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RESEARCH PAPER

In vitro and *in vivo* evaluation of the anti-angiogenic actions of 4-hydroxybenzyl alcohol

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BACKGROUND AND PURPOSE
4-Hydroxybenzyl alcohol (HBA) is a phenolic plant compound, which has been shown to influence many cellular mechanisms. In the present study, we analysed *in vitro* and *in vivo* the anti-angiogenic actions of this pleiotropic agent.

EXPERIMENTAL APPROACH
Migration and protein expression of HBA- and vehicle-treated endothelial-like eEND2 cells was assessed by cell migration assay and Western blot analyses. HBA action on vascular sprouting was analysed in an aortic ring assay. *In vivo* anti-angiogenic actions of HBA were studied in the dorsal skinfold chamber model of endometriosis in mice.

KEY RESULTS
Western blot analyses demonstrated that HBA inhibited proliferation of eEND2 cells, as indicated by down-regulation of proliferating cell nuclear antigen expression, and reduced expression of vascular endothelial growth factor and matrix metalloproteinase 9. HBA suppressed the migration of eEND2 cells, accompanied by inhibition of actin filament reorganization, revealed by fluorescence staining of the cytoskeleton. In addition, HBA reduced vascular sprouting in the aortic ring assay. Finally, we found, in the dorsal skinfold chamber model *in vivo* using intravital fluorescence microscopy, that HBA inhibited the vascularization of developing endometriotic lesions, as indicated by a decreased functional capillary density of lesions in HBA-treated mice and a reduced lesion size, compared with control animals.

CONCLUSIONS AND IMPLICATIONS
HBA targets several angiogenic mechanisms and therefore represents a promising anti-angiogenic agent for the treatment of angiogenic diseases, such as endometriosis.

Abbreviations
BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethylsulphoxide; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HBA, 4-hydroxybenzyl alcohol; LDH, lactate dehydrogenase; MMP9, matrix metalloproteinase 9; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; ROI, region of interest; VEGF, vascular endothelial growth factor; WST, water-soluble tetrazolium

Introduction

4-Hydroxybenzyl alcohol (HBA) is a naturally occurring phenolic compound, found in many plants, including carrots (Kobayashi *et al.*, 2003), *Gymnadenia conopsea* (Cai *et al.*, 2006) and *Gastrodia elata* Blume (Yu *et al.*, 2005). The last is a member of the Orchidaceae and has been used as a traditional herbal medicine for the treatment of headache, tetanus and

epilepsy in Oriental countries for centuries due to its analgesic, sedative and anti-convulsant effects (Hsieh *et al.*, 2000; Yu *et al.*, 2005). More recently, it has become evident that these beneficial effects can be attributed to the pharmacological activity of HBA.

HBA is a typical pleiotropic agent and affects many cellular mechanisms. HBA has been shown to ameliorate cerebral ischaemic injury due to the inhibition of apoptotic pathways and the up-regulation of protein disulphide isomerase without inducing any toxic effects at doses up to 200 mg·kg^{-1} (Descamps *et al.*, 2009; Yu *et al.*, 2010). It exerts an anxiolytic-like action via the activation of the 5-hydroxytryptaminergic nervous system (Jung *et al.*, 2006). Furthermore, HBA is a potent inhibitor of lipid peroxidation and protein oxidation, indicating its anti-oxidative properties as a free radical scavenger (Dhiman *et al.*, 2009). In addition, HBA exhibits an anti-inflammatory activity, which is thought to be due to the suppression of cellular nitric oxide production (Lim *et al.*, 2007).

Of interest, HBA has recently also been shown to inhibit the development of new blood vessels in the chick chorioallantoic membrane assay (Lim *et al.*, 2007). Accordingly, HBA may represent a potential anti-angiogenic agent for the treatment of various angiogenic diseases such as cancer (Folkman, 2002), rheumatoid arthritis (Szekanecz *et al.*, 2010), diabetic retinopathy (Crawford *et al.*, 2009), psoriasis (Heidenreich *et al.*, 2009) and endometriosis (Laschke and Menger, 2007). However, the mechanisms underlying the anti-angiogenic action of HBA remain to be determined. Furthermore, the capability of HBA to inhibit the development of new microvascular networks needs confirmation *in vivo* using appropriate angiogenesis models.

Therefore, the aim of the present study was to investigate the HBA actions on cell viability, migration and protein expression of endothelial-like eEND2 cells. We also used the aortic ring assay to study *in vitro* the effect of HBA on vascular sprout formation. Finally, we analysed, using the dorsal skinfold chamber model of endometriosis (Laschke *et al.*, 2005), whether HBA is capable of inhibiting angiogenesis *in vivo* in developing endometriotic lesions.

Methods

Cell culture

Murine endothelial-like eEND2 cells (kind gift from the Department of Surgery, Malmö Hospital, Lund University, Malmö, Sweden) were used for the *in vitro* experiments. They were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS), 2 mM·L-glutamine, 100 U·mL^{-1} penicillin and 0.1 mg·mL^{-1} streptomycin (PAA) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were grown to 80–90% confluence and subjected to no more than five cell passages after cryostorage.

Water-soluble tetrazolium (WST)-1 assay

To assess the actions of HBA on the viability of cultured eEND2 cells, an *in vitro* colorimetric assay (WST-1 assay; Roche, Mannheim, Germany) was performed. This assay is based on the cleavage of the slightly red-coloured WST salt to dark red-coloured formazan by mitochondrial dehydrogenases in viable cells (Hamasaki *et al.*, 1996; Ishiyama *et al.*, 1996). Formazan is then excreted into the culture medium, and the absorbance can be measured with a microplate reader. The WST-1 assay was carried out according to the manufacturer's instructions. The cells were cultured in presence of 1–100 mM HBA. As positive control, we cultured cells in presence of 1–100 μM of the well-established anti-angiogenic compound TNP-470 (Kruger and Figg, 2000). Cells exposed to vehicle dimethylsulphoxide (DMSO) served as control. All experiments were performed in quadruplicate. After 24 h, 10 μL of WST-1 reagent per 100 μL medium were added into each well. After 30 min at 37°C, absorption was measured at 450 nm with 620 nm as reference using a microplate reader and corrected to blank values (wells without cells).

Lactate dehydrogenase (LDH) assay

To assess the cytotoxicity of HBA, we performed a LDH assay by means of the Cytotoxicity Detection Kit^{PLUS} (Roche) according to the manufacturer's instructions. This assay measures the activity of LDH released from damaged cells in the medium. For this purpose, eEND2 cells were cultured in presence of 1–100 mM HBA or 1–100 μM TNP-470. Cells exposed to vehicle (DMSO) served as control. All experiments were performed in quadruplicate. After 24 h, 100 μL of reaction mix per 100 μL medium was added into each well. After 10 min at room temperature in the dark, absorption was measured at 492 nm with 620 nm as reference after addition of 50 μL stop solution using a microplate reader and corrected to blank values (wells without cells).

Cell migration assay

The migration capability of eEND2 cells exposed for 24 h to 10 or 25 mM HBA or vehicle (DMSO; control) was assessed using 24-well chemotaxis chambers and polyvinylpyrrolidone-coated polycarbonate filters with an 8 μm pore size (BD Biosciences, Heidelberg, Germany). For this purpose, 500 μL of a cell suspension containing 2×10^5 HBA-treated or vehicle-treated cells in DMEM was added to each of the upper wells. The lower wells were filled with DMEM supplemented with 1% FCS. The chamber was then incubated for 5 h at 37°C in a humidified atmosphere with 5% CO_2. After incubation, non-migrated cells were removed from the upper surface of the filters, and migrated cells, which were adherent to the lower surface, were fixed with methanol and stained with Dade Diff-Quick (Dade Diagnostika GmbH, München, Germany). The number of these migrated cells was counted in 10 randomly chosen microscopic regions of interest (ROIs) at 20× magnification and is given as cells per ROI. All migration assays were performed in quadruplicate.

Phalloidin staining of filamentous actin

To analyse the effect of HBA on intracellular actin filament reorganization, aliquots of eEND2 cells were plated on sterile coverslips on a 24-well plate (1×10^5 per well) and cultured in DMEM with 10% FCS. After 24 h, medium was changed, and 10 mM HBA, 25 mM HBA or the vehicle DMSO was added for another 24 h incubation. Then cells were washed with

phosphate-buffered saline (PBS) twice and fixed in 4% formaldehyde/PBS (Rotifix; Roth, Karlsruhe, Germany). After PBS washing, the fixed cells were treated for 5 min with 0.1% Triton-x-100/PBS (Roth) for permeabilization. Non-specific binding was blocked by incubation with 1% bovine serum albumin (BSA)/PBS for 30 min. After washing with PBS twice, the cytoskeleton of the eEND2 cells was labelled with Alexa-568 nm–conjugated phalloidin (Invitrogen, Darmstadt, Germany) in a concentration of 1:100 in PBS for 30 min. Cell nuclei were stained with Hoechst 33258 (1:500; Sigma-Aldrich, Taufkirchen, Germany). The coverslips were mounted with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany) and analysed using a BX60 microscope (Olympus, Hamburg, Germany).

Western blot analysis

To investigate the action of HBA on protein expression of proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP9) in cultured eEND2 cells, the cells were exposed to 10 and 25 mM HBA (Sigma-Aldrich) or vehicle (DMSO; control). All experiments were performed in quadruplicate. After 24 h, the cells were harvested with accutase (PAA) and stored in liquid nitrogen for Western blot analysis from whole-cell extracts. For this purpose, membranes were incubated for 2 h with a mouse-monoclonal anti-PCNA antibody (1:20 000; DAKO, Hamburg, Germany), a rabbit-polyclonal anti-VEGF antibody (#147; 1:100; Santa Cruz, Heidelberg, Germany) and a rabbit-polyclonal anti-MMP9 antibody (1:300; Abcam, Cambridge, UK) followed by the corresponding horseradish peroxidase (HRP)–conjugated secondary antibodies (1:5 000; GE Healthcare Amersham, Freiburg, Germany). Protein expression was visualized using luminol-enhanced chemiluminescence and exposure of membranes to blue light–sensitive autoradiography film (Hyperfilm ECL, GE Healthcare Amersham). Signals were densitometrically assessed (Geldoc, Quantity one software; Bio-Rad, München, Germany).

Aortic ring assay

To study *in vitro* the effect of HBA and TNP-470 on vascular sprout formation, an aortic ring assay was performed, as described previously (Ehrmantraut *et al.*, 2010). Briefly, aortic rings from four male Sprague–Dawley rats (250 g body weight) were embedded in 200 µL Matrigel (BD Matrigel™ Matrix; BD Biosciences) in 48-well tissue culture grade plates, and the Matrigel was allowed to polymerize for 20 min at 37°C and 5% CO_2. The wells were then overlaid with 800 µL of DMEM (10% FCS, 100 U·mL^{-1} penicillin, 0.1 mg·mL^{-1} streptomycin) supplemented with 10 or 25 mM HBA, 10 or 25 µM TNP-470 or vehicle (DMSO) respectively. Subsequently, the rings were maintained at 37°C and 5% CO_2 for 6 days with medium change at day 3. All assays were done in sextuplicate. Vascular sprouting from each ring was examined by phase-contrast microscopy (BZ-8000; Keyence, Osaka, Japan) and quantitatively analysed by means of the software package CapImage (version 8.5; Zeintl, Heidelberg, Germany). Quantitative analysis included the determination of the area (mm^2) and the maximal length (µm) of the outer aortic vessel sprouting.

In vivo *endometriosis model*

To confirm the inhibitory effect of HBA on the development of new microvascular networks *in vivo*, we used the dorsal skinfold chamber model of endometriosis (Laschke *et al.*, 2005). This model has already been used to study the anti-angiogenic activity of various compounds, including rapamycin (Laschke *et al.* 2006a), cyclooxygenase-2 inhibitor (Laschke *et al.*, 2007) and epigallocatechin-3-gallate (Laschke *et al.*, 2008).

All animal care and experimental procedures were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 Rev. 1985). In the present study, the model was adapted to C57BL/6 mice with a body weight of 22–25 g. The mice were housed one per cage and had free access to tap water and standard pellet food (Altromin, Lage, Germany). Endometrial tissue was harvested from four donor C57BL/6 mice. The implantation procedure of the chamber has been described previously in detail (Menger *et al.*, 2002). After implantation, the animals were allowed to recover from anesthesia and surgical trauma for 72 h. Subsequently, the cover glass of the dorsal skinfold chamber was temporarily removed, and three endometrial fragments with a comparable initial size of 0.6–0.7 mm^2 were placed onto the striated muscle tissue within each chamber (n = 15). Subsequently, vascularization of the developing endometriotic lesions was analysed by means of intravital fluorescence microscopy. For this purpose, animals were randomized to a control and a treatment group. A group of seven animals was given daily i.p. injections of 100 mg·kg^{-1} HBA (dissolved in DMSO; Sigma-Aldrich). The dose of 100 mg·kg^{-1} HBA has been used in previous studies for the effective reduction of inflammation and pain in mice (Lim *et al.*, 2007). Moreover, it has been shown that HBA can be administered *in vivo* at doses up to 200 mg·kg^{-1} without inducing any toxic effects (Descamps *et al.*, 2009). Eight vehicle-treated animals served as controls.

For intravital fluorescence microscopy, 0.1 mL 5% fluorescein isothiocyanate (FITC)–labelled dextran 150 000 (contrast enhancement by staining of blood plasma) were injected i.v. via the retrobulbary space. Intravital fluorescence microscopy was performed using a Zeiss Axiotech microscope (Zeiss, Oberkochen, Germany) with a 100 W mercury lamp attached to an epi-illumination filter block for blue, green and ultraviolet light. The microscopic images were recorded by a charge-coupled device video camera (FK6990; Pieper, Schwerte, Germany) and transferred to a DVD system for off-line evaluation. By means of 5×, 10× and 20× long-distance objectives (Zeiss) magnifications of ×115, ×230 and ×460 were achieved on a 14 inch video screen (KV-14CT1E; Sony, Tokyo, Japan).

The microscopic images were analysed quantitatively offline by means of the computer-assisted image analysis system CapImage (Zeintl). Intravital fluorescence microscopic analyses were performed directly after endometrium transplantation and on days 3, 6, 10 and 14. The analyses included the determination of the size of developing endometriotic lesions (in % of the initial size) and the functional capillary density, that is, the length of red blood cell–perfused microvessels per observation area (in cm·cm^{-2}).

In an additional set of experiments, we tested potential toxic effects of HBA on normal blood vessels in empty dorsal skinfold chambers of four mice. For this purpose, the animals were given daily i.p. injections of 100 mg·kg^{-1} HBA, and intravital fluorescence microscopic analyses were performed at day 0 (onset of HBA-treatment), 3, 6, 10 and 14, as described above. By means of CapImage, we assessed in each animal the diameter (in μm) and the centerline red blood cell velocity (in μm·s^{-1}) of arterioles and venules as well as the functional density (in cm·cm^{-2}) of capillaries in five randomly chosen observation areas.

Histology and immunohistochemistry

At the end of the *in vivo* experiments, formalin-fixed specimens of the dorsal skinfold chamber preparations were embedded in paraffin. Sections (4 μm) were cut and stained with haematoxylin and eosin according to standard procedures. Immunohistochemical staining of apoptotic cells was performed by a rabbit polyclonal anti-cleaved caspase-3 antibody as primary antibody (1:100; New England Biolabs, Frankfurt, Germany). This was followed by a peroxidase-conjugated goat anti-rabbit antibody (1:200; Dianova GmbH, Hamburg, Germany). 3,3′-Diaminobenzidine was used as chromogen. The sections were counterstained with hemalaun and examined by light microscopy (BX60; Olympus).

Statistics

All values are expressed as means ± SEM. After testing the data for normal distribution and equal variance, differences between two groups were analysed by the unpaired Student's *t*-test, and differences between multiple groups were analysed by ANOVA followed by the appropriate *post hoc* comparison. To test for time effects in the individual groups, ANOVA for repeated measures was applied. This was followed by the Student–Newman–Keuls test, including the correction of the α error according to Bonferroni probabilities to compensate for multiple comparisons (SigmaStat; Jandel Corporation, San Rafael, CA). Statistical significance was accepted for a value of $P < 0.05$.

Materials

HBA and TNP-470 were supplied by Sigma-Aldrich. Nomenclature of drugs and molecular targets in the present study follows Alexander *et al*. (2009).

Results

HBA action on endothelial cell viability and integrity

When applying the WST-1 assay to eEND2 cells incubated with a range of concentrations of HBA, we found an obvious loss in cell viability with a LD_{50} of ~50 mM (Figure 1D). In line with these findings, cytotoxicity of HBA progressively increased between 1 and 100 mM, as indicated by an enhanced LDH release from damaged eEND2 cells (Figure 1E). Using the well-established anti-angiogenic compound TNP-470 as positive control for our WST-1 and LDH assays, we found that a similar loss in cell viability and increase of

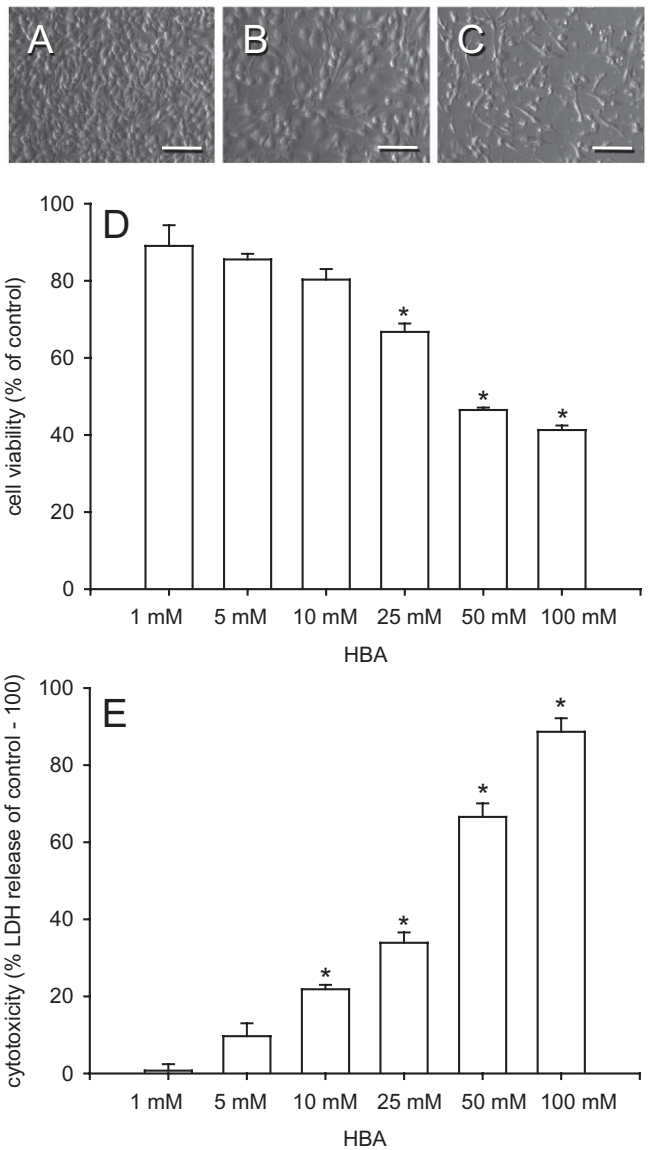

Figure 1

(A–C) Microscopic images of eEND2 cells, which were cultured for 24 h in DMEM supplemented with vehicle (A, control), 10 mM (B) or 25 mM HBA (C). Scale bars: 100 μm. (D, E) Cell viability (% of control) of eEND2 cells, which were exposed for 24 h to different concentrations (1–100 mM) of HBA, as assessed by the WST-1 assay (D). Cytotoxicity of HBA (% increase of LDH release over control), as assessed by the LDH assay (E). Data shown are means ± SEM. *$P <$ 0.05 vs. control; $n = 4$. DMEM, Dulbecco's modified Eagle's medium; HBA, 4-hydroxybenzyl alcohol; WST-1, water-soluble tetrazolium-1; LDH, lactate dehydrogenase.

cytotoxicity could be achieved with TNP-470 doses, which were 1000-fold lower than our HBA doses (Figure 2A and B). This shows that both compounds exert comparable dose-dependent effects on eEND2 cell viability and integrity but over different dose ranges. Based on our results, we decided to use HBA concentrations of 10 and 25 mM for further *in vitro* analyses. At these concentrations, eEND2 cells still exhibited a viability of 80% and 67% when compared with that of

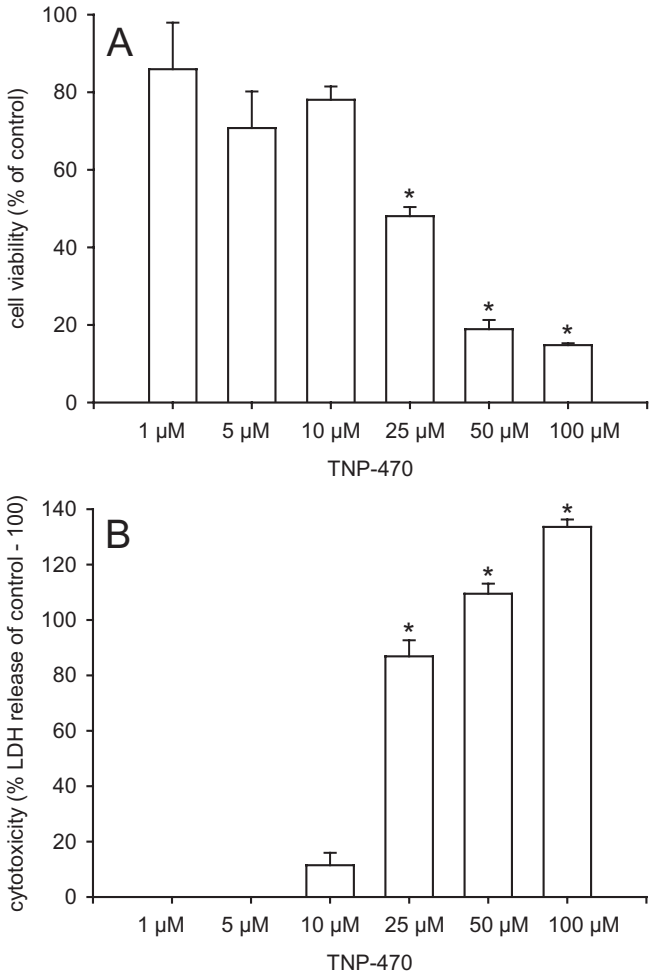

Figure 2

Cell viability (% of control) of eEND2 cells, which were exposed for 24 h to different concentrations (1–100 μM) of TNP-470, as assessed by the WST-1 assay (A). Cytotoxicity of TNP-470 increase of LDH release over control), as assessed by the LDH assay (B). Data shown are means ± SEM. *$P < 0.05$ versus control. $n = 4$. WST-1, water-soluble tetrazolium-1; LDH, lactate dehydrogenase.

vehicle-treated control cells. In addition, the cells were able to recover completely after HBA exposure, exhibiting a viability of 100% (10 mM) and 102% (25 mM) after cultivation for 7 days in HBA-free DMEM. Of interest, we found that treatment with 10 and 25 mM HBA caused characteristic changes in cell morphology (Figure 1A–C). In comparison with control cells (Figure 1A), cells exposed to 10 mM HBA appeared to be swollen and exhibited long cellular extensions (Figure 1B). In contrast, exposure to 25 mM HBA resulted in the formation of small spindle-shaped cells (Figure 1C).

HBA actions on endothelial cell migration and actin filament reorganization

In the migration assay, chemotactic stimulation with 1% FCS promoted migration of vehicle-treated eEND2 cells through polycarbonate filters (Figure 3A). This migratory activity was markedly decreased in HBA-treated eEND2 cells (Figure 3B and C), as indicated by a significantly lower number of cells that migrated through the filters to the lower membrane surface compared with controls (Figure 3D). Notably, this effect was most pronounced in cells exposed to 10 mM HBA (Figure 3D).

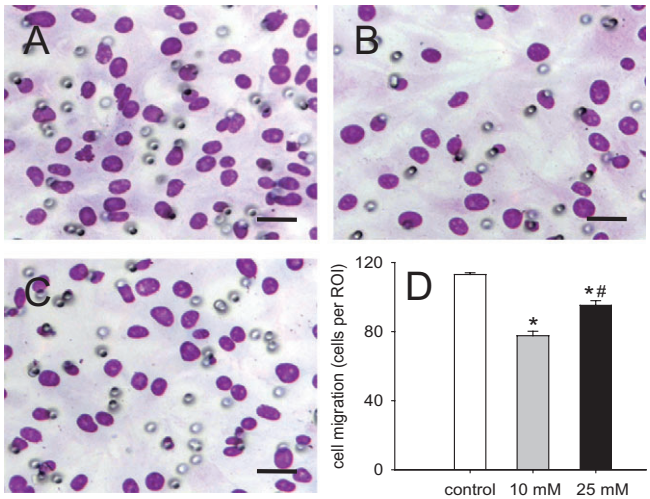

Figure 3

(A–C) Light microscopic images of eEND2 cells, which have migrated through the 8 μm pores of the polycarbonate filters to the lower membrane surface of the cell migration assay. The cells were cultured for 24 h in DMEM supplemented with vehicle (A, control), 10 mM (B) or 25 mM HBA (C). Scale bars: 40 μm. (D) Cell migration (cells per ROI) of eEND2 cells, which were cultured for 24 h in DMEM supplemented with vehicle (control), 10 or 25 mM HBA, as assessed by the cell migration assay. Data shown are means ± SEM. *$P < 0.05$ versus control; #$P < 0.05$ versus 10 mM HBA. $n = 4$. DMEM, Dulbecco's modified Eagle's medium; HBA, 4-hydroxybenzyl alcohol.

Because the reorganization of actin filaments is crucially involved in the process of cellular migration (Lauffenburger and Horwitz, 1996; Abe *et al.*, 2002), we further analysed the cytoskeleton of HBA-treated and vehicle-treated eEND2 cells by phalloidin staining. By this, we could demonstrate that vehicle-treated control cells exhibited a physiological morphology of the cytoskeleton system with typical filamentous actin (Figure 4A). In contrast, treatment of eEND2 cells with 10 mM HBA resulted in a massive perinuclear aggregation of filamentous actin (Figure 4B). This was also found in cells, which were exposed to 25 mM HBA. However, these cells were considerably smaller (Figure 4C), which may explain their improved capability of passing the 8 μm pores in the migration assay when compared to 10 mM HBA-treated cells.

HBA actions on endothelial cell protein expression

Western blot analyses revealed that eEND2 cells, cultured in DMEM supplemented with 10 or 25 mM HBA, exhibited a significantly decreased expression of the proliferation marker PCNA compared with controls (Figure 5). Moreover, expression of important proteins involved in the angiogenic process, that is, VEGF and MMP9, was also significantly reduced in HBA-treated cells (Figure 5).

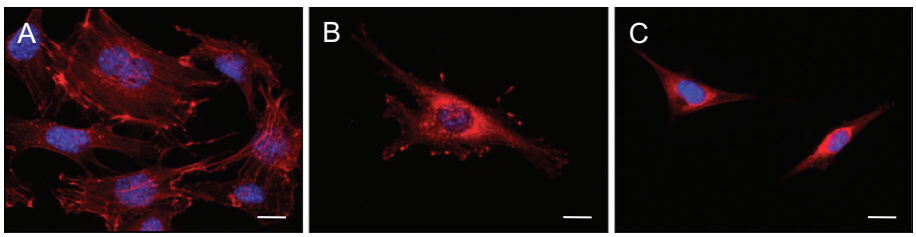

Figure 4

Fluorescence microscopic images of eEND2 cells, which were cultured for 24 h in DMEM supplemented with vehicle (A, control), 10 mM (B) or 25 mM HBA (C). The cells were stained with Alexa-568 nm–conjugated phalloidin (red) for the detection of the cytoskeleton and the cell nucleus marker Hoechst 33258 (blue). Scale bars: 23 μm. DMEM, Dulbecco's modified Eagle's medium; HBA, 4-hydroxybenzyl alcohol.

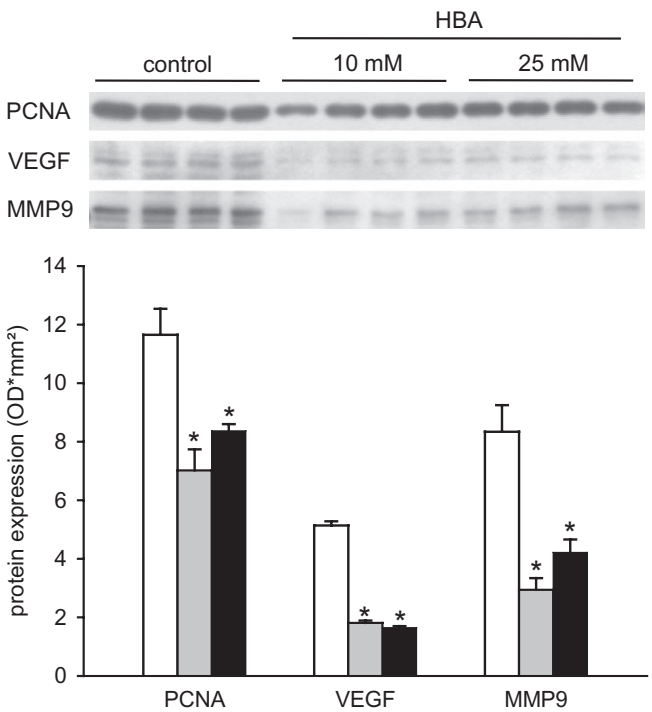

Figure 5

Western blot analysis of PCNA, VEGF and MMP9 protein expression [shown as optical density (OD) × mm^2] of eEND2 cells, which were cultured for 24 h in DMEM supplemented with vehicle (control, white bars), 10 mM (grey bars) or 25 mM (black bars) HBA. Data shown are means ± SEM. *P < 0.05 versus control, n = 4. DMEM, Dulbecco's modified Eagle's medium; HBA, 4-hydroxybenzyl alcohol.

HBA actions on vascular sprout formation

Incubation of rat aortic rings in Matrigel stimulates the formation of vascular sprouts growing outside the aortic wall, resulting in a dense network of tubular vessel structures (Figure 6A). Accordingly, aortic rings of vehicle-treated control rings exhibited a sprout area of 7.0 ± 0.7 mm^2 and a maximal sprout length of 1210 ± 65 μm after 6 days. Aortic rings, which were treated with 10 and 25 μM of the anti-angiogenic compound TNP-470, presented with a significantly reduced sprout area (2.6 ± 0.3 mm^2 and 2.3 ± 0.3 mm^2) and maximal sprout length (735 ± 86 μm and 755 ± 58 μm) when compared with controls (Figure 6B). In contrast, vascular sprouting was completely suppressed in Matrigel-embedded aortic rings exposed to 10 mM and 25 mM HBA respectively (P < 0.05) (Figure 6C). Taking our results on HBA and TNP-470 cytotoxicity into account, these findings indicate that HBA even exhibits a stronger anti-angiogenic potency than TNP-470 when used in doses exerting comparable effects on cell viability and cytotoxicity.

HBA action on vascularization of endometriotic lesions

To analyse the anti-angiogenic action of HBA *in vivo*, we used the dorsal skinfold chamber model of endometriosis in mice. In both HBA-treated and vehicle-treated animals, transplanted endometrial fragments exhibited a comparable initial size of ~0.6–0.7 mm^2, excluding size-dependent differences in the developing endometriotic lesions. Using intravital fluorescence microscopy, newly formed microvessels could be observed in both groups at day 3 after endometrium transplantation onto the host striated muscle tissue within the chamber. Throughout the further time course of the experiment, an increasing number of microvessels developed within the lesions, finally resulting in the formation of glomerulum-like microvascular networks (Figure 7A and B). In line with the *in vitro* results, this angiogenic process was inhibited in HBA-treated animals, as indicated by a significantly decreased functional capillary density at days 10 and 14 when compared with vehicle-treated control mice (Figure 7B and C). Moreover, HBA-treated animals also presented with a significantly reduced lesion size at day 14 in comparison to controls (Figure 7D). Importantly, our analyses of empty dorsal skinfold chambers additionally demonstrated that the treatment with HBA did not exert any toxic effects on normal blood vessels under physiological conditions *in vivo*, as indicated by unchanged arteriolar and venular microhaemodynamic parameters and a constant functional capillary density over time (Table 1).

Histological examination of the dorsal skinfold chamber preparations at day 14 showed that, in both groups, transplantation of isolated endometrium onto the host striated muscle tissue had resulted in the development of typical endometriotic lesions, composed of endometrial glands, which were surrounded by a vascularized endometrial stroma (Figure 7E and F). Interestingly, immunohistochemical detection of cleaved caspase-3 further revealed that HBA-treated

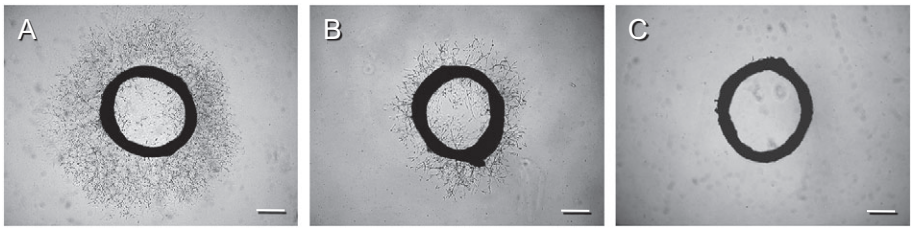

Figure 6

Phase-contrast microscopic images of rat aortic rings after 6 days of incubation in DMEM supplemented with vehicle (A), 10 μM TNP-470 (B) or 10 mM HBA (C) respectively. Scale bars: 575 μm. DMEM, Dulbecco's modified Eagle's medium; HBA, 4-hydroxybenzyl alcohol.

Table 1

Diameter and centerline red blood cell velocity of arterioles and venules as well as functional density of capillaries within empty dorsal skinfold chambers of HBA-treated mice

	Day 0	Day 3	Day 6	Day 10	Day 14
Diameter (μm)					
Arterioles	45.3 ± 1.4	47.8 ± 3.0	50.7 ± 6.0	58.3 ± 5.3	53.9 ± 4.4
Venules	67.0 ± 7.1	60.1 ± 12.1	62.6 ± 14.6	64.1 ± 12.9	50.5 ± 5.1
Centerline red blood cell velocity ($\mu m \cdot s^{-1}$)					
Arterioles	1092 ± 117	1113 ± 141	1165 ± 62	1170 ± 26	892 ± 89
Venules	497 ± 167	696 ± 179	669 ± 132	832 ± 74	547 ± 139
Functional density ($cm \cdot cm^{-2}$)					
Capillaries	192 ± 2	188 ± 5	184 ± 3	186 ± 1	190 ± 2

Data shown are means ± SEM; $n = 4$.

endometriotic lesions exhibited an increased number of apoptotic stromal and glandular cells when compared to vehicle-treated controls (Figure 7G and H).

Discussion and conclusions

During the last few years, the phenolic plant compound HBA has been identified as a promising pharmacological agent, which exhibits anti-oxidative, anti-inflammatory and anti-nociceptive activity. Recently, HBA has also been shown to inhibit the development of new blood vessels in the chorio-allantoic membrane assay (Lim *et al.*, 2007). Here, we provide for the first time a systematic analysis of the mechanisms underlying this anti-angiogenic action of HBA.

Interestingly, we found that HBA is capable of inhibiting several steps of the angiogenic process. Accordingly, HBA may be more effective in suppressing the development of new blood vessels than more selective inhibitors, which only block one distinct angiogenic target. Angiogenesis is characterized by the coordinated sequence of humoral and cellular interactions (Risau, 1997; Carmeliet, 2000). Upon stimulation by growth factors such as VEGF, vascular endothelial cells produce and release MMPs, resulting in the degradation of their surrounding basement membrane. This enables the cells to migrate into the interstitium, resulting in the formation of capillary buds and sprouts. These sprouts are highly organized structures (Ruhrberg *et al.*, 2002; Gerhardt *et al.*, 2003; Gerhardt and Betsholtz, 2005) and on activation by VEGF, specialized endothelial cells at their tips extend multiple filopodia, which determine the growth direction of the sprouts (Gerhardt *et al.*, 2003). These tip cells are followed by endothelial stalk cells with high proliferating activity, promoting sprout elongation and tube formation (Gerhardt *et al.*, 2003). Finally, the sprouts interconnect with each other to form new microvascular networks. The wall of these networks is then again stabilized by the recruitment of smooth muscle cells, pericytes and fibroblasts, as well as the production of extracellular matrix compounds.

Our *in vitro* analyses of eEND2 cells showed that HBA inhibited the initiation of the angiogenic process by down-regulating VEGF and MMP9 expression and by affecting endothelial cell migration. By fluorescence staining of the actin cytoskeleton system, we could demonstrate that this was caused through the inhibition of actin filament reorganization, which is known to be crucially involved in cellular migration (Lauffenburger and Horwitz, 1996; Abe *et al.*, 2002). The staining of individual cells further revealed that cells, exposed to 25 mM HBA were considerably smaller when compared with cells treated with 10 mM HBA. This may explain their improved capability of passing the 8 μm pores in the migration assay and, thus, our finding that the lower HBA dose seemed to be more effective for the inhibition of

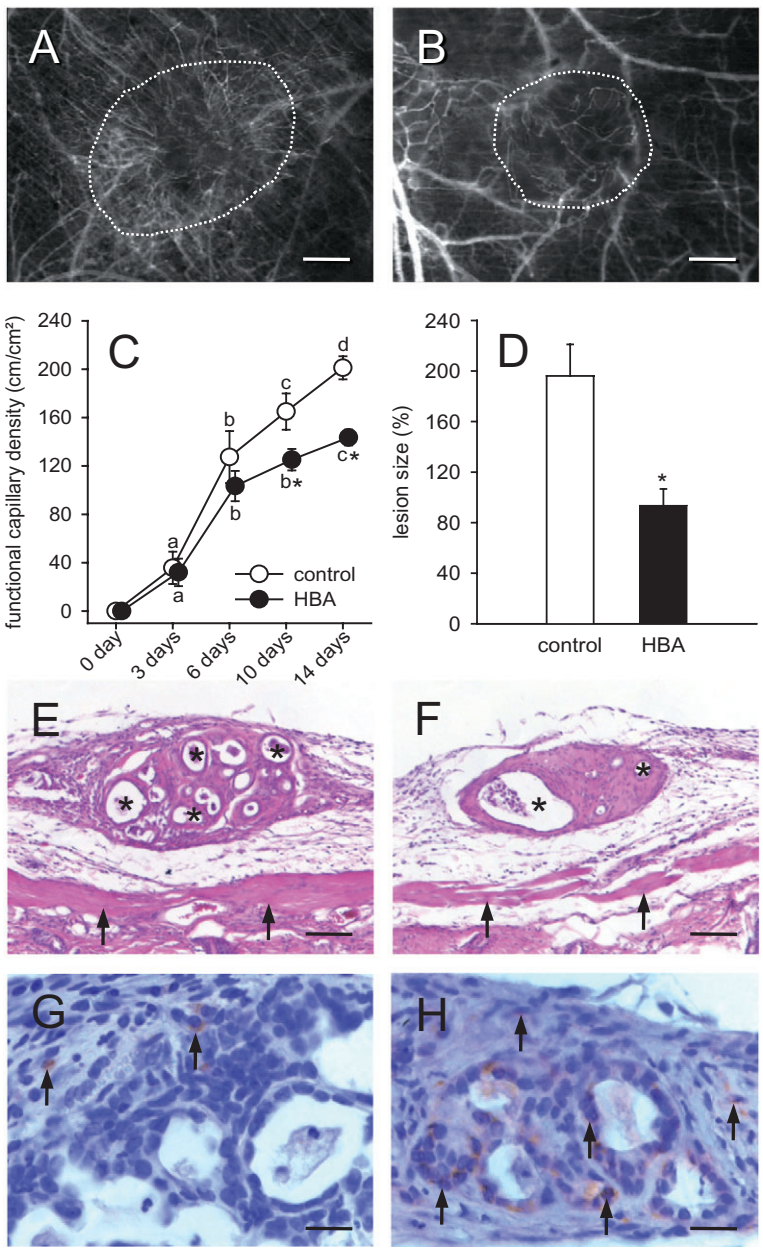

Figure 7

(A, B) Intravital fluorescence microscopic images of endometriotic lesions (borders marked by dotted line) at day 14 after endometrium transplantation into the dorsal skinfold chamber of a vehicle-treated control mouse (A) and an HBA-treated animal (B). Both endometriotic lesions exhibit newly developed microvascular networks with a glomerulum-like angioarchitecture. However, the HBA-treated endometriotic lesion presents with a markedly reduced functional capillary density and lesion size (B). Blue light epi-illumination with contrast enhancement by 5% FITC-labelled dextran 150 000 i.v. Scale bars: 275 µm. (C, D) Functional capillary density ($cm \cdot cm^{-2}$) (C) and final lesion size (%) at day 14 (D) of endometriotic lesions in dorsal skinfold chambers of vehicle-treated control animals and HBA-treated C57BL/6 mice, as assessed by intravital fluorescence microscopy and computer-assisted off-line analysis. Data shown are means ± SEM. $^{a}P < 0.05$ versus day 0 within each individual group; $^{b}P < 0.05$ versus days 0 and 3 within each individual group; $^{c}P < 0.05$ versus days 0, 3 and 6 within each individual group; $^{d}P < 0.05$ versus days 0, 3, 6 and 10 within each individual group; $*P < 0.05$ versus control, $n = 7–8$. (E, F) Haematoxylin–eosin stained cross sections of endometriotic lesions at day 14 after endometrium transplantation onto the striated muscle tissue (arrows) within the dorsal skinfold chamber of a vehicle-treated control mouse (E) and a HBA-treated animal (F). Both endometriotic lesions are characterized by cyst-like dilated endometrial glands (asterisks), which are surrounded by a vascularized endometrial stroma. Scale bars: 85 µm. (G, H) Immunohistochemical detection of cleaved caspase-3-positive apoptotic cells (arrows) within endometriotic lesions of a vehicle-treated control mouse (G) and a HBA-treated animal (H) at day 14 after endometrium transplantation. Note that the HBA-treated lesion (H) exhibits an increased number of apoptotic stromal and glandular cells when compared with the vehicle-treated control (G). Scale bars = 20 µm. HBA, 4-hydroxybenzyl alcohol; FITC, fluorescein isothiocyanate.

endothelial cell migration. In addition, HBA suppressed endothelial cell proliferation, as indicated by a significantly decreased expression of PCNA in HBA-treated eEND2 cells compared with controls. Based on these findings, we speculated that HBA treatment may also inhibit the formation and growth of vascular sprouts.

To test our hypothesis, we next performed an aortic ring assay. This approach is considered to come closest to mimic angiogenesis under *in vivo* conditions, because the aortic ring not only consists of endothelial cells but also includes the surrounding non-endothelial mural cells (Auerbach *et al.*, 2003). In addition, the endothelial cells of the ring are not pre-selected by passaging and thus are not in a proliferative state at the time of explantation (Auerbach *et al.*, 2003). In line with the results of our cell experiments, we found that the outer aortic sprouting of HBA-treated rings was completely suppressed compared with vehicle-treated control rings. Taken together, these *in vitro* findings indicated that HBA might be used for the treatment of angiogenic diseases, such as endometriosis.

Endometriosis is a frequent gynaecological disease, which is defined by the presence and proliferation of endometrial tissue at ectopic sites, also referred to as endometriotic lesions (Giudice and Kao, 2004). Comparable with tumours or metastases, an adequate vascularization is a major prerequisite for survival and growth of these lesions (Groothuis *et al.*, 2005; Laschke and Menger, 2007). Over the last few years, we found that the transplantation of endometrial tissue into dorsal skinfold chambers was a suitable model to study the effects of anti-angiogenic agents on endometriosis (Laschke *et al.*, 2006a,b; 2007; 2008). Accordingly, we used this approach in the present study to confirm *in vivo* the anti-angiogenic activities of HBA observed *in vitro*. For this purpose, we used a dose of 100 $mg \cdot kg^{-1}$ HBA, which corresponds to an *in vitro* dose of ~13 mM, assuming a complete uptake of the drug in the systemic circulation. We found that HBA inhibited the formation of new microvascular networks in endometriotic lesions, as shown by a significantly decreased functional capillary density of lesions in HBA-treated animals at days 10 and 14 compared with that of vehicle-treated controls. This was associated with an increased number of apoptotic stromal and glandular cells and a markedly reduced lesion size, indicating that the survival and growth of endometriotic lesions is closely linked to the extent of vascularization. However, in contrast to our *in vitro* results, we found a less pronounced inhibition of angiogenesis in our *in vivo* experiments. This may be due to the fact that we administered HBA in our experiments by i.p. injections. Accordingly, we cannot exclude that a certain fraction of HBA did not reach the systemic circulation. Thus, further studies focusing on the bioavailability of HBA and testing different routes of application may be useful to identify the optimal doses, which inhibit even more effectively angiogenesis *in vivo*.

In summary, the present study demonstrates that the phenolic plant compound HBA inhibited the development of new blood vessels by targeting several angiogenic mechanisms, including expression of angiogenic growth factors and MMPs, endothelial cell proliferation and migration as well as vascular sprouting and network formation. Accordingly, HBA may represent a promising anti-angiogenic agent for the treatment of angiogenic diseases, such as endometriosis.

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Conflicts of interest

None.

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