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Hydrazinocurcumin, a Novel Synthetic Curcumin Derivative, Is a Potent Inhibitor of Endothelial Cell Proliferation

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Abstract—Curcumin and some of its derivatives were known as in vivo inhibitors of angiogenesis. In present study, a novel curcumin derivative, named hydrazinocurcumin (HC) was synthesized and examined for its biological activities. HC potently inhibited the proliferation of bovine aortic endothelial cells (BAECs) at a nanomolar concentration ($IC_{50} = 520$ nM) without cytotoxicity. In vivo and in vitro angiogenesis experiments showed HC as a new candidate for anti-angiogenic agent.

Introduction

Angiogenesis is the process of new blood vessel formation by endothelial cells.[1] The process is complex and involves several distinct steps such as membrane degradation by proteolytic enzymes secreted by the endothelial cells, chemotactic migration toward the stimulus, proliferation of these cells, and formation of vascular loops.[2] Each step of the process is tightly controlled by regulatory factors that stimulate or inhibit angiogenesis. However, these elaborated control mechanisms are often disordered in several pathologic diseases including cancer.[3] Extensive studies have shown that angiogenesis is a crucial process for the outgrowth of cancer cells and their spreading into other tissues. Therefore, the specific inhibition of angiogenesis is considered as a powerful way to inhibit tumor growth and metastasis. Several angiogenesis inhibitors from natural products and chemical libraries have been developed for this purpose and some of them, including TNP-470, are undergoing clinical trials for cancer therapy.[4–6]

Curcumin, a natural product found in the rhizome of *Curcuma longa*, is a potent chemopreventive agent that has been entered into the phase I clinical trials for chemoprevention by National Cancer Institute.[7] Curcumin showed a potent anti-carcinogenic activity against a broad range of tumor types including skin, forestomach, duodenal, and colon carcinogenesis.[8–11] It has been postulated that the broad spectrum of anti-carcinogenic activity of curcumin may be due in part to angiogenesis inhibition.[12,13] Curcumin and some of its derivatives including demethoxycurcumin (DC) and tetrahydrocurcumin (THC) were known as potent inhibitors of angiogenesis.[14] DC as well as THC, a murine metabolite of curcumin, showed a significant inhibition of corneal neovascularization, but the potential of the activity was relatively weaker than that of curcumin.

Extensive studies on structure–activity relationship of curcuminoids have revealed that phenol ring structures of the compound may be essential for the anti-oxidant activity,[15] and diketone moiety of THC also involves

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antioxidative mechanism of the compound.[16] In addition, phenolic hydroxyl or methoxyl groups were modified to enhance the potency of other biological activities of curcumin including the induction of Phase 2 detoxification enzymes and the inhibition of HIV-1 integrase.[17,18] These studies suggest that diketone moiety of curcumin may be essentially involved in its broad-range biological activities.

We have developed several new synthetic derivatives of curcumin to enhance the biological activity, especially, anti-angiogenic activity of the compound. The potencies of newly synthesized curcumin derivatives were evaluated using several angiogenesis assays including endothelial cell proliferation, chemoinvasion, capillary tube formation, and in vivo angiogenesis of chorioallantoic membrane of chick embryo. Among the synthetic derivatives, hydrazinocurcumin (HC) showed the most potent inhibitory activity against the proliferation of BAECs. The synthesis, structure determination, and anti-angiogenic activity of HC will be described.

Results and Discussion

Chemistry

The general procedures for synthesis of derivatives are shown in Schemes 1 and 2. Curcumin (**1a,** R_1, $R_2 = OCH_3$) and its natural analogues including demethoxycurcumin (**1b**, $R_1 = OCH_3$; $R_2 = H$ or vice versa) and bisdemethoxycurcumin (**1c**, $R_1 = H$; $R_2 = H$) were prepared from the purification of commercially available curcuminoids using preparative thin layer chromatography. Diketone moieties of each curcumin analogue were replaced with hydrazine derivatives in the presence of acetic acid. One-step coupling of **1** with hydraziniumdihydrochloride and hydrazinobenzoic acid gave compounds **2** to **3**. Each compound was purified with preparative TLC and HPLC, and was assayed for anti-angiogenic activity.

Structure determination of HC

HC (**2a**), a synthetic analogue of curcumin was obtained as pale yellow gum which analyzed for $C_{21}H_{20}N_2O_4$ by HRMS, thus 13 degrees of unsaturation. The structure of this compound was determined by combined 2D NMR experiments as well as comparison of spectral data with those obtained for curcumin (Table 1). All of the signals of protons and carbons of the benzene ring were readily assigned on the basis of the results of ^1H COSY, gHSQC, and gHMBC experiments. However, considerable differences were observed for the NMR signals corresponding to the protons and carbons at the side chain. The ^{13}C NMR spectra showed a broad signal at δ 131.1 (d) which correlated to both of the protons at δ 7.09 and 6.94 in the gHSQC spectra. The ^1H COSY data revealed that these protons were coupled to each other with very large coupling constant ($J = 16.1$ Hz).

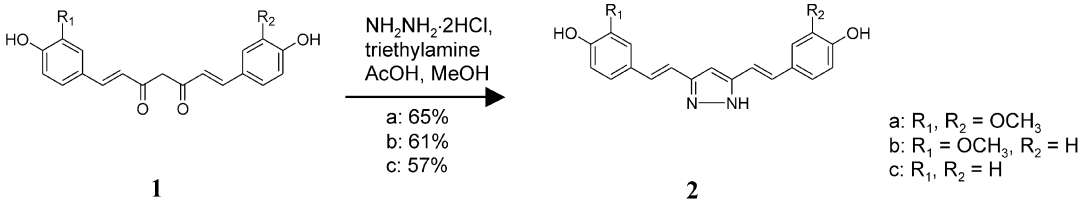
Therefore, these were thought to be the olefinic protons of a *trans*-double bond. This interpretation was supported by ROESY experiments in which cross peaks were observed between both of the olefinic protons and aromatic ones at δ 7.16 and 6.97.

The NMR data of HC contained another signals of an additional methine group; δ_H 6.64, δ_C 99.9. Since the intensity of the proton signal was almost half of others, this methine must be corresponded to the C-10 symmetric center of the molecule. Although signal of the C-9 carbon was not observed due to the rapid tautomerization between two forms of a pyrazole moiety, the upfield shift of the C-10 methine carbon in the ^{13}C NMR data indicated the placement of an olefinic carbon bearing an electronegative atom at adjacent location. Data supporting this interpretation were provided by 2D NMR experiments in which the gHMBC correlation between H-8 and C-10 as well as the ROESY cross peaks between H-8 and H-10 were observed. Thus, the structure of HC was defined as an analogue of curcumin bearing a pyrazole moiety in the middle of chain.

Anti-angiogenic activity of HC

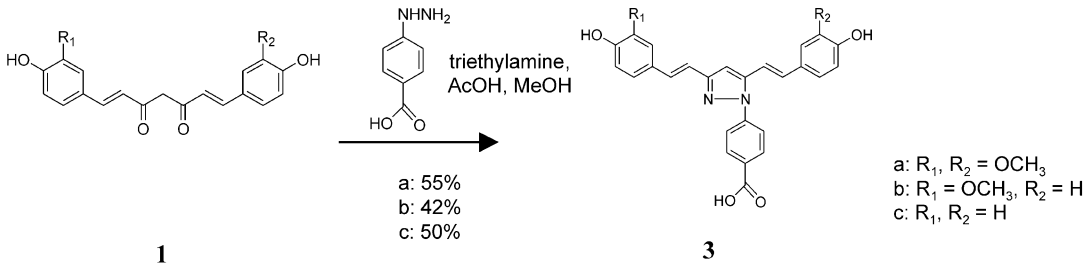
To evaluate anti-angiogenic activities of synthetic curcumin derivatives, we first examined the effect of compounds on endothelial cell proliferation using MTT colorimetric assay. Among the six derivatives tested, HC showed the most potent growth inhibitory activity against BAECs with an IC_{50} of 0.52 μM (Table 2). The inhibitory potency of HC against BAECs proliferation was over 30-fold higher than that of curcumin. Hydrazine derivative with bulky benzoic acid (**3**) also showed an enhanced anti-proliferative activity against BAECs. However, the potency of **3** was relatively weaker than that of HC.

Next, we investigated to determine the cell line specificity of HC on its anti-proliferative activity. Several epithelial and fibroblast cells including, HT29, colon carcinoma, NIH3T3, normal fibroblast, Chang, normal liver cells, and BAECs, were examined and the result showed that HC inhibited the proliferation of each cell lines with a different activity spectrum (Fig. 1). Inter-



1 **2**

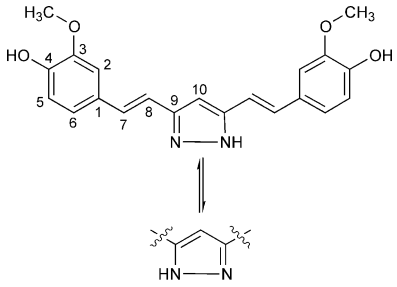
Scheme 1.



1 3

Scheme 2.

Table 1. The structure of HC



	^{1}H	^{13}C	HMBC	ROESY
1		129.5 s		
2	7.16, 2H, br s	110.0 d	3, 4, 6, 7	7, 8, OMe
3		148.8 s		
4		148.0 s		
5	6.78, 2H, d (7.8)	116.1 d	1, 3	6
6	6.97, 2H, dd (7.8, 1.5)	121.2 d	2, 4, 7	5, 7
7	7.09, 2H, d (16.1)	131.1 d	2, 6	2, 6, 10
8	6.94, 2H, d (16.1)	131.1 d	10	2, 10
9		N. A.[a]		
10	6.64, 1H, s	99.9 d		7, 8
OMe	3.86, 6H, s	56.2 d	3	2

[a]Signal was not observed.

Table 2. IC_{50} values of curcuminoids and synthetic derivatives for BAECs

	R_1	R_2	BAECs, IC_{50} (M)[a]
Curcuminoids, **1**	OCH_3	OCH_3	$1.5\pm0.3\times10^{-5}$
	OCH_3	H	$2.2\pm0.5\times10^{-5}$
	H	H	$5.3\pm0.6\times10^{-5}$
Hydrazino curcuminoids, **2**	OCH_3	OCH_3	$5.2\pm0.4\times10^{-7}$
	OCH_3	H	$1.8\pm0.3\times10^{-6}$
	H	H	$5.8\pm0.2\times10^{-6}$
Hydrazinobenzoyl curcuminoids, **3**	OCH_3	OCH_3	$9.3\pm0.4\times10^{-7}$
	OCH_3	H	$2.4\pm0.1\times10^{-6}$
	H	H	$8.7\pm0.2\times10^{-6}$

[a]IC_{50} values are the mean±SE from three independent experiments.

estingly, there was high endothelial cell specificity on the anti-proliferative activity of HC. Specificity factors over 20 were obtained versus HT29, colorectal carcinoma cells. Other normal cell lines also showed a degree of different sensitivity to HC with BAECs. In contrast to HC, curcumin, the parental compound, inhibited the proliferation of these cells in relatively non-selective manner (data not shown). These data suggest that HC inhibits cellular proliferation with an endothelial cell specificity.

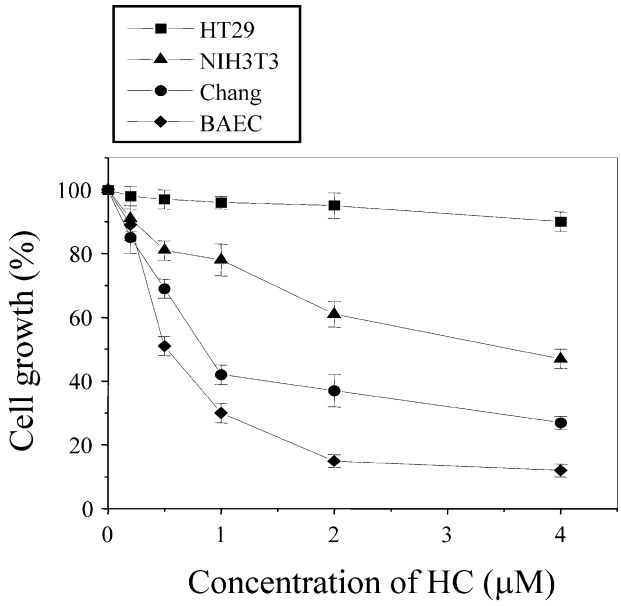


Figure 1. Effect of HC on the growth of various cell lines. Cell growth was measured using MTT colorimetric assay. Data represent mean ±SE from three independent experiments.

Table 3. Inhibitory effect of HC on in vivo neovascularization of CAM

Drug	Inhibited egg/live egg	Inhibition ratio (%)
Empty	0/19	0
Retinoic acid (1 μg/egg)	11/13	85
HC (10 μg/egg)	11/15	73

Endothelial cell invasion and the formation of tubular structures as well as cellular proliferation are essential steps for angiogenic process. Thus, we next investigated the effect of HC on BAECs invasion and tube formation using well established in vitro assays. Basic fibroblast growth factor (bFGF) was used as a chemoattractant or an angiogenic factor. HC potently inhibited bFGF-induced BAECs invasion and tube formation at nanomolar concentrations (Fig. 2A–C). Trypan blue staining was performed in parallel with in vitro angiogenesis assays and cytotoxicity was not shown at concentrations used in this study (Fig. 2D).

In vivo experimental angiogenesis was performed using chorioallantoic membrane (CAM) from growing chick embryo. HC-loaded thermanox coverslip was placed on CAM surface and neovascularized zones were observed

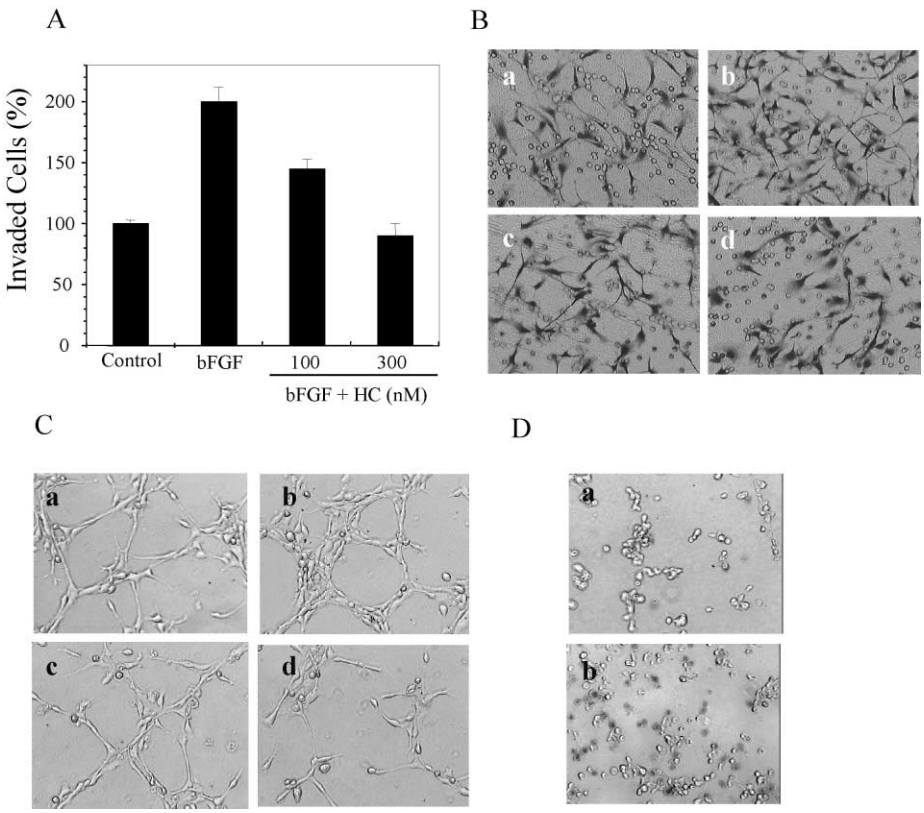


Figure 2. Effect of HC on in vitro angiogenesis. (A) Inhibitory activity of HC on endothelial cell invasion. Serum-starved BAECs left in serum-free medium (Control) or treated with bFGF in the presence or absence of HC were used for invasion assay. Data represent mean ±SE from three independent experiments. (B) Microscopic observation of invaded cells (×100 magnification). (a) Control; (b) bFGF alone; (c) bFGF + HC (100 nM); (d) bFGF + HC (300 nM). (C) Effect of HC on tube forming ability of BAECs. (a) Control; (b) bFGF alone; (c) bFGF + HC (100 nM); (d) bFGF + HC (300 nM). (D) Trypan blue staining to evaluate in vitro toxicity of HC on tube-formed endothelial cells. Staurosporin, a cytotoxic agent, was used as an indicator of in vitro toxicity. (a) bFGF + HC (300 nM); (b) bFGF + staurosporin (10 μM).

under microscope. The inhibition of the angiogenesis of retinoic acid used as the positive control was 85% ($n = 13$), and that of empty coverslips was 0% ($n = 19$). However, HC effectively inhibited the neovascularization of chick embryo (73%, $n = 15$) without affecting any pre-existing vessels (Table 3). These results indicate that HC potently inhibits angiogenesis without affecting endothelial cell viability, and suggest that the compound can be developed as a novel anti-angiogenic agent.

Conclusion

Data presented here showed a novel synthetic analogue of curcumin with altered potency of anti-angiogenic activity, and its chemical and physical properties were characterized using several analytical methods. Biological activities of HC also were evaluated through in vivo and in vitro angiogenesis assays. Our results demonstrate HC as a new candidate for anti-angiogenic agent.

Experimental

General procedures

Curcuminoids (mixture of curcumin, demethoxycurcumin, and bisdemethoxycurcumin) were obtained by Sigma (St. Louis, MO). Chromatography purification: Silica gel column chromatography (Merck, Darmstadt, Germany), thin-layer chromatography (TLC, Merck), and high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) were used to perform general purification procedures. Spectral analysis: The HRFAB-MS spectra were obtained using a Jeol JMS-HX 110 mass spectrometer. The NMR spectra were recorded in $CDCl_3$ solutions on a Varian Unity 500 spectrometer. The proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. All chemical shifts were recorded according to an internal Me_4Si. All solvents used were of spectral grade or distilled from glass prior to use.

Hydrazinocurcumin (2a). Purified **1a** (10 mg, 0.027 mmol) was dissolved in methanol (2 mL), and hydraziniumdihydrochloride (14 mg, 0.135 mmol) and triethylamine (18.8 μL, 0.135 mmol) were added to the solution. In the presence of catalytic amount of acetic acid, the reaction mixture was incubated for 24 h at room temperature with gentle stirring. The solvent was evaporated in vacuo and the residue purified by preparative TLC ($CHCl_3$/MeOH = 6:1; $R_f = 0.4$) and HPLC (semipreparative C18 column; acetonitrile:H_2O = 50:50; flow rate = 3 mL/min; retention time: 21 min) gave **2a** as pale yellow gum (6.19 mg, 65%) which analyzed by HRFAB-MS. Exact mass calcd for $C_{21}H_{20}N_2O_4$: 364.1423, observed (M + H) 365.1501.

Hydrazinodemethoxycurcumin (2b). Utilizing the same protocol as described for the synthesis of **2a**, **2b** (5.9 mg, 61%) was obtained from **1b** (10 mg, 0.029 mmol), hydraziniumdihydrochloride (14 mg, 0.135 mmol), and triethylamine (18.8 μL, 0.135 mmol). Preparative TLC ($CHCl_3$/MeOH = 6:1; R_f = 0.32) gave **2b** as a pale yellow gum which analyzed by HRFAB-MS. Exact mass calcd for $C_{20}H_{18}N_2O_3$: 334.1317, observed (M + H) 335.1393.

Hydrazinobisdemethoxycurcumin (2c). Utilizing the same protocol as described for the synthesis of **2a**, **2c** (5.5 mg, 57%) was obtained from **1c** (10 mg, 0.032 mmol), hydraziniumdihydrochloride (14 mg, 0.135 mmol), and triethylamine (18.8 μL, 0.135 mmol). Preparative TLC ($CHCl_3$/MeOH = 6:1; R_f = 0.26) gave **2c** as a pale yellow gum which analyzed by HRFAB-MS. Exact mass calcd for $C_{19}H_{16}N_2O_2$: 304.1212, observed (M + H) 305.1291.

Hydrazinobenzoylcurcumin (3a). To a solution of **1a** (10 mg, 0.027 mmol) in methanol (2 mL) was added 4-hydrazinobenzoic acid (20 mg, 0.135 mmol), triethylamine (18.8 μL, 0.135 mmol), and catalytic amount of acetic acid. After incubation for 24 h, the solvent was evaporated in vacuo and the residue purified by preparative TLC ($CHCl_3$/MeOH = 4:1; R_f, = 0.5) and HPLC (semipreparative C18 column; acetonitrile/H_2O = 50:50; flow rate = 3 mL/min; retention time: 15 min) gave **3a** as a dark orange powder (7.18 mg, 55%) which analyzed by HRFAB-MS. Exact mass calcd for $C_{28}H_{24}N_2O_6$: 484.1634, observed (M + H) 485.1715.

Hydrazinobenzoyldemethoxycurcumin (3b). Utilizing the same protocol as described for the synthesis of **3a**, **3b** (5.52 mg, 42%) was obtained from **1b** (10 mg, 0.029 mmol), 4-hydrazinobenzoic acid (20 mg, 0.135 mmol), and triethylamine (18.8 μL, 0.135 mmol). Preparative TLC ($CHCl_3$/MeOH = 4:1; R_f, = 0.41) gave **3b** as a dark orange powder which analyzed by HRFAB-MS. Exact mass calcd for $C_{27}H_{22}N_2O_5$: 454.1529, observed (M + H) 455.1611.

Hydrazinobenzoylbisdemethoxycurcumin (3c). Utilizing the same protocol as described for the synthesis of **3a**, **3c** (6.78 mg, 50%) was obtained from **1c** (10 mg, 0.032 mmol), 4-hydrazinobenzoic acid (20 mg, 0.135 mmol), and triethylamine (18.8 μL, 0.135 mmol). Preparative TLC ($CHCl_3$/MeOH = 4:1; R_f, = 0.34) gave **3c** as a dark orange powder which analyzed by HRFAB-MS. Exact mass calcd for $C_{26}H_{20}N_2O_4$: 424.1423, observed (M + H) 425.1501.

Cell culture and growth assay

Early passages (5–7 passages) of bovine aortic endothelial cells (BAECs) were kindly provided by Dr. Jo at the NIH of Korea. BAECs were grown in MEM supplemented with 10% fetal bovine serum. HT29 (colon carcinoma), NIH3T3 (mouse normal fibroblast), and Chang (normal liver) cells were maintained in RPMI1640 containing 10% fetal bovine serum. All cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Cell growth assay was carried out using MTT colorimetric assay. Exponentially growing cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. The cells were incubated in growth media for 24 h. Various concentrations of curcumin derivatives were added to each well and incubated for up to 72 h. After 72 h, 50 μL of MTT (2 mg/mL stock solution, Sigma) was added and the plate was incubated for an additional 4 h. After removal of the culture supernatants, 150 μL of DMSO was added. The plate was read at 540nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Chemoinvasion assay

The invasiveness of the endothelial cells was performed in vitro using a Transwell chamber system with polycarbonate filter inserts. The lower side of the filter was coated with 10 μL of gelatin (1 mg/mL), whereas the upper side was coated with 10 μL of Matrigel (3 mg/mL). Exponentially growing cells (1×10^5 cells) were placed in the upper part of the filter and HC was applied to the lower part for 30 min at room temperature before seeding. The chamber was then incubated at 37 °C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting whole cells on the lower side of the filter using an optical microscope at ×100 magnification.

Tube formation assay

Matrigel (250 μL, 10 mg/mL) was placed in a 24-well culture plates and polymerized for 30 min at 37 °C. The BAECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/mL). Then, HC was added and incubation was continued for 6–18 h. The morphological changes in the cells and tubes formed were observed under a microscope and photographed at ×100 magnification using JVC digital camera (Victor, Yokohama, Japan).

Chorioallantoic membrane (CAM) assay

Fertilized chick eggs were kept in a humidified incubator at 37 °C for 3 days. About 2 mL of egg albumin was then removed with a hypodermic needle allowing the CAM and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out and removed and the shell membrane peeled away. At the stage of a 4.5-day old chick embryo, a HC (10 μg/egg)-loaded thermanox coverslip was air-dried and applied to the CAM surface. Two days later, 2 mL of 10% fat emulsion was injected into the chorioallantois and the CAM was observed under a microscope. Since retinoic acid (RA) is known as an anti-angiogenic compound, 1 μg/egg RA was used as a positive control for anti-angiogenic responses. When the CAM treated with a sample showing an avascular zone to a similar degree of RA-treated CAM that had little vessels compared to empty coverslip, the response was scored as positive, and calculated based on the percentage of positive eggs to the total number of eggs tested.

Acknowledgements

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