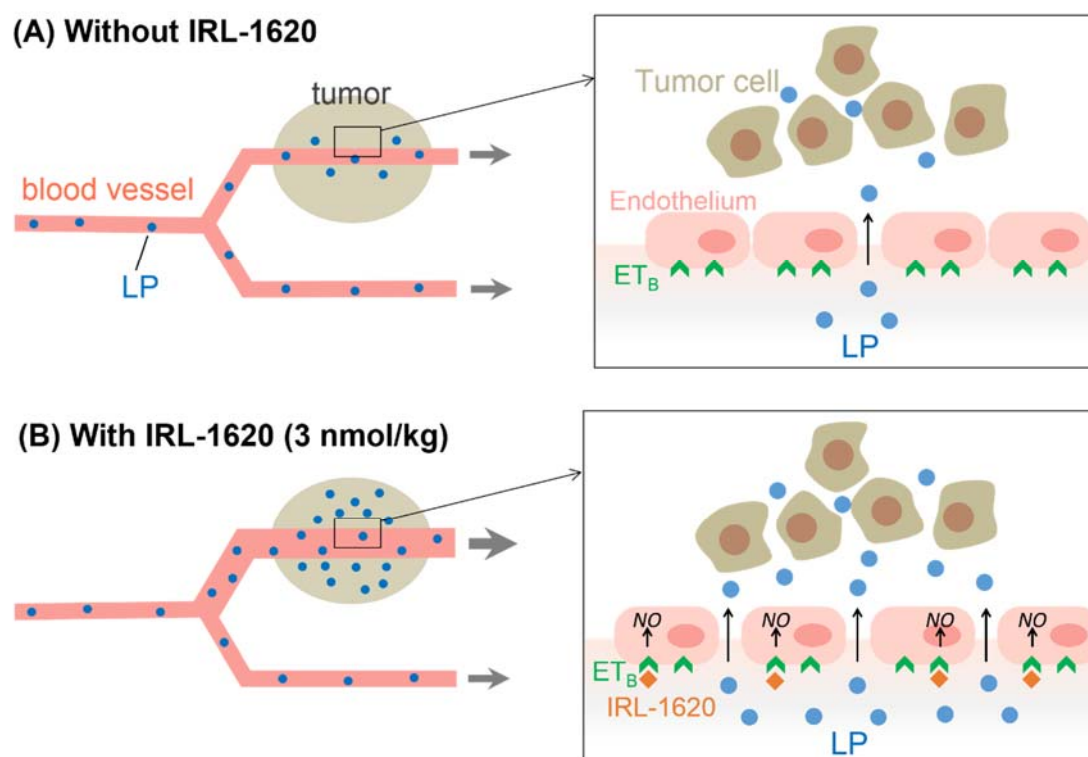


Figure 2-5. Expected mechanism of enhancement in LP tumor accumulation produced by co-injected IRL-1620.

2.4 Summary

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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Chapter 3 Evaluation of Synergistic Effect of L-Arginine on the Anticancer Activity of Doxorubicin by Using Co-culture System

3.1 Introduction

Macrophages constitute a large portion (up to 50%) of tumor mass and these macrophages are called tumor associated macrophages (TAM). [1, 2] TAM changes their phenotype with the stage of cancer development. In the cancer-initiation stage, TAM is in a type I phenotype (M1) and has anti-tumor activity. With the development of cancer, TAM is converted to type II phenotype (M2) which promotes the tumor development. [1] Anti-tumor effect of M1 results from the secretion of soluble factors such as nitric oxide (NO), reactive oxygen species, and inflammatory cytokines.[3, 4] NO is produced by one of the isoforms of nitric oxide synthase (NOS), inducible NOS (iNOS) expressed in M1. NO secreted from M1 shows anti-tumor effect due to the cytotoxicity of reactive nitrogen species derived from NO as well as inhibition of the anti-apoptotic survival signaling. [26]

L-Arginine (Arg) is the substrate of iNOS to produce NO in M1 [5, 6] and NO production is known to increase by increasing the Arg concentration in the outer media.[7] Thus, it can be hypothesized that the Arg supplementation would suppress the tumorigenesis. Two research groups confirmed the suppression of the tumorigenesis by the Arg supplementation both in animal models [8] and human clinical trial. [9] Recently, polyarginine-containing nanoparticles was reported to

retard the tumor growth in animal models. [6]

We examined whether the sensitivity of cancer cells to anticancer drugs is enhanced by the macrophage mediated NO release from Arg (Fig. 3-1A). NO has been reported to raise the sensitivity of cancer cells toward anti-cancer drugs such as cisplatin. [11, 12] This sensitization in cancer cells was attributed to the inhibition of the anti-apoptotic survival signaling by NO. [13] The combination of NO donors and anti-cancer drugs has been examined in animal studies¹¹ and clinical trials [14, 15] and positive results were observed in some of the studies. In these studies, synthetic NO donors such as NONOates and nitrate esters have been used. These molecules release NO spontaneously or enzymatically without the help of TAM, thereby rapidly raise the concentration of NO. The rapid release of NO would show higher anti-cancer effect whereas it has a risk of systemic hypotension. [16] In this sense, Arg is a mild NO donor without the side effect.

Jiang et al. recently reported synergetic effect of anti-cancer activity of Arg and Dox by using Dox-loaded dendrimers chemically modified with Arg both in vitro and in vivo.⁷ In their formulation, Arg would be released from the dendrimer by enzymatic reaction in macrophage after endocytotic uptake and iNOS would contribute to the NO production, although it was remained to be examined. The purpose of the present research is to clarify the contribution of Arg and iNOS in the synergy of anti-cancer effect and obtain more insight for future development for co-administration formulation. Thus, we simply used Arg as NO donor, and Arg and Dox were separately added to the co-culture system of macrophage and cancer cells (Fig. 3-1B).

This co-culture system was originally reported by Soma et al to see the effect of NO produced from macrophage on the anti-cancer effect of Dox. [17]

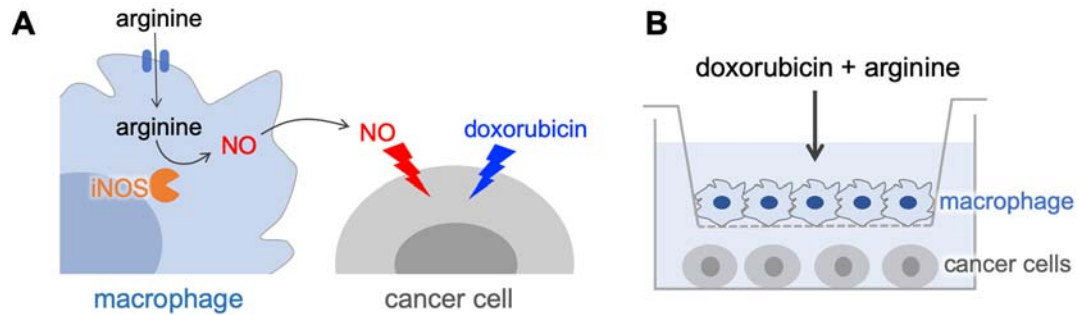


Figure 3-1. (A) Schematic illustration of synergy of NO produced from macrophage with anti-cancer drug, doxorubicin. (B) Experimental setup to prove the synergy mimicking the tumor environment.

3.2 Experimental section

3.2.1 Materials and reagents

L-arginine hydrochloride, doxorubicin, N^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), lipopolysaccharides (LPS) from *Escherichia coli* were all purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Polycarbonate cell culture inserts in multi-dishes were obtained from Thermo Scientific™ Nunc™ (MA, U.S.A.). Trypsin - EDTA (0.25%) and L-arginine deficient SILAC Dulbecco's modified Eagle's medium (DMEM) were obtained from Thermo Scientific (MA, USA).

3.2.2 Cell line

Murine macrophage cell RAW 264.7, A549 human pulmonary adenocarcinoma cell, CT-26 murine colon adenocarcinoma cell, and B16 mouse melanoma cell were all obtained from ATCC (Manassas, USA) and cultured in DMEM from ATCC (Manassas, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, USA). The cells were cultured in a humidified atmosphere containing 5% CO₂ in air at 37 °C.

3.2.3 Measurement of nitrite oxide release

Macrophages RAW 264.7 or cancer cells were seeded into 24-well plate (50000 cells/well) and incubated for 24 h. Then the medium was removed and cells were incubated with fresh L-arginine deficient medium with or without the stimulation of LPS (1 µg/mL) for 6 h. After that, cells were incubated with various concentrations of

Arg for another 48h. The supernatant of the cultured medium was collected and centrifuged at 1500×g for 15 min. The amount of NO which reflected by nitrite was quantified by using the Griess Reagent Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured at 540 nm using Infinite M Plex microplate reader (TECAN, Switzerland).

3.2.4 Measurement of the inhibition of cell growth

The experiments were conducted in polycarbonate cell culture inserts which consisted of two compartments separated by a porous membrane (0.4 µm pore size, catalog number 140620, Thermo Scientific Nunc). Cancer cells (A549, B16, CT-26) were cultured in 50000 cells/well at the lower chamber and RAW 264.7 were seeded into the upper chamber in 80000 cells/well. After 24 h of culture at 37 °C, LPS (1 µg/mL) and L-NAME (2 mM) were added to RAW 264.7 in the upper chamber and incubated for 6 h. Then various Arg and Dox were added in the upper chamber and incubated for 48 h. After separating RAW 264.7 and the cancer cells, the medium was removed and cells were incubated with fresh medium containing Cell Counting kit-8 reagent (Dojindo, Kumamoto, Japan) for another 4 h. The absorbance was measured at 450 nm by using Infinite M Plex microplate reader (TECAN, Switzerland).

3.2.5 Statistical analysis

All data are expressed as mean ± SD. Data were evaluated using one-way analysis of variance and statistical analysis was performed via the GraphPad Prism software. A value of $p \leq 0.05$ was considered significant.

3.3 Results and Discussion

3.3.1 NO production from Arg in macrophage and cancer cells

NO production ability of macrophage and cancer cells from Arg in response to LPS stimulation was examined. First, the monocyte derived cells were stimulated with LPS (1 $\mu\text{g/mL}$) for 6 h to induce the expression of iNOS. [18] LPS-stimulated cells were regarded as a model of M1 cells. [19] Then, the cells were incubated in the presence of Arg for 48 h. The produced NO was detected by Griese reagent, which detects NO_2^- in media derived from the oxidization of NO.[20] As shown in Fig. 3-2, Raw 264.7 macrophage produced NO with the increase of Arg concentration, which is consistent with previous reports that exogenous Arg could produce NO in the activated macrophages. [8] In contrast, NO production was not increased in all three cancer cell lines even at higher concentrations of Arg. It was reported that for the induction of iNOS in cancer cells, inflammatory cytokines were required in addition to LPS.[21] The obtained results confirmed that macrophage can be activated by simple stimulation and can be a source of NO by using Arg as a substrate.

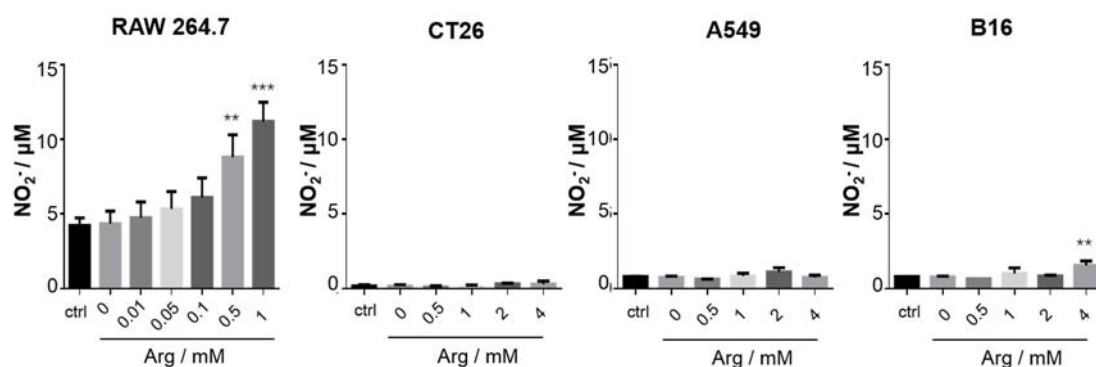


Figure 3-2. NO production from RAW 264.7 macrophage and cancer cells (CT26, A549 and B16). After 6 h incubation with LPS (1 $\mu\text{g/mL}$), Arg was added to the cells and incubated for another 48 h. Data are expressed as mean $\pm$ SD (n = 3). ** p < 0.01, *** p < 0.001 compared with ctrl

(without LPS stimulation).

3.3.2 Arg augmented the cytotoxicity of Dox to cancer cell

We examined the effect of NO production of macrophages on the cytotoxicity of Dox toward co-cultured cancer cells, which imitates the tumor environment (Fig. 3-1B). The cancer cells were cultured in the lower chamber. As shown in Fig. 3-3, co-culturing with LPS-activated macrophages reduced around 30% in the viability of the three kinds of cancer cells. This reduction of the viability resulted from secreted soluble factors from the macrophages. A similar level of viability reduction in cancer cells was reported previously in the co-culture system.[22] When Dox alone was added to the macrophages in the upper chamber, the viability of the three cancer cell lines was further reduced. Thus, the reduction of the cancer cell viability by Dox indicated the penetration of Dox through the macrophage to reach the cancer cells in the lower chamber. In contrast, the addition of Arg alone in the upper chamber did not affect the viability of the cancer cells, indicating that the NO concentration produced from the macrophages was mild and would not induce cytotoxicity. However, the simultaneous addition of Dox with Arg in the upper chamber further reduced the viability of the three cancer cells comparing with those treated with Dox alone, indicating a synergetic effect in combination of Dox and Arg. It is notable that the co-cultured macrophages tolerated the addition of Dox and/or Arg probably due to a lower sensitivity of macrophages against Dox (Fig. 3-3).

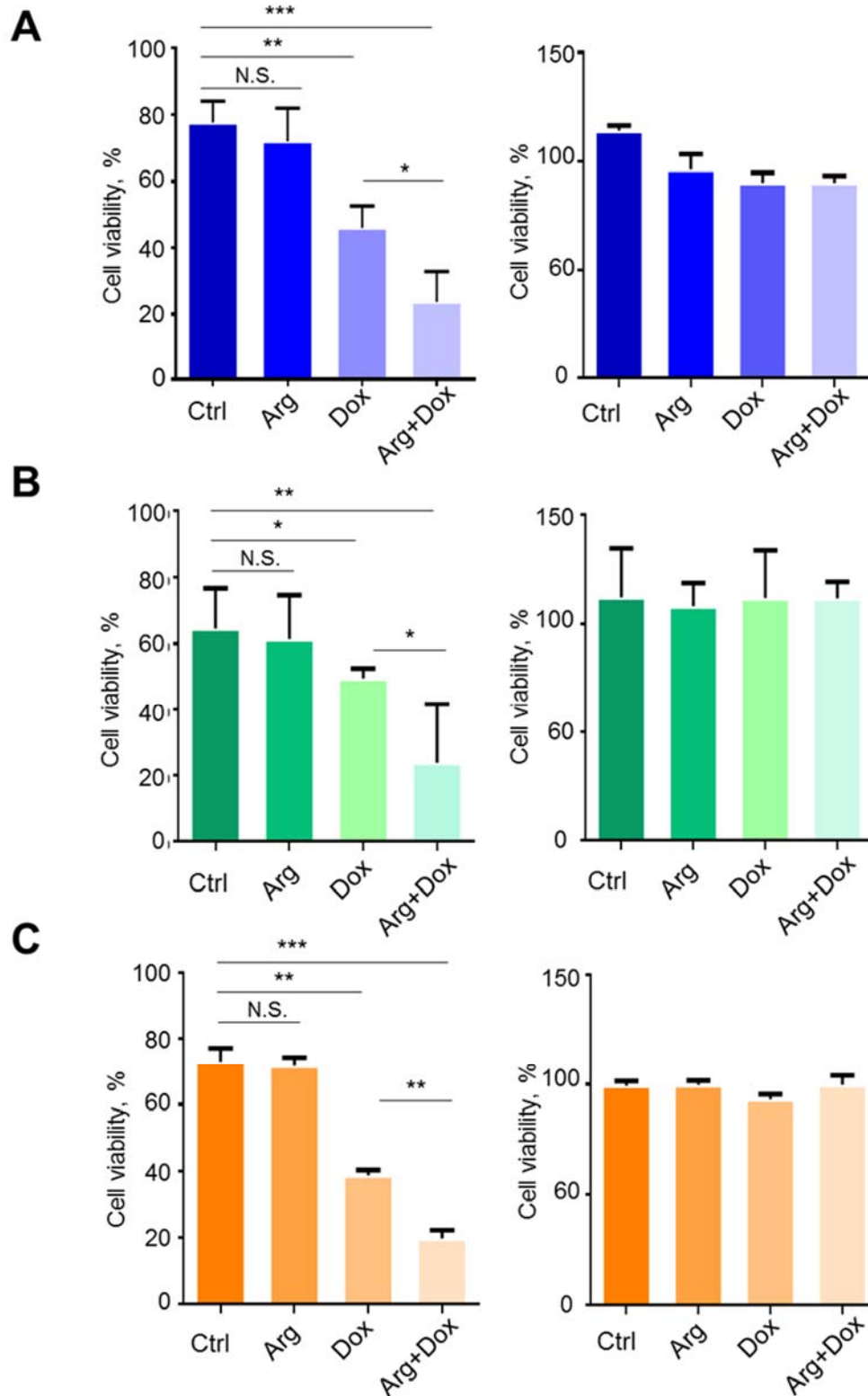


Figure 3-3. Synergistic effect of Arg with Dox on the cytotoxicity of (A) CT26, (B) A549, (C) B16 cells (left rows), and co-cultured Raw 264.7 (right rows). After six hours stimulation of Raw264.7 with LPS (1 $\mu\text{g/mL}$), Arg (1 mM) and Dox (1 μM for CT26 and A549, 1.5 μM for B16) were added to Raw 264.7 in the upper chamber and incubated for another 48 h. Dox concentration was optimized for each cell line to clearly see the synergetic effect between Dox and Arg. Results are

expressed as mean $\pm$ SD (n = 3). * p<0.05, ** p<0.01, *** p < 0.001, N.S.: not significant.

3.3.3 NO is essential in the synergistic anticancer effect

To examine the contribution of NO synthesis from Arg in the enhancement of the cytotoxicity of Dox, NOS inhibitor L-NAME was added to the coculture system. As shown in Fig. 3-4, inhibition of NOS abrogated the synergetic effect of Dox with Arg. The mechanism of this tumoricidal effect augmentation probably due to the increased sensitization of cancer cells to Dox by NO. [23, 24] Meanwhile, inhibition of DNA repair by the reactive nitrogen oxide species derived from NO may also contribute the drug efficacy augmentation.[25]

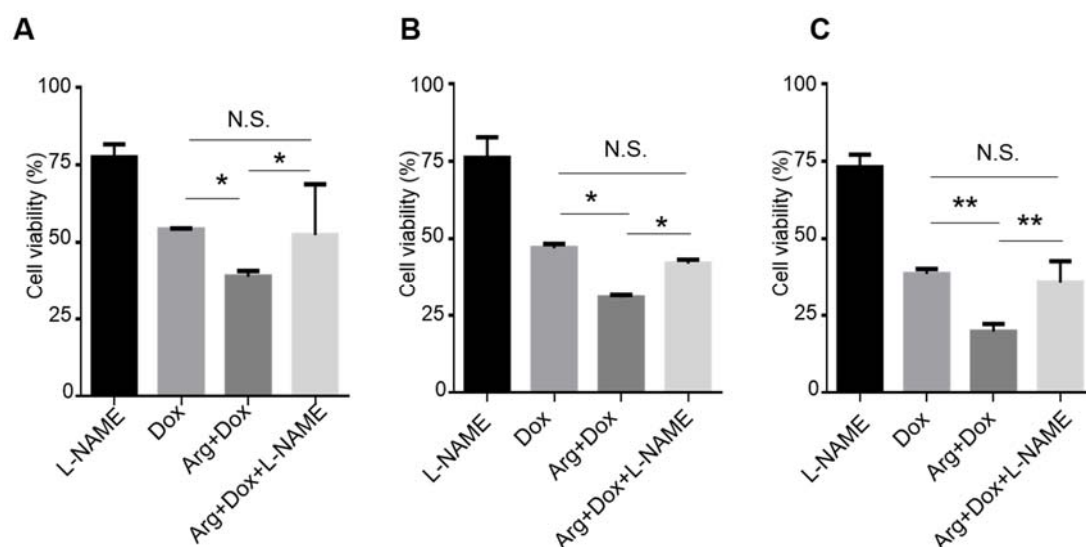


Figure 3-4. Effect of NOS inhibition on the synergistic effect of Arg and Dox in the co-culture system (A) C T26, (B) A549, and (C) B16. The experimental procedures were same with Fig. 3, except for L-NAME (2 mM) was added to Raw 264.7 in the upper chamber together with LPS. Results are expressed as mean $\pm$ SD (n = 3). * p<0.05, ** p<0.01, N.S.: not significant.

3.3.4 The sequence in the addition of Dox and Arg is critical for the augmentation in the Dox effect

For the application of the present synergy to the therapy, we should consider the timing of the administration of Dox and Arg. We assessed the effect of the sequence of the addition of Dox and Arg in the upper chamber. As shown in Fig. 3-5, a sequence of Arg-first (6 h prior to Dox) showed a similar augmentation effect with the simultaneous addition of Arg and Dox. However, the opposite sequence (Dox-first; 6 h prior to Arg) abolished the augmentation effect. The disappearance of the synergetic effect would be explained by the reported fact that Dox deteriorates the macrophage's ability to produce NO,[16] which will reduce total amount NO released from the macrophages. According to the obtained results, simultaneous or Arg-first would be the suitable timing to use Arg as an enhancer of Dox cytotoxicity.

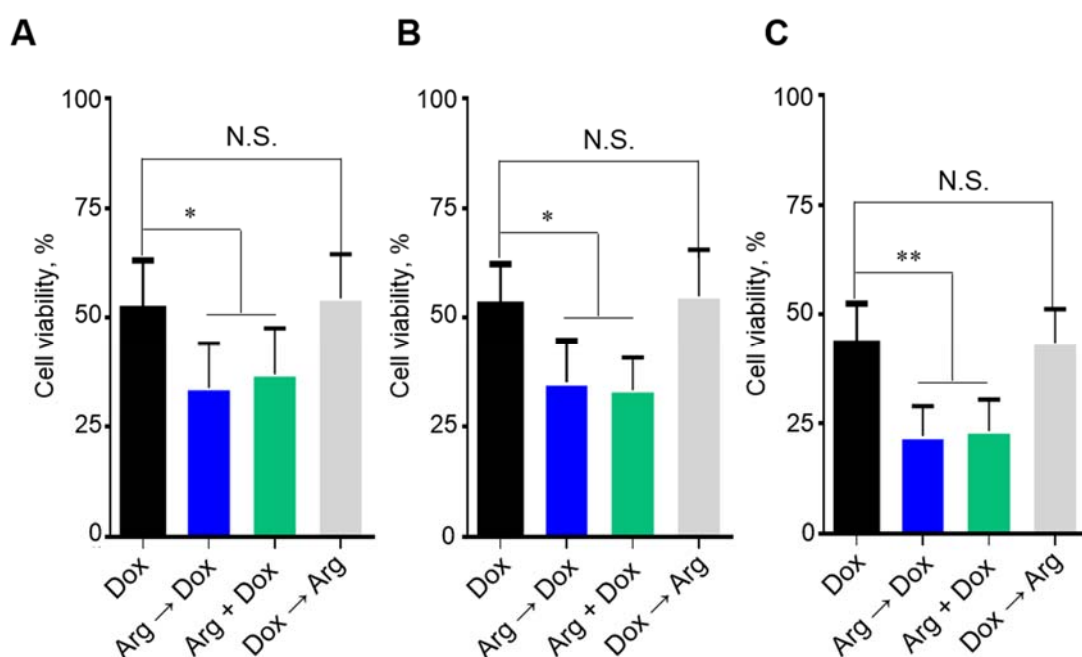


Figure 3-5. The sequence of Arg and Dox addition on the synergetic effect. (A) CT26, (B) A549, and (C) B16. Arg → Dox represents Arg was added 6 h prior to Dox; Arg + Dox represents simultaneous addition; Dox → Arg represents Dox was added 6 h prior to Arg. Other experimental conditions were same with Fig. 3-3. Results are expressed as mean $\pm$ SD (n = 3). * $p < 0.05$, ** $p < 0.01$, N.S.: not significant.

3.4 Summary

Here we clarified that NO produced from macrophage by using Arg as an iNOS substrate augmented cytotoxicity of Dox to cancer cells by using co-culture system. This mechanism would be a basis of synergetic anti-cancer effect of Arg and Dox in vivo reported by Jiang et al using Dox-loaded Arg-modified dendrimers. [7] The present strategy which utilizes TAM as a source of NO would enhance the efficacy of anti-cancer drugs without amplifying their side effects.

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Chapter 4 Preparation of PEGylated Liposome that co-encapsulated Arg and Dox

4.1 Introduction

In chapter 3, the author found the synergistic anticancer effect from a mild NO donor Arg and chemotherapy drug Dox. In order to improve its practical application and further augment its effectiveness, the author sought to utilize the EPR effect of the tumor environment to co-incorporate Arg and Dox into one liposome. In this chapter, the author reported the developed liposome and analyzed the its properties from basic characters and the NO production capability to the drug encapsulation efficiency and the stability *in vitro* and *in vivo* condition. Here, the author showed for the first time that co-encapsulating Arg and Dox liposome was successfully prepared, and this developed liposome showed comparable encapsulation efficiency (EE%) and storage stability with conventional Dox-LP. This liposome is going to be used to test its improved anticancer effect *in vivo*. This strategy will be promising for the augment specific, therapeutic effects of anticancer nanomedicine.

4.2 Experimental section

4.2.1 Materials & reagents

Hydrogenated soybean phosphatidylcholine (HSPC) and N-(carbonyl-methoxypolyethyleneglycol2000)-1,2-distearoyl-snglycero-3-phosphoethanolamine (DSPE-PEG) were purchased from NOF Corporation (Tokyo, Japan). Cholesterol was obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 1,1'-Dioctadecyltetramethyl indotricarbocyanine iodide (XenoLight DiR) was supplied by Caliper Life Sciences (Hopkinton, MA, USA). L-arginine hydrochloride, Doxorubicin, Lipopolysaccharides (LPS) from *Escherichia coli* were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). L-arginine deficient SILAC Dulbecco's modified Eagle's medium (DMEM) were obtained from Thermo Scientific (MA, USA).

4.2.2 Cell line

RAW 264.7 macrophage was purchased from American Type Culture Collection (ATCC) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) (all were purchased from Gibco Invitrogen Co., Grand Island, NY, USA). The cells were maintained in a humidified incubator containing 5% CO₂ in air at 37 °C.

4.2.3 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).

4.2.4 Preparation of L-arginine-Dox liposomes (Arg-Dox-LP)

The preparation of liposome lipid-thin film was the same with chapter 2. The dried lipid film was hydrated with 250 mM Ammonium sulphate buffer (pH 5.5) alone or combined with 250 mM Arg, by intermittently heating and vortexing at 60°C for 30 min. LPs were subsequently extruded through 200 nm (21 times) and then 50 nm polycarbonate membranes (41 times). The external solution was changed by ultracentrifugation ($400,000 \times g$) for 30 min at 4 °C with 10 mM HEPES buffer containing 5% glucose. Then, the liposome solution was mixed with doxorubicin, heated at 55 °C for 1 h to remote load the doxorubicin into the liposome. The unincorporated doxorubicin was replaced by ultracentrifugation ($400,000 \times g$) for 30 min at 4 °C with the HEPES buffer. The obtained liposome was stored at 4°C. The average hydrodynamic diameter and ζ potential were determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Worcestershire, UK).

4.2.5 Encapsulation efficiency (EE%) and the stability of Arg-Dox-LP

The encapsulation efficiency (EE %) was calculated using below formula:

$$\text{Encapsulation efficiency (EE \%)} = (\text{Wt} - \text{Wo} / \text{Wt}) \times 100 \%$$

where Wt is the total amount of drug in the initial suspension and Wo is the quantity

of drugs detected in the supernatant after ultracentrifugation to remove the liposomes. Concentrations of the drug in the supernatant were quantitatively analyzed using the spectrophotometer at the absorbance of 495nm. To determine the storage stability of Arg-Dox-LP, the sample solution (pH7.4) was stored at 4 °C for 24 and 48 h. At each time point, the sample was treated as described above and calculated the released drug from the supernatant. The drug release rate was calculated using the formula:

$$\text{Drug release rate (\%)} = (W_o/W_t) \times 100 \%$$

4.2.6 Measurement the release of nitrite oxide

Macrophages RAW 264.7 were seeded into 24-well plate (50000 cells/well) and incubated for 24 h. After that, the medium was removed and cells were incubated with fresh L-arginine deficient medium with or without the stimulation of LPS (1 µg/mL) for 6 h. Next, cells were incubated with various concentrations of Arg-LP for another 48h. The supernatant of the cultured medium was collected and centrifugated at 1500×g for 15 min. The amount of NO which reflected by nitrite was quantified by using the Griess Reagent Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured at 540 nm using Infinite M Plex microplate reader (TECAN, Switzerland).

4.2.7 Measurement of the cytotoxicity of Arg-LP

Macrophages RAW 264.7 were seeded into 96-well plate (5000 cells/well) and incubated for 24 h. Then indicated concentrations of Arg-LP were added to the RAW

264.7 cell and incubated for another 48h. After that, Cell Counting kit 8 reagent (Dojindo, Kumamoto, Japan) was added into the cell and incubated for 4 h. The absorbance was measured at 450 nm by using Infinite M Plex microplate reader (TECAN, Switzerland).

4.2.8 Blood circulation stability

To compare the blood circulation stability of Arg-Dox-LP with the only Dox- LP, 6-week-old BALB/c mice (n = 18) were randomly divided into six groups. Each group of mice was i.v. injected with Arg-Dox-LP or Dox-LP. At 1, 3, 6, 12, 24 and 48 h after administration, blood was collected from the mice in each group. The blood was allowed to clot for 30 min at room temperature and at 4 °C for another 24 h, and the serum was separated through centrifugation at 4 °C and 3,000 rpm for 20 min. The number of liposomes in the serum was determined by the fluorescence intensity of each sample, using the fluorescence intensity of DiR. The standard calibration curve was obtained through a serial dilution of the original liposomes. A blank serum sample without liposome injection was analyzed to determine the autofluorescence of serum, which was subtracted from the fluorescence intensities of the injected samples during the calculation. The liposomes are represented as the percentage of injected dose per mL of serum.

4.2.9 Statistical analysis

All data are expressed as mean $\pm$ SD. Data were evaluated using one-way analysis of variance and statistical analysis was performed via the GraphPad Prism software. A value of $p \leq 0.05$ was considered significant.

4.3 Results and discussion

4.3.1 Preparation of Arg containing liposome

Liposomes with a membrane of phospholipids was used for effective medication. Arg is a water-soluble agent which can be incorporated into PEGylated liposome during the extrusion process. The obtained liposomes were suspended in neutral buffer at 4 °C, which is well below the gel-liquid crystalline phase transition temperature of the matrix lipid, HSPC (53 °C).[1] The characteristics of the liposomes are showed in Table 4.1. Their diameters (around 100 nm) were suitable for tumor accumulation by the EPR effect. [2]

Table 4.1 Characteristics of liposomes.

Sample	diameter (nm)	PDI	ζ-potential (mV)
LP	105	0.05	-19
Arg-LP	101	0.05	-18

4.3.2 The NO production ability of Arg containing liposome (Arg-LP) in macrophage and its cytotoxicity

In chapter 3, NO production ability of macrophage from Arg in response to LPS stimulation was confirmed. Here, the author sought to evaluate the NO production capacity of Arg after encapsulated into the liposome. The experiment scheme is the same with before that cells were first stimulated with LPS (1 µg/mL) for 6 h to

activate the expression of iNOS. Then, the cells were incubated with indicated amount of Arg-LP which represent by Arg concentration for 48 h. The produced NO was detected by Griese reagent, As shown in Fig. 4.1A, after incorporated into liposome, Arg still induced NO production from activated macrophages in a concentration dependent manner. This result revealed that Arg-LP could be a promising source of NO to TAM. Meanwhile, the cytotoxicity of the Arg-LP to macrophages was also examined. Different amount of Arg-LP represented by Arg concentration were incubated with macrophages for 48h, the Cell Counting kit-8 reagent was used to evaluate the cell viability. Results (Fig. 4.1B) showed that up to 2mM Arg containing LP, there is no significant toxic effect. It indicates that the Arg-LP will not affect to macrophage viability.

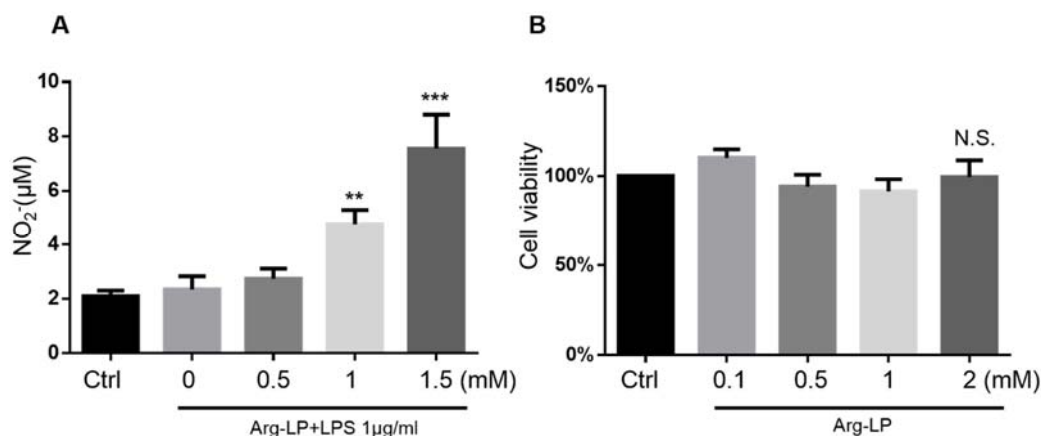


Figure 4.1 (A) NO production from Arg containing liposome in RAW 264.7 macrophage. After 6 h incubation with LPS (1 μg/mL), Arg-LP was added to the cells and incubated for another 48 h. (B) The cytotoxicity of Arg-LP on macrophages after 48h incubation. Data are expressed as mean ± SD (n = 3). ** p < 0.01, *** p < 0.001 compared with control.

4.3.3 Basic characteristics and EE% of the developed Arg-Dox-LP

Based on the synergistic anti-cancer effect of Arg and Dox observed in chapter 3 and the aim to achieve efficient drug delivery to avoid short-term repeat administration, the author sought to co-encapsulate Arg and Dox into one liposome through the reported remote loading method. The detailed Arg-Dox-LP preparing methods were mentioned in experimental part. Arg and Ammonium sulphate buffer (pH 5.5) was first incorporated into liposome during the extrusion process, after replaced the external solution of the prepared-LP with HEPES buffer (pH 7.4) to create an internal and external pH gradient, Dox was introduced through remote loading procedure as described in experimental part. The characteristics of prepared LP was first examined and showed in Table 4.2. The diameter, polydispersity index (PDI) of these two liposomes were closely similar before and after the remote loading. Moreover, the encapsulation efficiency (EE%) of Dox was evaluated compared with the only Dox containing LP. The table 4.2 showed that, by introducing of Arg, the EE% of Dox still reserved. These results indicated that further introducing Arg is acceptable to the original Dox containing LP.

Table 4.2 Characteristics and EE% of developed liposomes.

Sample	diameter (nm)		PDI		ζ -potential (mV)	EE%
	Before	After	Before	After		
Dox-LP	94	102	0.05	0.1	-19	82.2%
Arg-Dox-LP	78	87	0.05	0.17	-18	77%

4.3.4 The stability of Arg-Dox-LP

In order to confirm the stability and drug release rate of the developed Arg-Dox-LP above, the author measured these properties by incubated the LP with HEPES buffer (pH 7.4) at room temperature for 24h and 48h respectively through the method described in experimental section. The results (Table 4.3) showed that incorporating Arg did not affect the LP stability compared with conventional Dox-LP. Therefore, the first step that co-incorporating Arg and Dox liposome was successfully prepared.

Table 4.3 The stability of the developed liposomes.

Sample	Drug release rate (pH 7.4)	
	24h	48h
Dox-LP	2%	2.4%
Arg-Dox-LP	2.7%	3.5%

4.3.5 Blood circulation of Arg-Dox-LP

The blood circulation half-lives of Arg-Dox-LP and Dox-LP were evaluated after i.v. administration into mice. Fig. 4.2 shows that the blood clearance profiles of Arg-Dox-LP is comparable with Dox-LP. Meanwhile, it was estimated that the blood half-life was about to be 12 h for both liposomes, which is accordance with the previous reports of PEGylated liposomes in mice.[3] Thus, the introducing of Arg into Dox-LP did not affect its blood circulation stability. According to the results obtained above, Arg-Dox-LP is a promising nanoparticle to be a new modality for enhancing the anti-cancer therapy.

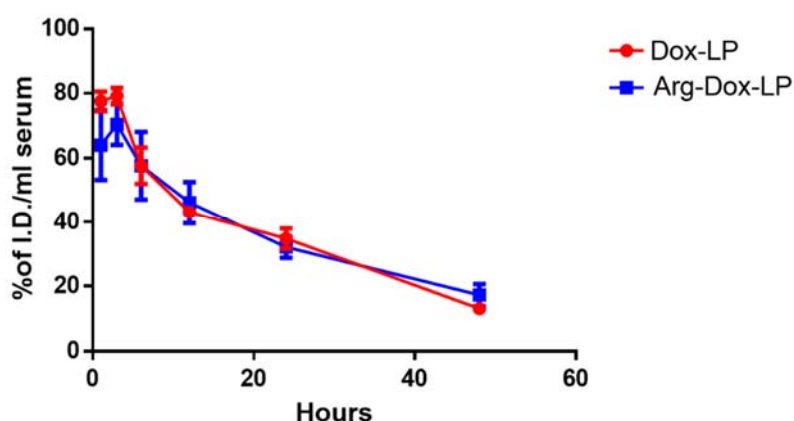


Figure 4.2 Concentration change of liposomes in mouse serum after intravenous injection into mice. Data are expressed as mean $\pm$ SD (n = 3).

4.4 Summary

In this chapter, the author developed a co-encapsulating Arg and Dox PEGylated liposome, named Arg-Dox-LP. The prepared Arg-Dox-LP had about 100 nm in diameter which was expected to be applied in tumor accumulation via the EPR effect.

The NO release measurement revealed that after enclosed by the phospholipid membrane, the capacity of Arg to convert to NO still reserved. Meanwhile, the author confirmed that by incorporating Arg, the EE% of Dox and the in vitro or in vivo stability of liposome not be affected. Therefore, the author developed a promising liposome, co-incorporate two agents which showed a synergistic effect. It is expected to open up new technological and pharmaceutical innovations for anticancer therapy.

4.5 Reference

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Chapter 5

Conclusions

The complexities and special structures of tumor microenvironment (TME) set great obstacles to the cancer therapies. Researchers are now coming to the appreciation that making use of the TME by developed techniques to treat solid tumors. Among those strategies, selective modifying blood flow to tumors and combining conventional drugs with active molecules produced in the TME seem to be effective and promising. In this thesis, aiming to enhance the tumor drug delivery, the author applied a tumor specific vasodilator IRL-1620 combined with a representative nanoparticle PEGylated liposome. The author found that optimized dose of IRL-1620 increased tumor accumulation of liposome in the mice colon cancer model which provide a new option to enhance the nanomedicine's tumor delivery.

Moreover, with the purpose that further augment the effect of chemotherapy, the author utilized the effect of TAM in the TEM, employed an mild endogenous NO donor Arg to combined with a typical chemotherapeutic drug Dox. By a co-culturing system, the author observed a synergistic anticancer effect of Arg and Dox which provide a new effective combination strategy to treat tumor in an enhanced activity without amplification of the side effects. Meanwhile, the author found that the sequence of introducing the Arg and Dox matter the augmentation effect which also offer a new sight to the further application of this technique. Therefore, based on the observation above, the author established a strategy that co-incorporated Arg and Dox

into one liposome to further enhance its delivery efficiency. The developed Arg-Dox-LP was successfully prepared which showed comparable characteristics with conventional Dox-LP including the stability and EE%.

Perspective

In this thesis, based on the special properties of TME, the author developed two approaches which aims to improve the tumor drug delivery system and enhance the drug's effectiveness. The strategy that utilizes the optimum dosage of a specific agonist of ET_B receptor IRL-1620 successfully boosted nearly two-fold of the nanoparticles' tumor accumulation. This approach could be expended to the chemotherapeutics' clinical application that improving tumor-specific drug delivery and decrease the unwanted side effect. Meanwhile, the strategy that employing a mild endogenous NO donor Arg at a lower dosage synergistically enhanced the anticancer effect of Dox also could be further applied. The author found that the drug introducing sequence matters the synergic effect which gives a new sight to the drug effectiveness development. To co-encapsulating of Arg and Dox into PEGylated-LP is a potential strategy that combined the synergistic anticancer effect with the EPR effect to maximum augment the drug effectiveness. This strategy also needs extended experiment to evaluate its anticancer effect on mice tumor model. We expect that this liposome is promising to show enhanced drug delivery and anticancer effect.

Acknowledgement

Completion of this thesis is a milestone of my three years' research study at Kyushu University and specifically within the Katayama Laboratory. I wish to show my profound gratitude to multiple people who supported and encouraged me to pursue my research and the dissertation, without the help of them I could not complete my doctoral thesis.

First and foremost, I would like to say a very big thank you to my supervisor, Professor Yoshiki Katayama, Director of Systems Life Sciences Kyushu University. I clearly remember the first day when I entered the Lab, he encouraged me to “enjoy the science life”. I wish to express my sincere gratitude to him for the continuous support of my Ph.D. study and the related research, for his motivation, and immense knowledge.

I would like to thank my doctoral thesis committee members; Professor Noriho Kamiya and Professor Yoshinori Katakura, for their invaluable advice and questions.

My heartfelt thanks also go to Associate Professor Takeshi Mori, my research advisor, for his guidance and help in all the time of my research and writing of this thesis, also for his warm support when I needed most. With his extraordinary patience and consistent assistance made me go further professionally and personally.

I am sincerely grateful for Associate Professor Akihiro Kishimura's valuable suggestion on my experimental procedures during discussion and presentation.

Then I pleased to acknowledge my S-DDS group members, Dr. Hikari Sato, Dr. Takuma Yoshikawa, Mr. Thanh Nam Le, Ms. Xizheng Sun, Ms. Yukina Mori, Mr.

Khanh Quoc Phan, Ms. Sayaka Inada, Mr. Takeshi Kishi, and Mr. Duong Viet Dung.

My research and study in the Kyushu University could not be realized without the financial support from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), which I should greatly appreciate.

Besides, I am extremely grateful for my classmates and friends for their wise advice, comments, and cooperation which give me a lot of care when I met difficulties during the pursuit of my Ph.D. study.

Last but not least, my thanks would go to my beloved family for their loving consideration, great confidence, and time in listening to me all through these years.

Haitao Feng
Kyushu University
July, 2020

Achievements

List of publications

1. **Haitao Feng**, Le Thanh Nam, Takuma Yoshikawa, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama, “Effect of an endothelin B receptor agonist on the tumor accumulation of nanocarriers”, *Biological and Pharmaceutical Bulletin*, 2020, in press.
2. **Haitao Feng**, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama, “Evaluation of Synergistic Effect of L-Arginine on the Anticancer Activity of Doxorubicin by Using Co-culture System”, *Analytical Sciences*, 2020, 20P200.

List of supplementary publications

1. Takuma Yoshikawa, Yukina Mori, **Haitao Feng**, Khanh Quoc Phan, Akihiro Kishimura, Jeong-Hun Kang, Takeshi Mori, Yoshiki Katayama, “Rapid and continuous accumulation of nitric oxide-releasing liposomes in tumors to augment the enhanced permeability and retention (EPR) effect”, *International Journal of Pharmaceutics*, 565 (2019) 481–487.
2. Takuma Yoshikawa, Khanh Q Phan, Hiroshi Tagawa, Koichi Sasaki, **Haitao Feng**, Akihiro Kishimura, Takeshi Mori, Yoshiki Katayama, “Modification of nitric oxide donors onto a monoclonal antibody boosts accumulation in solid tumors”, *International Journal of Pharmaceutics*, 583 (2020) 119352.

List of presentations and awards.

1. “Employing an endothelin B receptor agonist to enhance tumor accumulation of nanocarriers” 2019 第 41 回日本バイオマテリアル学会大会 ハイライト発表.
2. 3rd Student Seminar on Chemistry for Molecular System, Kyushu University **Best Presentation Award**
3. 2nd Student Seminar on Chemistry for Molecular System, Kyushu University **Best Presentation Award**