Pharm Res (2015) 32:2318–2327
DOI 10.1007/s11095-015-1623-4

RESEARCH PAPER

Enhanced Anti-Angiogenic Effect of Low Molecular Weight Heparin-Bile Acid Conjugates by Co-Administration of a Selective COX-2 Inhibitor

Ji-young Kim • Seung Woo Chung • Sang Yoon Kim • Youngro Byun

Received: 11 September 2014 / Accepted: 6 January 2015 / Published online: 15 January 2015

ABSTRACT

Purpose To overcome definite limitations of angiogenesis inhibitors such as insufficient therapeutic efficacy as a single drug and resisting or conflicting effect under chronic treatment, it is required to develop a new regimen to improve the therapeutic effect.

Methods The combination effect of a multi-targeting angiogenesis inhibitor (LHT7) and a selective cyclooxygenase-2 inhibitor (celecoxib) on neovascularization in tumor growth was studied both *in vitro* and *vivo* experiments.

Results While hypoxia-mediated COX-2 overexpression and macrophage recruitment were observed at LHT7-treated tumor tissues, it was well-controlled by the combination of celecoxib and LHT7. On the other hand, the *in vitro* tube formation and the *in vivo* tumor vessel formation and structure were inhibited by either LHT7 or celecoxib, but the inhibition effect was further enhanced by using them together. However, the combination therapy did not further enhance the inhibitory effect on tumor growth in terms of volume compared to single drug uses, which attributed not to increased cellular apoptosis but to decreased cell proliferation.

Conclusions COX-2 inhibition could enhance the therapeutic effect of anti-angiogenic drugs both by inhibiting the inflammatory reactions induced by hypoxia and by altering the vascular stabilization that is mediated by an assembly with mural cells.

J.-y. Kim • S. W. Chung • Y. Byun (✉)
Research Institute of Pharmaceutical Sciences, College of Pharmacy Seoul National University, Seoul 151-742, South Korea
e-mail: yrbyun@snu.ac.kr

S. Y. Kim
Department of Otolaryngology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul 138-736, South Korea

S. Y. Kim
Biomedical Research Institute, Korea Institute of Science and Technology, Seoul 136-791, South Korea

Y. Byun
Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology Seoul National University, Seoul 151-742, South Korea

KEY WORDS angiogenesis · combination therapy · cyclooxygenase-2 · heparin conjugate · hypoxia

ABBREVIATIONS

COX-2	Cyclooxygenase-2
DCC	*N,N'*-dicyclohexylcarbodiimide
DMF	*N,N*-dimethylformide
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et-STC	Sodium ethylenediamine taurocholate
HoSu	*N*-hydroxylsuccinimide
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
LHT7	Low molecular weight heparin-taurocholic acid conjugate
LMWH	Low molecular weight heparin
PCNA	Proliferating cell nuclear antigen
TCA	Taurocholic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
α-SMA	Alpha smooth muscle actin

INTRODUCTION

Since Folkman raised the involvement of angiogenesis in the tumor development for the first time [1], the role of angiogenesis in tumor pathogenesis has been widely studied. As a result, a variety of angiogenesis inhibitors with different mechanisms have been developed and applied in the clinics together with other chemotherapeutics to treat cancer. However, it has shown its limitations to achieve a sustainable and successful clinical outcome [2, 3]. For example, first, its therapeutic efficacy is not sufficient to be used as a single drug. Thus, it should be combined with other cytotoxic drugs to treat cancer properly. Second, not every type of cancer was susceptible to anti-angiogenic therapy. Moreover, some types of cancer were

initially shrunken by anti-angiogenic therapy, but evolved with resistance traits under chronic inhibition of angiogenesis by eliciting evasive pathways from anti-angiogenic drug in different ways [4]. Even though diverse mechanisms, how cancers become resistant to anti-angiogenic drugs, are clarified, the clinical regimen how to overcome these obstacles has not developed yet.

It might be helpful to understand the close interactions between angiogenesis and inflammation in the tumor pathogenesis to overcome those limitations of anti-angiogenic drugs [5]. It is well-known that the inflammation and angiogenesis make a positive feedback cycle and closely cooperate on the further progression of cancer [6]. Inflammation is considered as a critical driving force for angiogenesis not only in the cancer but also in other diseases since it can produce a variety of cytokines and growth factors that are involved in the angiogenesis [7, 8]. Especially, in case of cancer, the enormous role of inflammatory factors as positive regulators over the whole period of carcinogenesis have been proposed. Among them, cyclooxygenase-2 (COX-2) and prostaglandins are known to be the most representative factors, and their expression can be used as an indicator for prognosis of cancer [9]. They can not only induce the initial vessel formation but also reinforce the vascular structure by assembling with vascular mural cells [10, 11]. In particular, in terms of evasive resistance against anti-angiogenic therapy, it was reported that these matured and vessels stabilized by mural cells do not respond to anti-angiogenic therapy since they pretend to function like a normal, healthy vessel by restoring tumor perfusion [12]. Thus, if the maturation and stabilization of immature vessels are properly inhibited by a pharmacological agent, the anti-angiogenic therapy might be continued for a much longer period with higher efficacy.

Moreover, it was recently reported that even though the primary tumor growth can be reduced by antiangiogenic therapy using sunitinib, which is a VEGFR/PDGFR kinase inhibitor, the pruning effect on tumor vessels might intensify the degree of hypoxia at the tumor tissue. This is because the delivery of oxygen to the tumor site is not sufficient under angiogenesis inhibition to meet the local demand for oxygen required for the excessive metabolism and proliferative rate of cancer cells. Consequently, under hypoxic and starved conditions for tumor growth, a pro-inflammatory response can be induced while generating favorable condition for invasive and aggressive tumors [13, 14]. In addition, the expression of COX-2 was also correlated with the hypoxia induced by anti-angiogenic therapy [15]. On the other hands, the angiogenic growth factors produced by cancer cells and endothelial cells could exacerbate COX-2 mediated inflammatory reaction in tumor tissues. For example, vascular endothelial growth factor (VEGF) is one of stimulator to induce the expression of COX-2 and prostaglandins in the endothelial cells [16]. Thus, the blockade of VEGF-mediated pathway in tumor tissues could improve the therapeutic efficacy by intervening both inflammation and angiogenesis.

Previously, we introduced a low molecular weight heparin-taurocholic acid conjugate (LHT7) as a multi-targeting angiogenesis inhibitor [17, 18]. While its binding affinity to multiple angiogenic growth factors was potentiated by introducing more number of sulfate and hydroxyl groups, its anticoagulant activity became negligible due to the chemical conjugation of LMWH with taurocholic acid at a molar ratio of 1:7 by intervening in the interaction of antithrombin III by steric hindrance [19]. However, the combination use of LHT7 with anti-inflammatory drugs can be a novel strategy not only to improve the therapeutic efficacy but also to prevent the adverse effects that might occur during anti-angiogenic therapy as mentioned above. Among a number of anti-inflammatory drugs, the selective COX-2 inhibitors might be the most promising agent to be utilized in cancer treatment due to its safety and efficacy. Since COX-2 has multiple functions in the overall carcinogenesis, selective COX-2 inhibitors including celecoxib and nimesulide have been widely used not only in cancer therapy but also in cancer prevention as a single and combination therapies [20, 21].

In this study, we have observed the induction of hypoxia and COX-2 expression at tumor tissues under angiogenesis inhibition by using LHT7. Then, the effect of COX-2 inhibition using a selective COX-2 inhibitor on inflammatory reactions, vascular structure and tumor growth was evaluated in a tumor xenograft model.

MATERIALS AND METHODS

Materials

LMWH (Fraxiparine®, 4500 Da) was obtained from Nanjing King-Friend Biochemical Pharmaceutical Company (Nanjing, China). Taurocholic acid (TCA), *N*,*N*'-dicyclohexylcarbodiimide (DCC), *N*-hydroxylsuccinimide (HoSu), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), formamide, methylcellulose, and Tween 20 were purchased from Sigma (St. Louis, MO). Dimethylformide (DMF) was obtained from Merck (Darmstadt, Germany).

Synthesis of LHT7

LHT7 was synthesized according to the previous study [17]. Briefly, ethylenediamine taurocholic acid (5 g) was dissolved in methanol (150 ml) in the presence of sodium hydroxide (5 g) and agitated for 2 h. The mixture was precipitated with cold acetonitrile, washed with cold acetonitrile and freeze-dried to obtain sodium ethylenediamine taurocholate (Et-STC). LMWH (500 mg) was dissolved in distilled water. Then,

HOSu (126.6 mg), EDAC (310 mg) and Et-STC (686 mg) were added consecutively into this solution. After an overnight reaction, LHT7 was finally obtained as a white powder after precipitation and lyophilization.

Tube Formation Assay

The *in vitro* endothelial tube formation assay was performed as described in previous studies [22]. Briefly, 1.5×10^4 human umbilical vein endothelial cells (HUVECs) in basal medium-2 containing VEGF (50 ng/mL; Peprotech, NJ) were added onto polymerized Matrigel (BD Bioscience, Billerica, MA). Then, the cells were treated with LHT7 (50 μg/ml), celecoxib (10 μg/ml), and the combination of those two drugs for 6 h. The number of branch points in each capillary-like tube in each well were counted and statistically analyzed ($n=3$).

In vivo Studies on Vascular Structure and Tumor Growth

Seven-weeks-old male C3H/HeN mice (Orient Bio Inc., Seungnam, South Korea) inoculated with 1×10^6 SCC7 murine squamous cancer cells to the right flank. At the same time, the mice were divided into two groups according to the experimental purpose: 1) dose-dependent induction of hypoxia of LHT7 at doses of 0, 0.5, 1, and 5 mg/kg/2 days; 2) combination effect of LHT7 (1 mg/kg/2 day; intravenous injection) and celecoxib (10 mg/kg/day; oral administration) on vascular structure and tumor growth ($n=5$). Two hundred microliter of celecoxib (Celebrex®, Pfizer, NY) suspended in 0.5% methylcellulose containing 0.025% Tween 20 as a stabilizer was administered by oral gavage. When the tumor volume of control was approximately 50–80 mm^3, tumor size was measured in two dimensions using slide calipers every 3 days. Tumor volume was calculated as $a\times b^2\times0.5$, where a is the largest and b is the smallest diameter. Body weight was also observed. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Seoul National University (Seoul, South Korea; approved animal experimental protocol number SNU-130830-2).

Hypoxic Probe Staining

After 25 days of the drug treatment, mice were intraperitoneally injected with pimonidazole hydrochloride (60 mg/kg body weight; Hypoxyprobe Inc., Burlington, MA) in order to detect the degree of hypoxia on tumor tissues ($n=3$). Then, tumor tissues were isolated, fixed in 4% formalin, embedded in paraffin, and sectioned at 4 μm for following studies. For the detection of hypoxic area bound with pimonidazole through thiol groups, the slides were stained with FITC-conjugated mouse monoclonal antibody to pimonidazole adducts. Then, the whole area of stained tissue was observed by tile scanning method using a confocal laser microscope (Carl Zeiss LSM710, Germany). For detection of COX-2 expression at hypoxic area, the slides were further incubated with anti-COX-2 antibody (Thermo Scientific, IL; dilution ratio at 1:200) followed by Alexa 594-conjugated anti-rabbit IgG secondary antibody (Life Science, OR) and counterstaining with Hoechst.

Vessel Staining with Lectin Perfusion

For microscopic imaging of functionalized vessels, FITC-lectin (100 μg) was intravenously administered to SCC7-tumor bearing mice to visualize microvessels ($n=3$). Five minutes after lectin injection, the tumor tissue was removed, and cryosectioned in 5 μm-thickness. After fixation in cold acetone for 10 min, the tissue slides were incubated with phycoerythrin-conjugated CD31 antibody (Life Science, OR) overnight. Then, after counter-staining by Hoechst, slides were imaged by confocal laser scanning microscopy (Carl Zeiss LSM710, Germany).

Immunohistological Evaluation

For the histological analysis, tumor tissues were labelled with anti-F4/80 (AbCam, Cambridge, MA; dilution ratio at 1:200), anti-alpha smooth muscle actin (α-SMA) (AbCam; dilution ratio at 1:200), anti-collagen IV (AbCam; dilution ratio at 1:500), and anti-PCNA (Thermo Scientific; dilution ratio at1:1600) following a general protocol of immunohistochemistry. After proper incubation with primary antibodies, in case of immunofluorescence for F4/80, the tissue sections were incubated with an Alexa 594-conjugated anti-rabbit IgG secondary antibody. On the other hand, tissue slides were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (to collagen IV antibody) and goat anti-mouse immunoglobulins (to anti-SMA antibody), and then developed with liquid 3, 3'-diaminbenzidine with counterstaining by hematoxylin. On the other hand, tumor tissues were also subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays according to the manufacturer's manual (Millipore Corporation, Billerica, MA).

Statistical Analysis

All data are reported as mean ± standard error of the mean (SEM). Statistical analysis of data was performed with the one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons procedure, in which several treatment groups were compared with a control. Differences of $p<0.05$ were considered significant.

RESULTS

Induction of Hypoxia and COX-2 Expression Under Anti-Angiogenic Therapy

We evaluated the inhibition effect of LHT7 on tumor growth in a dose-dependent manner (0, 0.5, 1, 5 mg/kg/day) in SCC7-bearing mice. After 25 days of the treatment, pimonidazole was injected in the mice before the sacrifice to detect hypoxic area on tumor tissues. There was a dose-dependent inhibition effect of LHT7 on tumor growth up to 1 mg/kg, however, no further increase in therapeutic effect was observed between 1 and 5 mg/kg (Fig. 1a). On the other hand, the degree of hypoxia was also increased in a dose-dependent manner. In the case of the control where mice were not treated with LHT7, the intensity of hypoxic area detected by FITC-conjugated pimonidazole was lowest (Fig. 1b).

The expression of COX-2 at the hypoxic area of the tumor tissues was observed by co-immunofluorescence. It was observed that the fluorescence image of FITC, which is conjugated to pimonidazole to detect hypoxic area, and Alexa594, which indicates the existence of COX-2, were well overlapped and correlated (Fig. 2). This result showed that the expression of COX-2 was significantly increased at hypoxic area of tumor tissue that was exposed to antiangiogenic therapy.

Combination effect of Celecoxib and LHT7 on Macrophage Recruitment on Tumor Tissue

The degree of inflammation at the tumor tissues was examined by quantifying the recruitment of macrophages through immunohistochemistry for F4/80. Compared to the control, where mice were not treated with any drug, the tumor tissues of the celecoxib-treated mice showed 20% decrease in the intensity of F4/80-positive cells at ($p > 0.05$, *vs.* control). However, it was observed that the recruitment of macrophage was increased by 66% at tumor tissues from LHT7-treated mice ($p < 0.05$, *vs.* control). Finally, the intensity of F4/80 in the group co-treated with celecoxib and LHT7 was observed to be similar with that of control and the celecoxib only-treated groups (Fig. 3a and b).

Combination Effect of Celecoxib and LHT7 on Vessel Formation *in vitro* and *vivo*

The drug effect on HUVEC activity was examined by *in vitro* tube formation assay. The drug effect was quantified by counting the number of tubular branches at every field. Even though tube formations in the celecoxib- and LHT7-treated groups were inhibited by 35.6 and 39.6%, respectively ($p > 0.05$, *vs.* control), the inhibition effect was significantly enhanced by the combination use of celecoxib and LHT7 (64.4%, $p < 0.05$, *vs.* control) (Fig. 4a and b).

The drug effect on vascular structure was also examined *in vivo* using tumor-bearing mice. The blood vessels in the tumor were stained by perfusion using FITC-labelled lectin and then further labelled with PE-conjugated CD31 antibodies (Fig. 5a). While the functionality of vessels was well maintained in the control, it was almost disturbed either by single or combination therapy with celecoxib and LHT7 without significant difference among drug-treated groups. On the

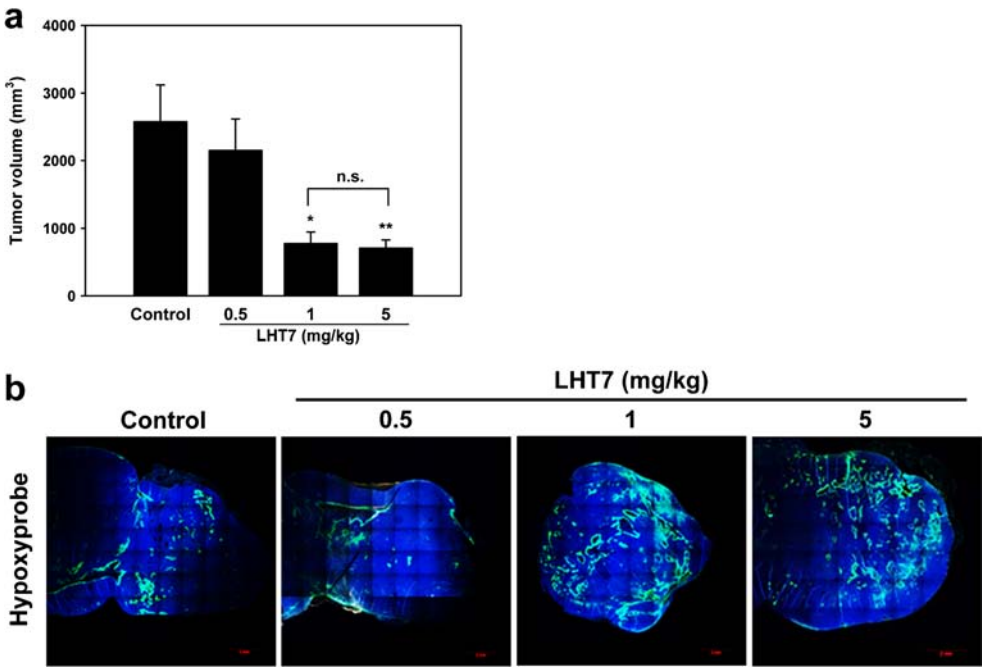

Fig. 1 Dose-dependent effect of LHT7 on tumor growth inhibition. (**a**) SCC7-bearing mice were treated with LHT7 at 0, 0.5, 1, and 5 mg/kg/2 day, and their tumor volume on day 25 were compared ($n = 5$). (**b**) Mice were intraperitoneally injected with Hypoxyprobe™-1 to detect hypoxic area at tumor tissues ($n = 3$). *$p < 0.05$ and **$p < 0.01$, *vs. control.*

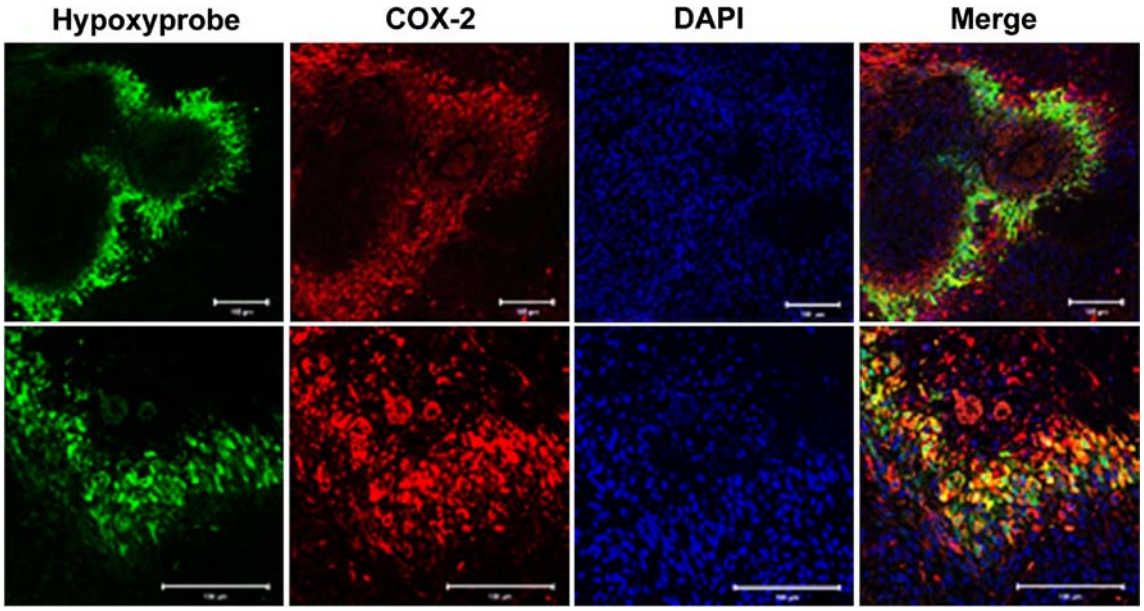

Fig. 2 COX-2 expression under hypoxia at tumor tissues. Tumor tissues were collected from SCC7-bearing mice after Hypoxyprobe™-1 injection and were further stained with anti-COX-2 antibodies ($n = 3$). The relationship between hypoxia and COX-2 expression was observed under confocal laser scanning microscope at magnification of 100 (*top panel*) and 200 (*bottom panel*) (*scale bar* = 200 μm).

other hand, the morphology of CD31-positive endothelial cells was observed a little bit different. Even though the number of endothelial cells was decreased either by celecoxib or LHT7, their combination use inhibited the proliferation of endothelial cells most significantly. In addition, while the CD31-positive endothelial cells in the control formed a regular structure, those of celecoxib-, LHT7-, and combination-treated groups were scattered and irregular.

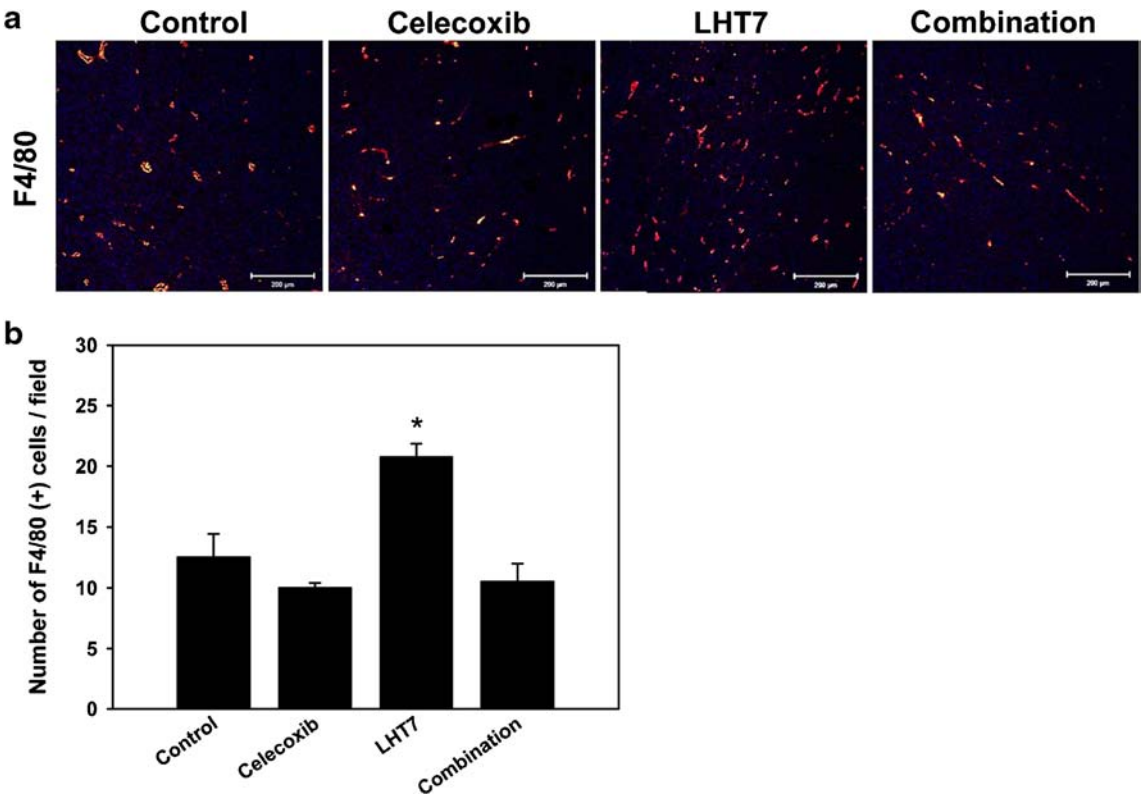

Fig. 3 The recruitment of macrophages under drug treatment with celecoxib, LHT7 and combination use of celecoxib and LHT7. (**a**) The drug effect on the recruitment of F4/80-positive macrophages was studied and (**b**) the intensity was quantitatively compared ($n = 3$) (*scale bar* = 200 μm). *$p < 0.05$, *vs. control.*

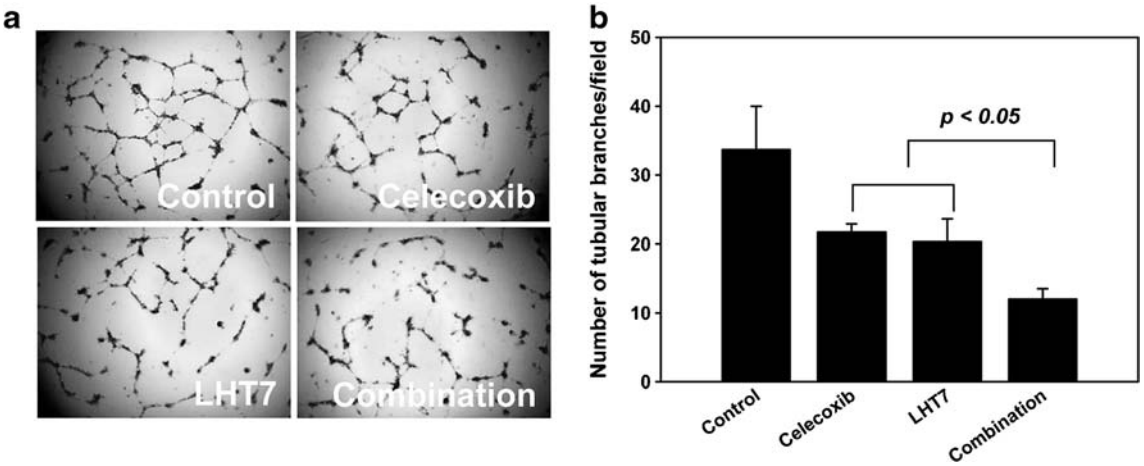

Fig. 4 The effect of celecoxib and LHT7 on *in vitro* tube formation using HUVECs. (**a**) The inhibition effect of celecoxib, LHT7, and combination of celecoxib and LHT7 on *in vitro* tubular formation was observed and photographed. (**b**) Then, the number of branch point per field was counted ($n = 3$).

To examine the combination effect of celecoxib and LHT7 on vascular stabilization in regard to defective assembly or recruitment of vascular mural cells, tumor tissues were immune-stained for α-SMA and collagen type IV. As shown in Fig. 5b, both α-SMA- and the collagen type IV-positive cells were observed to be abundant and well-organized in the control group. However, those of celecoxib- and LHT7-treated groups were sparse and discontinuous. Moreover, the intensities of two markers were weak and scattered throughout tumor tissues in the combination group.

The Effect of Combination Use of Celecoxib and LHT7 on Tumor Growth

The drug effect on tumor growth was evaluated in SCC7 bearing-mice. There was no significant difference in tumor volume between the celecoxib- and LHT7-groups at day 25 (Fig. 6a). Compared to the control, the tumor volume was decreased by 69.5 and 70.0%, respectively. Moreover, the combination use did not further enhance the inhibitory effect on tumor growth, compared to the single use of either celecoxib or LHT7 (77.5%; $p<0.05$, *vs.* control, $p>0.05$, *vs.* celecoxib- or LHT7-treated groups). The treatment with celecoxib, LHT7, or their combination did not affect body weight during the experiment period (Fig. 6b).

The immunohistochemistry of PCNA showed that while cell proliferation was inhibited by celecoxib- or LHT7-treatement (35.8 and 40.8%, respectively), the highest inhibition effect was achieved by the combination use (61.4%; $p<0.01$, *vs.* LH7- or celecoxib-treated groups) (Fig. 6c and e). However, in the TUNEL assay, even though the number of apoptotic cells was significantly increased in all drug-treated groups, there was no further increase in the combination group ($p>0.05$, *vs.* celecoxib-treated group) (Fig. 6a and f).

DISCUSSION

In the present study, we observed the induction of hypoxia and COX-2 expression by anti-angiogenic therapy using LHT7. In addition, COX-2 inhibition effects on anti-angiogenic therapy in regard to tumor vascular structure and tumor volume were evaluated. LHT7 was synthesized by conjugating a LMWH with taurocholic acid at a molar ratio 1:7 [17]. By this conjugated structure, while the anti-coagulant activity of LHT7 became negligible, its binding affinity to various angiogenic growth factors was potentiated by introducing more sulfate moieties to the molecule [18]. Thus, LHT7 was proven to be a potent anti-angiogenic drug by neutralizing multiple targets including VEGF, bFGF, and PDGF. This might guarantee the superiority of LHT7 as an angiogenesis inhibitor, compared to other drugs that target a specific single growth factor, because tumors treated with VEGF-targeted anti-angiogenic drugs might be able to shift from VEGF-dependence to other angiogenic pathways by producing alternative angiogenic growth factors including bFGF and PDGF.

Even though LHT7 is a multi-targeting angiogenesis inhibitor with a strong therapeutic efficacy, the tumor growth inhibition effect of LHT7 at the dose of 5 mg/kg was not further increased than that of 1 mg/kg dose. This might be due to the complexity of tumor pathogenesis that not only angiogenic factors but also other variety of factors are involved in cancer development. In other words, even though tumor growth can be inhibited by angiogenesis inhibition in some parts, this inhibition effect is not further enhanced at high dose of antiangiogenic drugs. Thus, it is common that antiangiogenic drugs are usually combined with other cytotoxic drugs in the clinical anticancer therapy. Moreover, while the tumor growth was inhibited by LHT7, the degree of hypoxia was

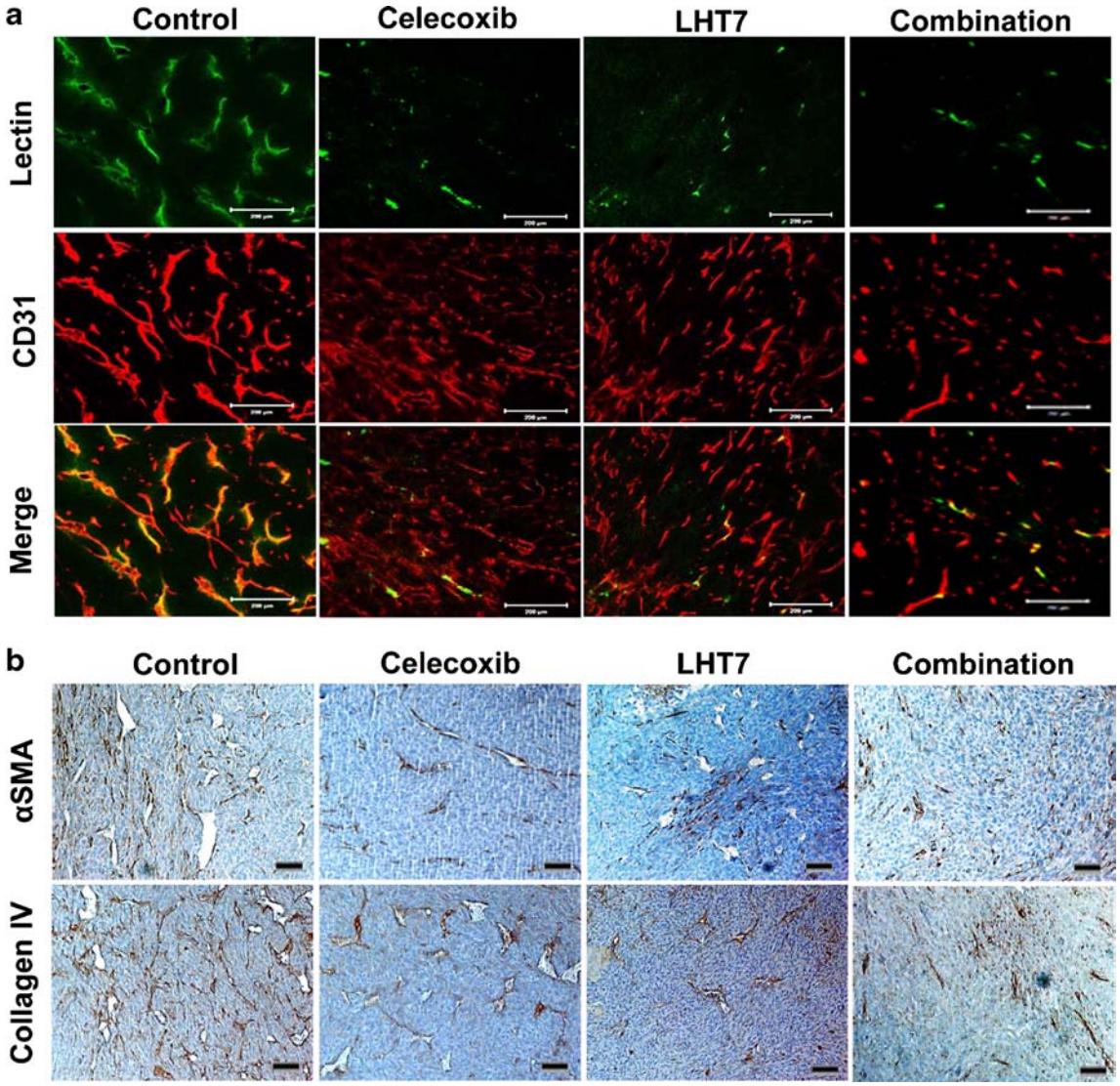

Fig. 5 The effect of celecoxib and LHT7 on *in vivo* vessel formation and structures. (**a**) The inhibition effect of celecoxib, LHT7, and combination of celecoxib and LHT7 on vessel formation and structure was studied using SCC7-bearing mice. Tumor vascular structures were imaged by FITC-labelled lectin-perfusion followed by immunofluorescence using PE-conjugated CD-31. (**b**) The recruitment of mural cells to vascular structure was observed in terms of α-SMA and collagen IV ($n = 3$) (*scale bar* = 200 μm).

dose-dependently increased. In concurrence with exacerbated hypoxia by LHT7 treatment, the expression of COX-2 was also increased at hypoxic area of tumor tissue. Since angiogenesis is initiated for tumor tissues to overcome the harsh conditions induced by insufficient blood supply, the compulsory inhibition of angiogenesis can bring a rather conflicting effect on cancer therapy by intensifying the degree of hypoxia [23]. Thus, contrary to our expectations on anti-angiogenic drugs as a cancer therapeutic, it can worsen the pathological niches at tumor tissues and accelerate the tumor progression in COX-2 mediated pathways under hypoxia [3, 4, 24]. As a result, anti-angiogenic therapy can elicit evasive resistance mechanisms systemically or locally, thus finally shortened the progression-free or overall survival time of anti-angiogenic therapy.

In this study, the recruitment of F4/80-positive macrophages was increased in the LHT7-treated group compared to the control. However, it was prevented by the treatment with celecoxib. These phenomena might be explained either by hypoxia or COX-2 effect on macrophage activity in tumor tissues. Previously, it was reported that macrophages are recruited in the hypoxic area of tumor tissue [25]. In addition, it was reported that the differentiation of monocytes into M2 type macrophages, which is known as tumor associated macrophages at tumor sites mediated by locally expressed COX-2 [26]. These M2 type macrophages can produce inflammatory cytokines and growth factors, which can facilitate the overall cancer progression. Thus, if COX-2 is more activated at tumor tissues by anti-angiogenic therapy, it can worsen the tumor microenvironment through COX-2 mediated

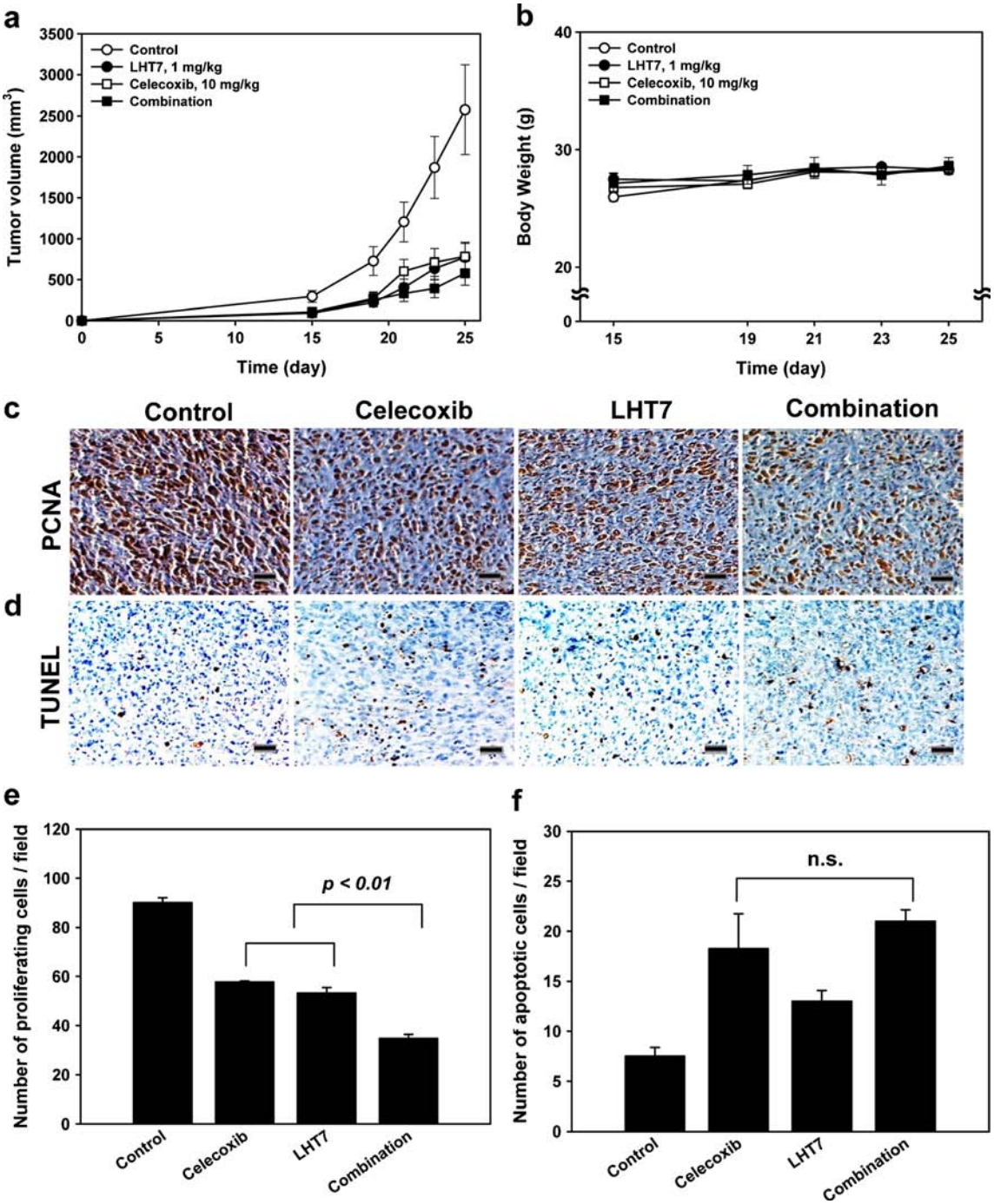

Fig. 6 The combination effect of celecoxib and LHT7 on tumor growth inhibition. SCC-7 bearing mice were treated with either celecoxib (10 mg/kg/day), or LHT7 (1 mg/kg/2 days), or combination of celecoxib and LHT7 ($n = 5$). (**a**) Tumor volume and (**b**) body weight change over the time were observed. The tumor tissues were isolated and examined by immunohistochemistry for PCNA (**c**, **e**) and TUNEL assay (**d**, **f**) (*scale bar* = 100 μm).

inflammatory reactions. This might provide a new rationale for the combination use of a selective COX-2 inhibitor with anti-angiogenic drugs.

In this context, we decided to study the combination effect of a multi-targeting anti-angiogenic drug (LHT7) and a selective COX-2 inhibitor (celecoxib) on vessel formation and stabilization and finally on tumor growth. *In vitro* tubular formation assay, even though a single use of celecoxib or LHT7 still inhibited the tube formation, their combined use further improved the inhibition effect. On the other hand, while the study on functional vessel formation by lectin perfusion showed no significant difference among the drug-treated groups, the morphology of CD31-positive endothelial cells at tumor vascular structure was affected by each drug regimen in

a different way. The number of endothelial cells was more significantly decreased by the combination use than that of single drug treatment. Moreover, the immunohistochemistry of α-SMA and collagen IV also shows that the combination use further improved the inhibitory effect on vascular mural cell recruitment to tumor sites than the single drug treatments. It is well known that new vessels are formed through a serial cascade including proliferation and recruitment of endothelial cells, tube formation and elongation by assembling endothelial cells, and finally maturation and stabilization by vascular mural cells [27, 28]. Thus, the results in this study showed that the combination use of LHT7 and celecoxib could inhibit tumor angiogenesis by intervening angiogenic process at multiple points. While LHT7 can disturb the extracellular action of angiogenic growth factors by capturing them, celecoxib can inhibit the intracellular activity of COX-2. On the whole, the combination use of LHT7 and celecoxib could inhibit the formation of finally functionalized vessels at tumor site.

However, the combination did not further enhance the tumor growth inhibition compared to either of the single drug groups. In addition, our drug regimen showed its therapeutic effect by retarding the tumor growth rather than by shrinking or diminishing the tumor tissues. This was also supported by the histological analysis, which showed that while cell proliferation was significantly inhibited by drug treatment with celecoxib and LHT7, the number of apoptotic cells was not significantly enhanced by combination therapy. Even though celecoxib can directly induce the apoptosis of cancer cells via cell cycle arrest at very high doses of celecoxib [20, 29], only 10 mg/kg of celecoxib was administered in the present study, an amount, which is not enough to induce cancer cell apoptosis but to retard cancer cell proliferation. Thus, the increased apoptosis in the tumor tissues of celecoxib-treated group might attribute to angiogenesis inhibition just as the LHT7-treatment would. In other words, the present regimens using celecoxib and LHT7 could not directly target cancer cells to eradicate, but rather intervened with tumor microenvironment to generate unfavorable conditions for cancer progression [10, 30]. This is because both LHT7 and celecoxib affect the tumor microenvironment to generate unfavorable conditions for cancer progression. PGE2 has been reported to trigger diverse intracellular signaling pathways, which can lead to cancer cell survival, angiogenesis and vessel maturation [31]. As a result, the anticancer effect of selective cyclooxygenase-2 inhibitors can be explained in terms of different mechanisms. That is, cyclooxygenase-2 inhibitors affects cancer progression either directly, being related to cancer cell survival, or indirectly, being related to angiogenesis, vessel maturation and ECM remodeling. In the single drug treatment group using celecoxib, the tumor growth inhibition can be attributed to the inhibition of cancer cell proliferation or angiogenesis. Thus while the combination use for primary tumor growth inhibition did not show a remarkable effect, this regimen could be applied as an adjuvant therapy to improve the efficacy of cancer chemotherapy using other cytotoxic drugs. COX-2 inhibitors have been used previously as an adjuvant therapy along with cytotoxic drugs like irinotecan and paclitaxel and radiotherapy [32, 33]. In addition, the combination use of cytotoxic drug with heparin-bile acid conjugates also showed a synergetic effect on cancer treatment [34]. Thus, when this regimen is combined with cytotoxc drug, the therapeutic effect might be enhanced more significantly with lower risk of resistance and longer period of duration. Moreover, it can be utilized for caner chemoprevention by slowing down overall carcinogenesis and extending the latency with a progression-free period.

CONCLUSION

In this study, we showed that the new anti-angiogenic regimen combined with selective COX-2 inhibitor is fully capable of being applied in clinics to prevent evasive resistance to cancer treatment which comes from the chronic anti-angiogenic therapy. Thus, we expect that this new regimen will improve the overall clinical therapeutic efficacy by prolonging the progression-free period and overall survival rate of cancer patients.

ACKNOWLEDGMENTS AND DISCLOSURES

This study was supported by grants from Basic Science Research Program (grant no. 2010–0027955) and the Bio & Medical Technology Development Program (grant no. 2012028833) through the National Research Foundation of Korea (NRF) funded by Korea government [MSIP].

REFERENCES

1. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285:1182–6.
2. Senninoand B, McDonald DM. Controlling escape from angiogenesis inhibitors. Nat Rev Cancer. 2012;12:699–709.
3. Bergersand G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 2008;8:592–603.
4. Loges S, Mazzone M, Hohensinner P, Carmeliet P. Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. Cancer Cell. 2009;15:167–70.
5. Ono M. Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. Cancer Sci. 2008;99:1501–6.
6. Sahin M, Sahin E, Gumuslu S. Cyclooxygenase-2 in cancer and angiogenesis. Angiology. 2009;60:242–53.
7. Coussensand LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7.
8. Kunduand JK, Surh YJ. Inflammation: gearing the journey to cancer. Mutat Res. 2008;659:15–30.
9. Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, *et al.* The COX-2/PGE2 pathway: key roles in the

hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis. 2009;30:377–86.
10. Lee A, Frischer J, Serur A, Huang J, Bae JO, Kornfield ZN, *et al.* Inhibition of cyclooxygenase-2 disrupts tumor vascular mural cell recruitment and survival signaling. Cancer Res. 2006;66:4378–84.
11. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, *et al.* Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature. 2007;446:765–70.
12. Fisher JC, Gander JW, Haley MJ, Hernandez SL, Huang J, Chang YJ, *et al.* Inhibition of cyclo-oxygenase 2 reduces tumor metastasis and inflammatory signaling during blockade of vascular endothelial growth factor. Vasc Cell. 2011;3:22.
13. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell. 2009;15:232–9.
14. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, *et al.* Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009;15:220–31.
15. Wang X, Zhang L, O'Neill A, Bahamon B, Alsop DC, Mier JW, *et al.* Cox-2 inhibition enhances the activity of sunitinib in human renal cell carcinoma xenografts. Br J Cancer. 2013;108:319–26.
16. Wu G, Luo J, Rana JS, Laham R, Sellke FW, Li J. Involvement of COX-2 in VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial cells. Cardiovasc Res. 2006;69:512–9.
17. Lee E, Kim YS, Bae SM, Kim SK, Jin S, Chung SW, *et al.* Polyproline-type helical-structured low-molecular weight heparin (LMWH)-taurocholate conjugate as a new angiogenesis inhibitor. Int J Cancer. 2009;124:2755–65.
18. Chung SW, Lee M, Bae SM, Park J, Jeon OC, Lee HS, *et al.* Potentiation of anti-angiogenic activity of heparin by blocking the ATIII-interacting pentasaccharide unit and increasing net anionic charge. Biomaterials. 2012;33:9070–9.
19. Chung SW, Bae SM, Lee M, Al-Hilal TA, Lee CK, Kim JK, *et al.* LHT7, a chemically modified heparin, inhibits multiple stages of angiogenesis by blocking VEGF, FGF2 and PDGF-B signaling pathways. Biomaterials. 2015;37:271–8.
20. Guptaand RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer. 2001;1:11–21.
21. Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer. 2006;6:130–40.
22. Park JW, Jeon OC, Kim SK, Al-Hilal TA, Jin SJ, Moon HT, *et al.* High antiangiogenic and low anticoagulant efficacy of orally active low molecular weight heparin derivatives. J Control Release. 2010;148:317–26.
23. Rapisardaand A, Melillo G. Role of the hypoxic tumor microenvironment in the resistance to anti-angiogenic therapies. Drug Resist Updat. 2009;12:74–80.
24. Ebos JM, Lee CR, Kerbel RS. Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy. Clin Cancer Res. 2009;15:5020–5.
25. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood. 2004;104:2224–34.
26. Nakanishi Y, Nakatsuji M, Seno H, Ishizu S, Akitake-Kawano R, Kanda K, *et al.* COX-2 inhibition alters the phenotype of tumor-associated macrophages from M2 to M1 in ApcMin/+ mouse polyps. Carcinogenesis. 2011;32:1333–9.
27. Carmelietand P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473:298–307.
28. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. Nat Rev Cancer. 2010;10:505–14.
29. Williams CS, Watson AJ, Sheng H, Helou R, Shao J, DuBois RN. Celecoxib prevents tumor growth in vivo without toxicity to normal gut: lack of correlation between in vitro and in vivo models. Cancer Res. 2000;60:6045–51.
30. Albiniand A, Sporn MB. The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer. 2007;7:139–47.
31. Gasparini G, Longo R, Sarmiento R, Morabito A. Inhibitors of cyclo-oxygenase 2: a new class of anticancer agents? Lancet Oncol. 2003;4:605–15.
32. Altorki NK, Keresztes RS, Port JL, Libby DM, Korst RJ, Flieder DB, *et al.* Celecoxib, a selective cyclo-oxygenase-2 inhibitor, enhances the response to preoperative paclitaxel and carboplatin in early-stage non-small-cell lung cancer. J Clin Oncol. 2003;21:2645–50.
33. Trifan OC, Durham WF, Salazar VS, Horton J, Levine BD, Zweifel BS, *et al.* Cyclooxygenase-2 inhibition with celecoxib enhances antitumor efficacy and reduces diarrhea side effect of CPT-11. Cancer Res. 2002;62:5778–84.
34. Park K, Lee GY, Park RW, Kim IS, Kim SY, Byun Y. Combination therapy of heparin-deoxycholic acid conjugate and doxorubicin against squamous cell carcinoma and B16F10 melanoma. Pharm Res. 2008;25:268–76.