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Inhibition of endothelial cell functions by novel potential cancer chemopreventive agents☆

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Abstract

Endothelial cells (EC) play a major role in tumor-induced neovascularization and bridge the gap between a microtumor and growth factors such as nutrients and oxygen supply required for expansion. Immortalized human microvascular endothelial cells (HMEC-1) were utilized to assess anti-endothelial effects of 10 novel potential cancer chemopreventive compounds from various sources that we have investigated previously in a human in vitro anti-angiogenic assay. These include the monoacylphloroglucinol isoaspidinol B, 1,2,5,7-tetrahydroxy-anthraquinone, peracetylated carnosic acid (PCA), isoxanthohumol, 2,2′,4′-trimethoxychalcone, 3′-bromo-2,4-dimethoxychalcone as well as four synthetic derivatives of lunularic acid, a bibenzyl found in mosses [Int. J. Cancer Prev. 1 (2004) 47]. EC proliferation was inhibited with half-maximal inhibitory concentrations from 0.3 to 49.6 μM, whereas EC migration was affected by most compounds at sub-micromolar concentrations. PCA and the bibenzyl derivative EC 1004 potently prevented differentiation of HMEC-1 into tubule-like structures. Overall, our data indicate that inhibition of endothelial cell function contributes to various extents to the chemopreventive or anti-angiogenic potential of these lead compounds.

Keywords: Anti-angiogenic; Anti-endothelial; Bibenzyls; Cancer chemoprevention; Chalcones; Human microvascular endothelial cells; Migration; Polyphenols; Proliferation; Tube formation; HCT-116; SK-BR3; NIH/3T3

Angiogenesis involves the formation of endothelial blood capillaries from already established microvasculature and is an obligatory step in carcinogenesis and for the formation of metastases. To identify novel inhibitors of angiogenesis, we have recently established a human in vitro anti-angiogenic model based on the natural wound healing abilities of superficial placental vessels embedded in fibrin gel [1]. This assay covers all crucial mechanistic events during the angiogenic cascade, starting from the release of growth factors to the final sprouting of capillaries from existing blood vessels [2]. We pre-selected 10 promising lead structures from a collection of more than 1000 natural products and their synthetic analogues, based on modulatory effects on carcinogen metabolism, anti-inflammatory, anti-hormonal, and

☆ *Abbreviations:* BDMC, 3′-bromo-2,4-dimethoxychalcone; CUR, curcumin; Cyp1A1, cytochrome P450 1A1; DMSO, dimethyl sulfoxide; EC, endothelial cells; ECM, extracellular matrix; EGCG, (−)-epigallocatechin gallate; FACS, fluorescence-activated cell sorting; HUVEC, human umbilical vein endothelial cells; HMEC-1, human microvascular endothelial cells; IC_{50}, half-maximal inhibitory concentration; ISO, isoaspidinol B; IXN, isoxanthohumol; MMPs, matrix metalloproteinases; MT1-MMP, membrane-type 1 matrix metalloproteinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCA, peracetylated carnosic acid; RES, resveratrol; ROS, reactive oxygen species; SRB, sulforhodamin B; THA, 1,2,5,7-tetrahydroxy-anthraquinone; TMC, 2,2′,4′-trimethoxychalcone; VEGF, vascular endothelial growth factor.

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anti-tumor promoting mechanisms (a summary and description of the test systems are given in Gerhauser et al. [3]). Most interesting compounds belonged to three major structural classes: polyphenolic compounds, including isoaspidinol B, a synthetic isomer of the monoacylphloroglucinol aspidinol found in ferns [4], which was identified as an inhibitor of cyclo-oxygenase 1 activity, 1,2,5,7-tetrahydroxyanthraquinone, which was isolated from a residue obtained during beer brewing and potently inhibited Cyp1A1 activity [5], peracetylated carnosic acid, which in genuine form is a typical constituent of rosemary [6], and isoxanthohumol, a prenylated flavonoid which was isolated from beer [7]. Further, two synthetic chalcones were tested, which were selected based on their potent modulation of carcinogen metabolism [8], as well as four synthetic derivatives of lunularic acid, a bibenzyl compound found in mosses [9]. Three known chemopreventive agents with anti-angiogenic activity were used as positive control substances: resveratrol found in grapes and red wine [10], curcumin, a major constituent of turmeric (*Curcuma longa* L.) and used as a colorant in many foods including curry [11], and (−)-epigallocatechin gallate, a main catechin of green tea [12–14]. All selected compounds were identified as potent inhibitors of in vitro angiogenesis and inhibited the formation of newly formed microcapillaries by 38–91% at concentrations of 0.1–10 μM [1] (Supplemental material Table S1).

Endothelial cells play a crucial role in angiogenesis, bridging the gap between a microtumor and the required factors for expansion [15]. Endothelial cells produce type IV collagenase as well as other members of the matrix metalloproteinase and serine protease family. After degradation of the basement membrane, they proliferate and migrate into the perivascular stroma, initiate capillary sprouting by forming capillary-like tubes, and thus supply a microtumor with essential nutrients and oxygen. Cells from the tumor vasculature are generally non-transformed and less prone to acquire drug resistance [16], therefore endothelial cell functions are considered as ideal targets for the prevention and control of tumor growth [17]. For our investigation, we utilized the human microvascular endothelial cell line HMEC-1. This cell line was established by Ades et al. [18] by transfection of human dermal microvascular endothelial cells with a PBR-322 based plasmid containing the coding region for the simian virus 40A gene product, large T-antigen, for immortalization. HMEC-1 retain morphologic, phenotypic, and functional characteristics of normal human microvascular endothelial cells, including cobblestone morphology, von Willenbrand factor and factor VIII expression. Except for constitutive expression of IL-6 and GM-CSF absent in primary cells, HMEC-1 exhibit major constitutive and inducible endothelial cell characteristics including induced expression of cell adhesion molecules and cytokines on stimulation with pro-inflammatory stimuli, as well as uptake of DiI-Ac-LDL and the formation of cord-like structures on Matrigel [19] and were therefore selected as a suitable in vitro model for the present studies.

Materials and methods

Chemicals. Cell culture materials were obtained from Gibco-BRL Life Technologies (Eggenstein, Germany). Fetal bovine serum was provided by Pan (Aidennach, Germany). Hydrocortisone, resveratrol, (−)-epigallocatechin gallate (EGCG), curcumin, and sulforhodamin B (SRB) were purchased from Sigma (Taufkirchen, Germany). Matrigel was obtained from BD Biosciences (Heidelberg, Germany).

Cell culture. Human microvascular endothelial cells (HMEC-1), kindly provided by Dr. F. Candal from the Center of Disease Control (CDC) in Atlanta (USA), were cultivated in MCBD 131 endothelial basal medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1 μg/ml hydrocortisone. The HCT-116 cell line was provided by B. Vogelstein, Johns Hopkins University (Baltimore, USA). Cells were cultivated in McCoy's medium supplemented with 5% FBS. ER-negative mammary tumor cells SK-BR3 were obtained from the Tumorbank at the German Cancer Research Center (Heidelberg, Germany) and maintained in RPMI 1640 containing 10% FBS. The murine fibroblast cell line NIH/3T3, generously provided by N. Hay from the University of Chicago (USA), was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 4 mM L-glutamine. All cell culture media contained 100 U/ml penicillin G sodium, 100 U/ml streptomycin sulfate, and 250 ng/ml amphotericin B. Cells were maintained at 37 °C/5% CO_2 in a humidified environment.

Cytotoxicity assay. HMEC-1 (3×10^4 cells/ml in MCBD 131 endothelial basal medium supplemented as described above), HCT-116 (2.5×10^4 cells/ml in McCoy's medium supplemented as described above), SK-BR3 (5×10^4 cells/ml in RPMI 1640 supplemented as described above), or NIH/3T3 cells (3×10^4 cells/ml in DMEM supplemented as described above), respectively, were cultured in 96-well plates (200 μl/well) and treated with the test compounds dissolved in DMSO (final DMSO concentration 0.5%) in a concentration range from 0.4 to 50 μM in duplicates. After treatment for 72 h, cytotoxic potential was evaluated by SRB staining according to Skehan et al. [20]. To assess potential cytotoxic effects in the endothelial cell migration assay, HMEC-1 were seeded in 96-well plates as described above. After 72 h incubation, confluent monolayers of cells were treated with the inhibitors for 18 h, and cytotoxic potential was evaluated by SRB staining. For the capillary-like tube formation assay, the effect on HMEC-1 proliferation was determined by seeding 1×10^5 cells/ml HMEC-1 in 96-well plates (200 μl/well) and treating them for 6 h as described.

FACS analysis. For flow cytometric analysis of cell cycle distribution, HMEC-1 were plated in 100 mm tissue culture dishes (3×10^5 cells/10 ml in MCBD 131 endothelial basal medium supplemented as described above) and treated with the test compounds (dissolved in DMSO) at their half-maximal inhibitory concentration (final DMSO concentration 0.1%). After 72 h, the supernatant with floating cells was collected, attached cells were harvested by trypsinization and pooled with the collected medium. The cells were counted using a hematocytometer, washed three times with PBS, and resuspended in 200 μl PBS. After fixation in 70% ice-cold ethanol, cells were stored at −20 °C. Cell cycle distribution was monitored by fluorescence-activated cell sorting analyses (FACS) after propidium iodine staining by standard techniques [21,22].

Endothelial cell migration assay. HMEC-1 migration was monitored using the wound healing assay described by Thaloor et al. [23]. Briefly, 3×10^4 cells/well/ml were seeded in 24-well plates using MCBD

131 medium supplemented as described above. After 72 h, the confluent monolayer was wounded mechanically by scraping with a P1000 pipet tip. The endothelial cell monolayer was incubated with media supplemented with the test compounds dissolved in DMSO (0.1% final DMSO concentration) at increasing concentrations, starting from 0.001 or 0.01 μM, respectively, for 18 h. Controls were treated with 0.1% DMSO. The extent of wound closure was evaluated by standardized digital images, acquired at time 0 and after 18 h with a color digital microscopic camera system (Leitz Diavert microscope, Leica, Bensheim; AxioCam, Carl Zeiss, Göttingen, Germany) with a resolution of 1300×1030 pixel at 40× magnification, and processed with AxioVision Rel. 3.1. software package (Carl Zeiss, Göttingen, Germany). The migration area (area covered by migrating cells) was measured using Adobe Photoshop 7.0 with histogram function in order to obtain the pixel area of migrated cells in relation to the scraped area in the picture taken at time 0, which was then converted to mm^2. The percentage of inhibition was calculated in comparison with the solvent control. Results are expressed as means ± standard deviation of three independent experiments.

Matrigel-based capillary tube formation assay. The formation of capillary-like structures by HMEC-1 on a basement membrane preparation was assessed as described previously [19]. Briefly, 24-well plates were coated with 0.1 ml/well Matrigel diluted 1:3 with MBCD 131 without supplements and incubated for 60 min at 37 °C. HMEC-1 (1×10^5 in 1 ml) were seeded onto the layer of Matrigel. The test compounds dissolved in DMSO were added to the cell suspension at a final concentration of 10 μM (final DMSO concentration 0.1%), and the cultures were maintained for 6 h at 37 °C. Three fields were selected randomly in each well to observe in vitro induced tubule formation and photographed using the camera system mentioned above. The degree of tube formation was scored visually by defining three levels of inhibition: (++) reduction of tube formation by more than 80% compared to the solvent control, (+) half-maximal inhibition, and (o) less then 50% inhibition.

Statistical analysis. Results are presented as means ± standard deviation of data originated from three independent experiments. For statistical evaluation Student's *t* test was applied. For the endothelial cell migration assay, paired Student's *t* test was performed comparing the migration area after 18 h to time 0. Values of $p < 0.005$ were considered as statistically significant and $p < 0.0005$ as highly significant.

Results

Our aim was to investigate anti-endothelial activities of several potential cancer chemopreventive compounds which we have identified previously as potential inhibitors of angiogenesis in a human in vitro anti-angiogenic assay [1]. (Chemical structures of inhibitors as well as the percentage of inhibition obtained in the human in vitro anti-angiogenesis assay at 1 and 10 μM concentrations are provided in the supplemental material Fig. S1 and Table S1, respectively.)

Inhibitory effects on cell proliferation

For the analysis of anti-proliferative and cytotoxic effects, HMEC-1 cells were treated for 72 h with the test compounds in a concentration range of 0.4–50 μM. Half-maximal inhibitory concentrations (IC_{50} values) are summarized in Table 1. Resveratrol inhibited HMEC-1 proliferation with an IC_{50} value of 9.4 μM and was less toxic than curcumin with an IC_{50} value of 3.0 μM (Fig. 1A). EGCG did not reveal anti-proliferative or cytotoxic effects at a concentration of 50 μM. Isoaspidinol B exerted the most potent dose-dependent anti-proliferative activity of all polyphenolic compounds tested with an IC_{50} value of 1.6 μM (Fig. 1B). Isoxanthohumol and the peracetylated form of carnosic acid were slightly less toxic (IC_{50}s of 4.8 and 6.9 μM, respectively), whereas THA similar to EGCG was considered as nontoxic (IC_{50} 49.6 μM). The synthetic chalcones TMC and BDMC revealed the strongest cytotoxic effects with IC_{50} values of 0.51 and 0.30 μM, respectively (Fig. 1C). The cytotoxic potential of lunularic acid derivatives

Table 1
Summary of anti-proliferative effects of tested lead compounds

	HMEC-1 IC_{50} (μM)[a]	HCT-116 IC_{50} (μM)[a]	SK-BR3 IC_{50} (μM)[a]	NIH/3T3 IC_{50} (μM)[a]
Polyphenolic compounds				
Resveratrol	9.4 ± 0.8[b]	12.6[c]	44.3[c]	18.7 ± 3.7[b]
Curcumin	3.0 ± 0.7	4.7	11.4	20.7 ± 1.8
EGCG	>50	46.5	>50	8.1 ± 0.6
Isoaspidinol B	1.6 ± 0.2	19	>50	>50
1,2,5,7-Tetrahydroxy-anthraquinone (THA)	49.6 ± 0.4	39.1	>50	47.8 ± 0.9
Peracetylated carnosic acid (PCA)	6.9 ± 0.7	24.3	49.1	8.3 ± 0.1
Isoxanthohumol	4.8 ± 0.5	35.5	>50	>50
Chalcones				
2,2′4′-Trimethoxychalcone (TMC)	0.51 ± 0.09	1.9	>5	16.5 ± 0.3
3′-Bromo-2,4-dimethoxychalcone (BDMC)	0.30 ± 0.01	1.1	2.2	14.9 ± 6.0
Lunularic acid derivatives				
EC 252	5.8 ± 0.7	6.3	9.8	7.6 ± 0.3
EC 1004	16.0 ± 2.3	>50	24.1	>50
EC 1001	3.0 ± 0.4	41.1	42.6	35.2 ± 2.0
EC 1021	>50	>50	>50	19.4 ± 1.5

[a] IC_{50}, half-maximal inhibitory concentration.
[b] Mean value ± standard deviation ($n = 3$).
[c] Mean value of two determinations.

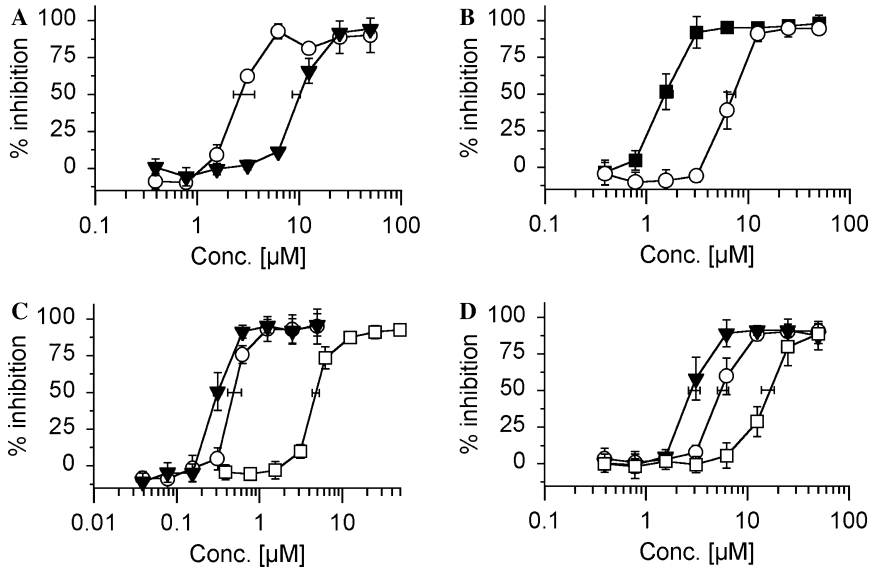

Fig. 1. Inhibition of HMEC-1 cell proliferation. HMEC-1 were cultured in 96-well plates and incubated with test compounds for 72 h. IC_{50} values were generated from eight serial dilutions tested in duplicates. Mean values from three independent experiments were computed, and standard deviations were indicated by horizontal (for IC_{50} values) and vertical error bars. Dose-inhibition curves obtained with (A) (▼) resveratrol and (○) curcumin; (B) (■) isoaspidinol B and (○) PCA; (C) (□) isoxanthohumol, (○) TMC, and (▼) BDMC; and (D) (○) EC 252, (▼) EC 1001, and (□) EC 1004.

was structure-dependent: the saturated bibenzyl EC 1001 demonstrated the highest activity (IC_{50} 3.0 μM), whereas the stilbene-like EC 1021 was not toxic up to a concentration of 50 μM. The two lunularic acid derivatives with a bromo-substituted thiophene ring system displayed intermediate growth-inhibitory potential with IC_{50} values of 5.8 and 16.0 μM, respectively (Fig. 1D).

To investigate whether the observed anti-proliferative effects were selective for endothelial cells, we additionally tested the compounds with three further cell lines, including the mismatch-repair deficient colorectal cancer epithelial cell line HCT-116, the Her-2 overexpressing estrogen receptor-negative human breast cancer cell line SK-BR3 and NIH/3T3 murine fibroblasts (Table 1). Resveratrol and curcumin demonstrated moderate selectivity for HMEC-1 (ratio of IC_{50} values: 1.3–6.9), whereas EGCG was most toxic for the fibroblast cell line. Interestingly, IC_{50} values for inhibition of HMEC-1 proliferation obtained with isoaspidinol B and the lunularic acid derivative EC 1001 were 12- to >30-fold lower than those observed with cultured HCT-116, SK-BR3, and NIH/3T3 cells, indicating a higher degree of selectivity for the endothelial cell line than resveratrol or curcumin. A similar, but weaker, association was observed with isoxanthohumol (ratio of IC_{50} values: 7.4–10).

Inhibition of HMEC-1 proliferation and cell cycle distribution were further analyzed by flow cytometry. Unsynchronized HMEC-1 cells were treated in the presence or absence of test compounds at their half-maximal inhibitory concentrations for 72 h (Table 2). For the untreated control we detected $5.0 \pm 1.7\%$ of total gated cells in sub-G_1 phase, $47.5 \pm 2.8\%$ in G_0/G_1-phase, $15.3 \pm 2.1\%$ in S-phase, and $25.3 \pm 3.2\%$ in G_2/M-phase. Resveratrol caused a weak arrest in S-phase. Similar results were observed previously with several human cancer cell lines [24]. For all other agents, the results did not indicate induction of apoptosis or cell cycle arrest in G_0/G_1-, S- or G_2/M-phase as a mechanism of endothelial cell growth inhibition.

Inhibition of endothelial cell migration

For the determination of potential anti-migratory activities, we tested the compounds in a wound closure assay [23]. After wounding with a pipet tip, solvent controls reformed a confluent monolayer within 18 h of incubation. Test compounds were added in a concentration range of 0.01–20 μM (Fig. 2). Results are summarized in Table 3. In the presence of resveratrol, a potent dose-dependent inhibition of endothelial cell migration was observed, and an IC_{50} value of 0.7 ± 0.3 μM was computed. EGCG and curcumin are known inhibitors of endothelial cell migration and reduced the dynamic process with IC_{50} values of 2.6 ± 0.6 μM and 0.3 ± 0.03 μM, respectively (Fig. 2A). Isoaspidinol B was identified as one of the most effective inhibitors and exerted a potent reduction of wound closure (IC_{50} 0.07 ± 0.01 μM), whereas two other polyphenols tested, THA and PCA, were slightly less active and reduced cell migration by 50%

Table 2
Flow cytometric determination of cell cycle distribution in HMEC-1[a]

	sub-G_1	G_0/G_1	S	G_2/M
Control	5.0 ± 1.7[b]	47.5 ± 2.8	15.3 ± 2.1	25.3 ± 3.2
Polyphenolic compounds				
Resveratrol	4.3 ± 1.4	41.4 ± 3.4	20.0 ± 2.8[c]	24.4 ± 3.2
Curcumin	6.2 ± 3.8	44.4 ± 2.3	15.1 ± 0.7	28.6 ± 2.7
EGCG	3.8 ± 1.3	48.5 ± 2.9	12.9 ± 2.4	28.3 ± 3.4
Isoaspidinol B	n.d.	n.d.	n.d.	n.d.
1,2,5,7-Tetrahydroxy-anthraquinone (THA)	4.5 ± 2.8	46.6 ± 2.9	15.7 ± 4.3	25.8 ± 4.6
Peracetylated carnosic acid (PCA)	3.4 ± 0.7	44.5 ± 4.4	18.7 ± 2.3	22.8 ± 2.4
Isoxanthohumol	5.7 ± 2.9	46.2 ± 3.8	16.9 ± 2.5	22.6 ± 3.7
Chalcones				
2,2′4′-Trimethoxychalcone (TMC)	4.7 ± 2.7	47.8 ± 1.0	14.4 ± 2.7	27.9 ± 3.3
3′-Bromo-2,4-dimethoxychalcone (BDMC)	4.0 ± 2.2	47.7 ± 0.3	14.4 ± 1.8	28.4 ± 2.9
Lunularic acid derivatives				
EC 252	3.9 ± 1.1	50.0 ± 1.7	14.5 ± 2.6	26.9 ± 2.6
EC 1004	2.8 ± 0.5	47.5 ± 1.7	15.5 ± 1.8	27.3 ± 1.3
EC 1001	4.5 ± 1.3	48.3 ± 2.8	14.7 ± 2.2	24.4 ± 4.8
EC 1021	3.9 ± 0.3	46.8 ± 4.7	17.6 ± 1.4	21.1 ± 2.7

n.d., not determined.
[a] Cells were treated with test compounds at their half-maximal inhibitory concentration for 72 h.
[b] Percentage of cells in the indicated cell cycle phase.
[c] Mean significantly different from control ($p = 0.038$) using Student's t test with $n = 4$.

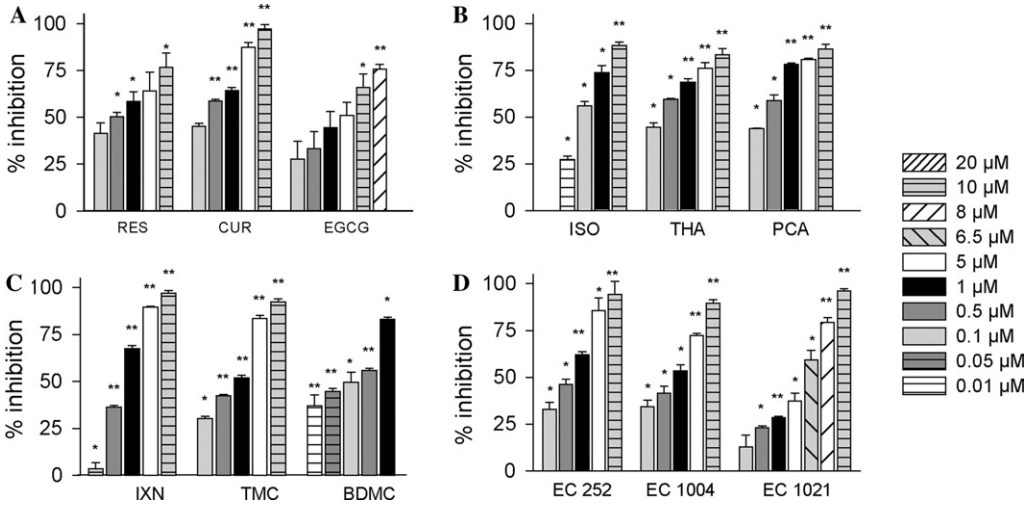

Fig. 2. Inhibition of HMEC-1 migration. HMEC-1 were seeded in 24-well plates and allowed to form a monolayer within 72 h. The monolayer was wounded mechanically by scraping with a pipet tip and incubated with the test compounds at the indicated concentrations for 18 h. *,**Mean significantly different from time 0 ($p < 0.005$, $p < 0.0005$, respectively) using paired Student's t test with $n = 3$.

at concentrations of 0.2 ± 0.04 μM and 0.4 ± 0.07 μM, respectively (Fig. 2B). The flavanone isoxanthohumol as well as the two chalcones TMC and BDMC were active in a similar concentration range with IC_{50} values of 0.6 ± 0.09 μM, 0.9 ± 0.05 μM, and 0.3 ± 0.06 μM (Fig. 2C). The lunularic acid derivatives EC 252 and EC 1004 with a bromo-substituted thiophene ring structure were identified as potent anti-migratory agents with IC_{50} values of 0.6 ± 0.1 μM and 0.9 ± 0.2 μM, whereas EC 1001 at 10 μM reduced cell migration by 35% only, and EC 1021 as about 10-fold less active than EC 252 (Fig. 2D).

Inhibition of capillary-like tube formation

The differentiation of endothelial cells into tube-like structures was investigated using Matrigel, a basement membrane preparation from the Engelbreth–Holm–Swarm mouse sarcoma. Untreated controls showed a complex network of tubes after a 6 h incubation period [19] as shown by representative photographs in Fig. 3. After treatment with test compounds, we noticed various levels of inhibition, which are summarized semi-quantitatively in Table 3. Resveratrol at a concentration of 10 μM reduced tube formation by about 50%, and we

Table 3
Influence of test compounds on endothelial cell migration and differentiation

	Migration		Tube formation at 10 μM
	IC_{50}-M (μM)[a]	IC_{50}-T (μM)[a]	
Polyphenolic compounds			
Resveratrol	0.7 ± 0.3	>50	+[b]
Curcumin	0.3 ± 0.03	>50	++
EGCG	2.6 ± 0.6	>50	+
Isoaspidinol B	0.07 ± 0.01	>50	+
1,2,5,7-Tetrahydroxy-anthraquinone (THA)	0.2 ± 0.04	>50	o
Peracetylated carnosic acid (PCA)	0.4 ± 0.07	>50	++
Isoxanthohumol	0.6 ± 0.09	>50	+
Chalcones			
2,2′4′-Trimethoxychalcone (TMC)	0.9 ± 0.05	>50	+
3′-Bromo-2,4-dimethoxychalcone (BDMC)	0.3 ± 0.06	>50	+
Lunularic acid derivatives			
EC 252	0.6 ± 0.1	>50	+
EC 1004	0.9 ± 0.2	>50	++
EC 1001	>10 (35)	>50	+
EC 1021	5.9 ± 0.3	>50	+

[a] Half-maximal inhibitory concentration for EC migration (M) and proliferation (T for toxicity) determined after an incubation period of 18 h; values in brackets indicate the percentage of inhibition at 10 μM concentration.
[b] Percentage inhibition of EC differentiation at 10 μM concentration; ++, >80% inhibition of tube formation compared to control; +, ~50% inhibition; o, no inhibition.

observed thinning of tube ends. Cells treated with 10 μM EGCG showed a similar pattern with incomplete tube formation. Curcumin, a known inhibitor of endothelial differentiation, was found to reduce capillary formation by more than 80% at a 10 μM concentration. A partial differentiation was observed with isoaspidinol B, whereas THA at 10 μM did not exert any inhibitory activity, resulting in an extensive tubular network after 6 h. PCA demonstrated similar activity as curcumin and almost completely suppressed capillary formation at 10 μM. After treatment with isoxanthohumol, we observed shortened, but strong, tubes. The chalcones TMC and BDMC were only moderate inhibitors of capillary-like tube formation, showing incomplete inhibition of endothelial differentiation. From the lunularic acid derivatives, EC 1004 inhibited capillary formation completely at a concentration of 10 μM, whereas EC 252, EC 1001, and EC 1004 showed lower activity.

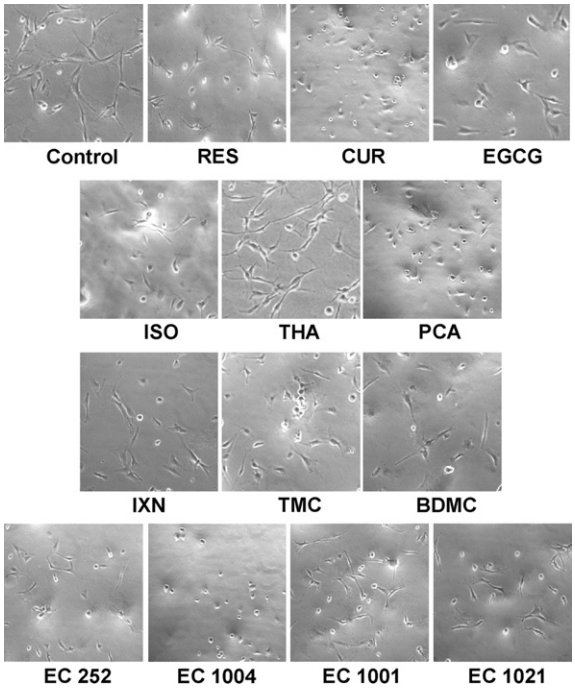

Fig. 3. Inhibition of HMEC-1 tube formation. HMEC-1 were cultured in 24-well plates coated with Matrigel and treated with test compounds at 10 μM concentration for 6 h. Pictures were acquired at 40× magnification.

Discussion

In the present study we have investigated inhibitory effects of potential chemopreventive agents on endothelial cell functions. In a series of complementary assay systems, the tested compounds exerted strong effects on endothelial cell proliferation, migration, and tube formation in vitro. Endothelial cells and microvasculature represent central target strategies in the prevention of angiogenesis-related diseases including cancer. A single intact capillary can supply thousands of tumor cells with essential nutrients. Moreover, their direct contact to blood circulation facilitates the delivery of compounds to their site of action. As an example, the flavonoid quercetin occurring in fruits and vegetables was found to possess anti-angiogenic potential with a strong focus on anti-endothelial activities [25,26]. Various other effective

chemopreventive compounds of natural or synthetic origin have been shown to inhibit the angiogenic cascade at the endothelial cell level [27], including three natural chemopreventive products selected as positive controls in our test systems, i.e., resveratrol, curcumin, and EGCG. Resveratrol as a potent inhibitor of angiogenesis [10,24] blocks the binding of the vascular endothelial growth factor (VEGF) to HUVEC and thus interrupts endothelial activation [28]. Curcumin, a diferuloylmethane derived from the spice turmeric (*Cucurma longa* L.), was identified as a very potent inhibitor of basic fibroblast growth factor (bFGF)-induced corneal neovascularization in mice in vivo [29]. Thaloor et al. [23] reported that curcumin dose-dependently inhibited endothelial cell proliferation, migration, and differentiation. These effects were based on inhibition of lytic matrix metalloproteinases (MMPs), which play an essential role for endothelial morphogenesis, tumor cell migration, and the formation of metastases [30]. EGCG derived from tea (*Thea chinenesis* L.) was identified in animal studies by Cao and Cao [13] as a potent anti-angiogenic agent in vivo. Potential mechanisms seemed to involve anti-endothelial effects with respect to growth, migration, and differentiation [31] due to dose-dependent inhibition of VEGF receptor binding [32]. Recently, EGCG was reported to inhibit membrane-type 1 matrix metalloproteinase (MT1-MMP), which generates an active form of MMP-2 from proMMP-2 [33].

In our investigations, isoaspidinol B, an isomer of the monoacylphloroglucinol aspidinol B found in ferns, revealed remarkable anti-endothelial activities, including inhibition of HMEC-1 proliferation, migration and tube formation. These activities may contribute to its potent anti-angiogenic activity [1]. Carnosic acid, isolated from *Rosmarinus officinalis* L., was described as a very strong anti-oxidant, scavenging hydroxyl- and peroxyl-radicals [34]. Since the formation of reactive oxygen species caused by hypoxia/reoxygenation contributes essentially to capillary neogenesis [35], anti-oxidant activity could be a possible mechanism of action of its peracetylated form PCA.

Interestingly, THA and the flavanone isoxanthohumol, both isolated from products generated during beer brewing, showed a strong reduction of capillary growth. Studies by Kim [36] suggest that flavonoids effectively inhibit VEGF/bFGF-stimulated expression and activity of MMPs including MMP-1, MT1-MMP, and pro-MMP2 activation and also decreased the induction of tissue inhibitors of MMPs (TIMPs). These matrix degrading proteinases facilitate endothelial cell migration. Further studies have to demonstrate whether similar mechanisms might contribute to the inhibitory activity of isoxanthohumol.

TMC and BDMC represent two synthetically derived chalcones. Nam et al. [37] have described 2′,5′-dihydroxychalcones as potential anti-angiogenic compounds with cytotoxic selectivity towards HUVECs. The activity profile strongly depended on the substitution pattern, with 2-chloro-2′,5′-dihydroxychalcone bearing an electron-withdrawing substituent on the B ring being the most potent compound. Since chalcones possess an α,β-unsaturated ketone (Michael reaction acceptor) moiety as a common structural entity, thiol reactivity could also be involved in anti-endothelial mechanisms. Madan et al. [38] demonstrated that inhibition of NF-κB by 2′-hydroxychalcone reduced the transcription of pro-angiogenic factors including VEGF and intercellular adhesion molecules.

Among the tested lunularic acid derivatives, belonging to two structurally different groups, EC 252 and EC 1004 carrying a bromo-substituted thiophene ring system revealed strong anti-migratory and moderate anti-proliferative potential. EC 1004 was not selective, but demonstrates relatively low toxicity for HMEC-1 and the other cell lines, considering its potent inhibitory effects on HMEC-1 migration and differentiation. In contrast, the bibenzyl derivatives EC 1001 and EC 1021 both exhibited weak to moderate inhibition of endothelial migration, but differed in their potential to inhibit endothelial cell proliferation. EC 1001 was identified as a quite selective inhibitor of HMEC-1 cell growth, whereas with EC 1021 we observe general low toxicity similar to EGCG. Interestingly, these compounds bear structural similarities to combretastatins derived from *Combretum caffrum* L. Combretastatins were found to shut down blood flow in tumor blood vessels by microtubule depolymerization [39]. A phosphate pro-drug of combretastatin A-4, CA-4-P, has entered clinical trials as a tumor vasculature-targeting agent [40,41].

In conclusion, we have demonstrated that inhibitory effects on endothelial cell functions might contribute to the potential chemopreventive and anti-angiogenic activities of the series of compounds tested. Further investigations on cellular targets and molecular mechanisms in vitro and in vivo are warranted to fully evaluate their preventive or therapeutic potential. Thus, in view of expectations that the suppression of abnormal capillary growth may provide new strategies, through the intake of dietary inhibitors, the risk of onset and progression of the angiogenic cascade may be delayed. Novel non-toxic compounds could halt progression of in situ tumors, i.e., micro-colonies of cancer cells which occur ubiquitously in the bodies of a ‘healthy’ population. Through intervention with anti-angiogenic agents, cancer could be delayed and probably turned into a manageable chronic disease [42].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.10.032.

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