

Antiangiogenic Properties of Lupulone, a Bitter Acid of Hop Cones

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Abstract. *Background: Angiogenesis is the result of intricate steps regulated by the balance between agonistic and antagonistic effectors. Disturbance of this balance leads to an 'angiogenic' switch critical for tumor development. Materials and Methods: Using human umbilical vein endothelial cells (HUVEC) the effects of lupulone were analyzed on proliferation induced by angiogenic growth factors, transmembrane cell migration toward fibronectin and formation of a network of tubular-like structures on Matrigel. Results: Lupulone (2.5-50 µg/ml) induced a concentration-dependent inhibition of HUVEC proliferation and chemotaxis. Lupulone caused a significant reduction of closed capillary-like structures in Matrigel indicating a strong inhibitory effect on neovascularisation. In mice receiving lupulone (20 mg/kg/day) in drinking water for 21 days, new vessel formation was reduced by 50% in matrigel plugs implanted under the skin when compared with controls. Conclusion: The present data demonstrate that lupulone is able to inhibit angiogenesis in vitro and in vivo. Lupulone emerges as a potential chemopreventive agent when considering its strong antiangiogenic properties.*

Cancer chemoprevention is defined as the use of pharmacological or natural agents in order to inhibit or delay the processes of tumor initiation, promotion, progression and extension (1). Tumor vascularization is mandatory for growth and metastatic dissemination and the inhibition of tumor angiogenesis represents an attractive strategy in cancer chemoprevention, a concept that is termed 'angioprevention'

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(2). Among potential chemopreventive agents, it was shown that two hop-derived constituents, humulone and xanthohumol exhibit antiangiogenic chemopreventive properties targeting endothelial cell migration and invasion (3, 4). Humulone is a bitter acid from hop. The bitter acids consist of α -acids (humulone) and β -acids (lupulone) (5). These components are extracted from hop cones (*Humulus lupulus* L.) and give the characteristic bitter taste and aroma of beers. Humulone was reported to have various potent biological activities including inhibition of angiogenesis (3), induction of apoptosis (6), inhibition of tumor promotion by phorbol ester (7) and suppression of cyclooxygenase-2 gene transcription (8). Other studies have reported on the proapoptotic and antibacterial effects of hop bitter acid extracts (9, 10), as well as on radical-scavenging and lipid peroxidation inhibitory activities (11). Recently, we demonstrated the chemopreventive efficacy of lupulone in a rat model of colon carcinogenesis (12).

In the present work, we aimed to assess the potential antiangiogenic properties of the hop β -acid lupulone. The effects of lupulone *in vitro* on different steps involved in the angiogenic process were investigated using human umbilical cord endothelial cells (HUVECs). The effects of lupulone were analysed, on basal proliferation and proliferation induced by angiogenic growth factors, on transmembrane cell migration toward fibronectin (FN) and on its ability to affect the formation of a network of tubular-like structures on Matrigel. In addition, lupulone was administered to mice in their drinking water and its angiopreventive effects on growth factor-induced angiogenesis in Matrigel plugs implanted under the skin of mice were evaluated.

Materials and Methods

Materials. Lupulone stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St-Quentin Fallavier, France) and diluted directly in cell culture medium (highest concentration of DMSO 0.1%). Human fibronectin (FN), human serum albumin (HSA) collagenase I and crystal violet were purchased from Sigma-

Aldrich. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from Tebu-bio (Le Perray en Yvelines, France). M199 supplemented with 25 mM HEPES, RPMI 1640, Universal culture supplement ITS™ (Insulin, Transferrin, Selenium), L-glutamine, phosphate-buffered saline (PBS) and gentamicin were obtained from Gibco-Invitrogen (Cergy Pontoise, France), while Endothelial Cell Growth Medium 2 (ECGM2), consisting of basal medium with supplements, hydrocortisone, heparin, ascorbic acid, long R3 IGF-1, bFGF, VEGF, and fetal calf serum (FCS), was obtained from PromoCell® GmbH (Heidelberg, Germany). The CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Inc., Eugene, OR, USA), and the Drabkin's reagent kit 525 (Sigma-Aldrich) were used according to the manufacturer's instructions. Matrigel™ Basement Membrane Matrix was obtained from BD Biosciences (Palo Alto, CA, USA). Transwell® cell culture insert migration chambers (8 µm pore size, 6.5 mm diameter) were from Becton Dickinson (Dickinson Labware Europe, Le Pont de Claix, France).

Male BALB/cJ mice (Janvier, Le Genest-St-Isle, France) were used for the angiogenesis test *in vivo*. Animal housing was maintained at a temperature of 21°C, and animal care was in accordance with institutional guidelines. Xylasin (Rompun®, Bayer Leverkusen, Germany), and ketamine (Ketalar®, Substantia Division Santé, Courbevoie, France) were used for anesthesia in the Matrigel plug experiments.

Isolation of lupulone. For the specific isolation of lupulone, an industrial by-product was used which is ordinarily discarded but which contains high amounts of β-acids. Hop paste was supplied by the brewery industry (Brasseries Kronenbourg, Obernai, France) and lupulone was isolated as described elsewhere (12).

Isolation of HUVECs. Human umbilical vein endothelial cells were isolated from human umbilical cords collected in sterile PBS (without Ca or Mg) supplemented with 11 mM glucose and 0.1% gentamicin (cord buffer). The traumatised areas were excised, the veins cannulated and perfused with prewarmed PBS to remove any trace of blood. The cords were then filled with a solution of 0.1% collagenase I and incubated at 37°C for 10 to 15 min. The effluent was flushed out with cord buffer and collected in 50 ml conical centrifugation tubes containing 5 ml of culture medium. After centrifugation at 200 xg for 5 min at 20°C the pelleted cells were resuspended in complete ECGM2, consisting of the basal medium plus the supplement growth factor mix and 0.1% gentamicin, and cultured on human FN-coated plates. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂, split every week at a 1/4 ratio and harvested for experiments during exponential growth. The cells were used up to the 4th passage. Cell death never exceeded 5% as assessed by Trypan blue dye exclusion. Experiments were performed in triplicate unless otherwise stated and carried out at least twice. Control contained concentrations of DMSO equivalent to those of the drug treatments.

Proliferation assays. Cells (6,000 cells per cm²) were plated onto FN-coated 96-well culture plates and cultured overnight in complete ECGM2. The culture medium was then replaced with fresh medium containing increasing concentrations of lupulone ranging from 2.5 to 50 µg/ml in a final volume of 200 µl. The plates were incubated for 5 days at 37°C. The medium was changed every second day and representative samples were removed, the

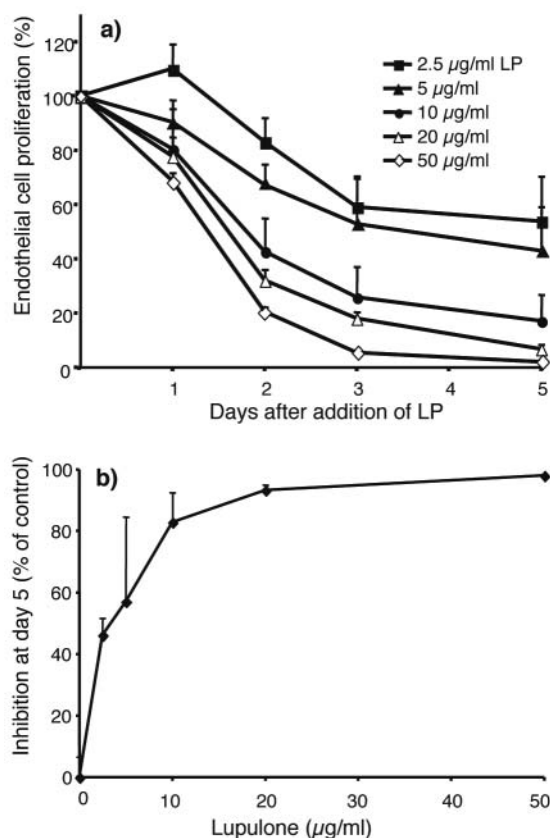


Figure 1. Effects of increasing concentrations of lupulone (LP) on the proliferation of HUVECs. a) Sparsely seeded cells were grown in the presence of lupulone (2.5 to 50 µg/ml) and proliferation estimated as described in Materials and Methods. The data are percentages of the value of the control set at 100% and are the mean ± SEM of at least two independent experiments performed in quadruplicate. b) Percentage of inhibition of proliferation at day 5 as a function of lupulone concentration. Results are the mean ± SEM of three independent experiments performed in triplicate.

medium discarded and the plates frozen at -20°C until analysis. Frozen cells were lysed by the addition of lysis buffer containing the fluorescent dye CyQUANT GR. The CyQUANT® assay relies on the use of an appropriate fluorescent dye that strongly fluoresces when bound to cellular nucleic acids (13). Fluorescence was measured using a microplate reader (Fluorolite Dynex Technologies, Issy les Moulineaux, France) with appropriate filters (excitation, 490 nm; emission, 535 nm). To test the antiproliferative effects of lupulone on VEGF and on bFGF induced proliferation, cells were grown in ECGM2 without growth factor supplements but with 2% FCS and 1% ITSTM and supplemented with 5 or 10 ng/ml of either VEGF or bFGF.

Cell migration assay. The ability of HUVECs to migrate was assayed in a modified Boyden chamber assay (14), using Transwell migration chambers. The lower compartment of the chamber was filled with 600 µl RPMI/M199 (v/v) containing 5 µg/ml of FN (positive control), or HSA (5 µg/ml) (negative control), and placed

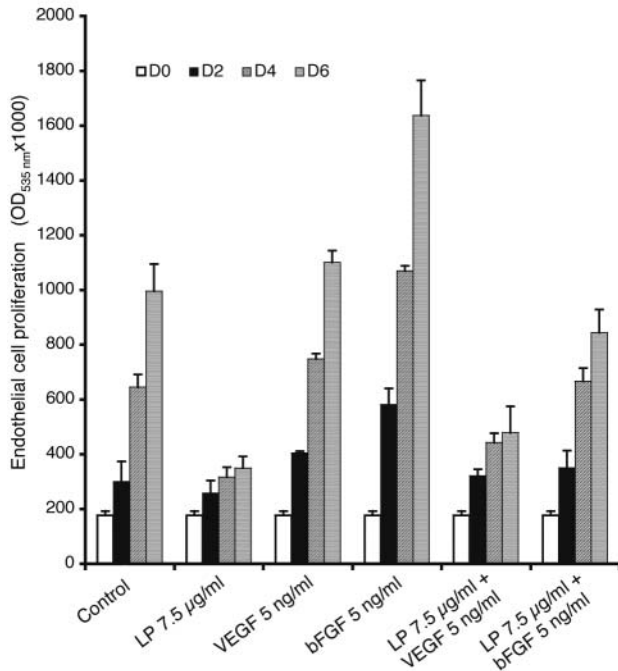


Figure 2. Inhibitory effects of lupulone on the proliferation of HUVECs induced by VEGF or bFGF. Cells were plated at 6,000 cells/cm² onto fibronectin-coated 96-well culture plates with or without 7.5 µg/ml lupulone and 5 ng/ml VEGF or bFGF. Proliferation was measured at day 0, 2, 4 and 6 after addition of lupulone. Results were quantified as described in Material and methods and are the mean ± SEM of three independent experiments performed in triplicate.

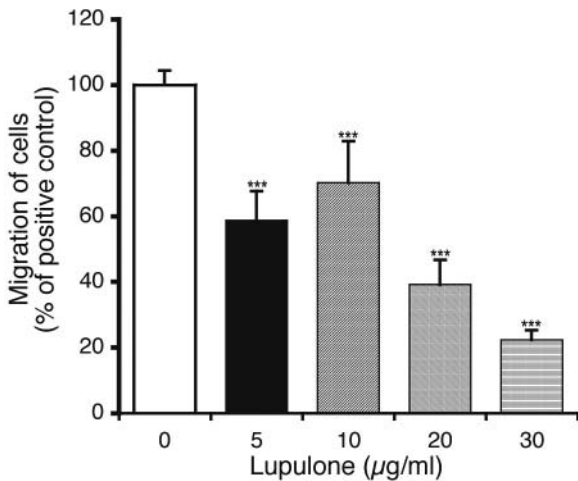


Figure 3. Modulation of transmembrane HUVECs migration toward fibronectin by lupulone. FN (5 µg/ml) in serum-free M199/RPMI (v/v) medium was placed in the lower chamber of the Transwell™ modified Boyden chamber. HUVECs (10⁵ cells per insert) in medium containing increasing concentrations of lupulone were placed in the upper cell culture insert and migration of cells through the porous membrane was quantified as described in Materials and Methods. Results expressed as a percentage of the positive control set at 100% are the mean ± SEM of three separate experiments with 2 chambers per experimental condition. ****p* < 0.001 vs. HUVEC migration in absence of lupulone.

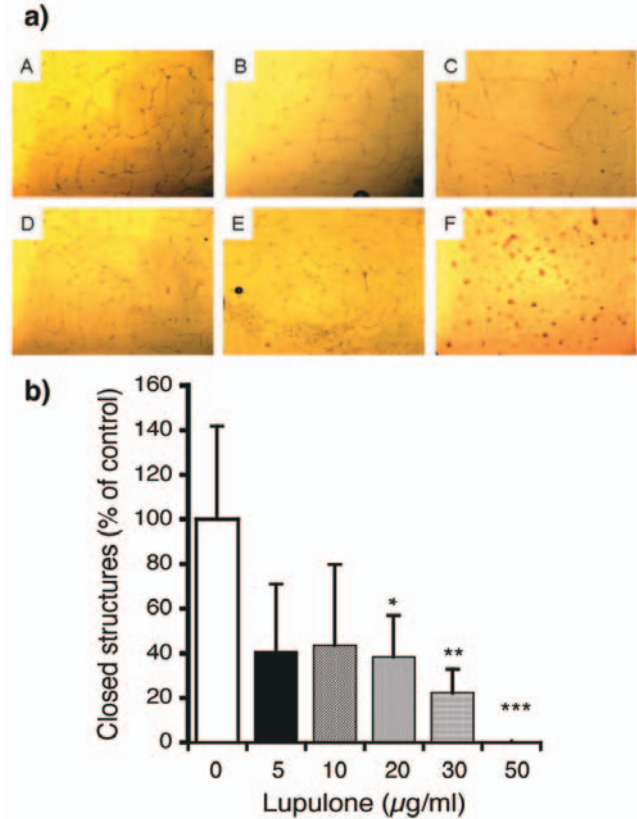


Figure 4. Effects of lupulone on capillary-like tube formation on Matrigel. HUVECs in ECGM2 were seeded on Matrigel as described in Materials and Methods and incubated for 24 h. a) Representative photomicrographs of capillary like structures formed by HUVECs exposed to no lupulone (A), or 5, 10, 20, 30 or 50 µg/ml lupulone (B to F) respectively. Original magnification x40. b) Closed capillary-like structures were counted in 5 randomly selected fields as described in Materials and Methods. Results are mean ± SEM of three separate experiments and expressed as percentage of the positive control set at 100%. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. capillary formation in the absence of lupulone.

at 37°C for 30 min. Cells (10⁵ in 100 µl) were seeded in the upper inserts and cultured without or with increasing concentrations of lupulone. Six hours after incubation at 37°C, non-migrated cells on the upper side of the membranes were removed with cotton swabs. Cells that moved to the lower side were fixed for 30 min in 2% paraformaldehyde solution at room temperature, and then stained with 0.1% crystal violet in 0.1 M sodium borate pH 9.0, containing 2% ethanol. The migrated cells from the total surface of the membranes were counted using an inverted light microscope at a x40 magnification (Nikon, Tokyo, Japan). Results were expressed as a percentage of the positive control set at 100%.

Matrigel™ morphogenesis assay. Unpolymerized Matrigel (350 µl/well) was placed in the wells of 24-well chilled plates with a cold pipette, avoiding bubbles, and allowed to polymerize for 30 minutes at 37°C (15). HUVECs (40,000 cells/well) were suspended in 500 µl of ECGM2 with lupulone concentrations ranging from 0 to 70 µg/ml.

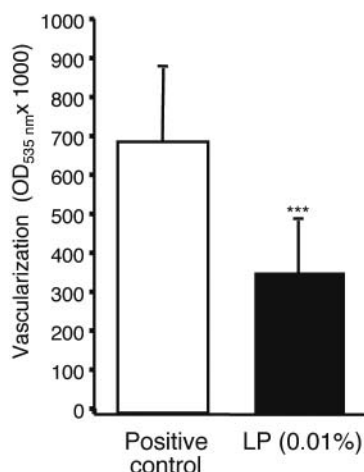


Figure 5. Inhibition of angiogenesis by lupulone in vivo in Matrigel plugs implanted in mice. Matrigel containing bFGF and standard heparin (500 ng and 100 µg/ml respectively) was subcutaneously injected in both flanks of mice. Lupulone was administered in the drinking water (0.01%) as described in Material and Methods. Control animals received the solvent (ethanol 0.1%) and vitamin C (0.001%) only. Results are the mean ± SEM of nine animals carrying 2 plugs each. *** $p < 0.001$.

The different cell suspensions were then carefully layered onto the Matrigel surface. Endothelial cell growth and two-dimensional organization were observed during the next 24 h through an inverted phase contrast light microscope. Capillary tube formation was evaluated by counting the number of capillary-like closed structures in 5 randomly selected microscopic fields 24 h after seeding (magnification x40) (15).

In vivo Matrigel plug assay. The Matrigel plug assay was performed as previously described (16) with some minor modifications. After one week's adaptation, 6-week-old male BALB/cJ mice were anesthetized with intramuscular xylasin/ketamine (8 mg/kg and 40 mg/kg respectively). A suspension of 500 µl Matrigel (High Concentration) supplemented with 500 ng/ml bFGF and 100 µg/ml heparin was slowly injected subcutaneously into both flanks. After injection, the matrigel rapidly formed a single solid gel plug.

The lupulone treated group (n=9) received lupulone (0.01%) plus 0.001% ascorbic acid (to prevent oxidation) in drinking water every day, starting 3 days before Matrigel injection and until the end of the experiment (3 weeks). The control group (n=9) identically received water containing the excipient (0.1% ethanol and 0.001% ascorbic acid). Plain Matrigel was implanted in non-treated negative controls (n=2) in order to eliminate eventual angiogenesis due to the experimental procedure. At day 22, matrigel plugs were collected and the extent of neovascularization was assessed by measuring the hemoglobin content using the Drabkin kit. The background value from negative control plugs was subtracted from positive controls and treated samples before comparison.

Statistical analysis. Data were reported as mean values ± SEM. The statistical analyses were performed using SigmaStat software (Systat Software GmbH, Erkrath, Germany). Comparison of two means was performed using Student's *t*-test. Other mean

comparisons were performed using the Tukey's multiple comparison test with adapted *p*-value. Differences with $p < 0.05$ were considered significant.

Results

Effects of lupulone on cell kinetics. The effects of lupulone (2.5 to 50 µg/ml) were measured on the proliferation of HUVECs in complete ECGM2 medium. The compound induced a time- and concentration-dependent inhibition of endothelial cell proliferation (Figure 1a). DMSO (0.1%) did not have significant effects on the proliferation of endothelial cells as compared to the control ($96\% \pm 7.5\%$ versus $100\% \pm 7\%$, mean ± SEM, n=3 separate experiments). Three days after addition of lupulone, an inhibition of $41\% \pm 12\%$ was observed for concentrations as low as 2.5 µg/ml lupulone. This inhibition reached $75\% \pm 8\%$ for 10 µg/ml and was near 100% for 50 µg/ml (mean ± SEM). On the basis of these results, for further proliferation experiments, a concentration of 7.5 µg/ml of lupulone, which reduced the proliferation by approximately 50%, was chosen (Figure 1b).

The effects of two growth factors, VEGF and bFGF on the lupulone-triggered inhibition of cell growth were assessed. The growth factors (5 or 10 ng/ml) were added to basal ECGM2 containing only 2% FCS and 1% ITS™. In the absence of lupulone, 5 ng/ml of VEGF did not significantly modify the basal growth of HUVECs, while the same concentration of bFGF enhanced the proliferation 1.6 fold (Figure 2). Lupulone (7.5 µg/ml) induced a $60\% \pm 10\%$ inhibition of intrinsic proliferation and a reduction of $48\% \pm 8\%$ of bFGF-induced proliferation at day 6 after addition of lupulone (mean ± SEM, $p < 0.01$). The presence of VEGF did not significantly alter the inhibitory effects of lupulone. Also, increasing the concentration of both growth factors to 10 ng/ml did not modify these results (data not shown).

Cell migration assay. We next investigated the ability of lupulone to affect HUVEC migration (Figure 3). Lupulone (5 to 30 µg/ml) caused a dose-dependent inhibition of cell chemotaxis toward FN. Up to 10 µg/ml lupulone inhibited the migration by $30\% \pm 13\%$. This inhibition reached $61\% \pm 8\%$ for 20 µg/ml, and $78\% \pm 3\%$ for 30 µg/ml lupulone (mean ± SEM, $p < 0.001$).

Matrigel™ morphogenesis assay. The morphogenic assay on Matrigel™ assesses the ability of endothelial cells to differentiate into capillary-like structures. Lupulone (5 to 50 µg/ml) induced a dose-dependent reduction of the number of closed capillary-like structures (Figure 4a). At concentrations as low as 5 µg/ml, lupulone inhibited the formation of closed structures by $60\% \pm 30\%$ (mean ± SEM, $p < 0.01$). This inhibition was $78\% \pm 10\%$ at 30 µg/ml and no closed structures were formed at 50 µg/ml (Figure 4a E and

F; Figure 4b). DMSO at a concentration corresponding to the highest concentration of lupulone did not affect the formation of capillary-like structures (data not shown).

The in vivo Matrigel™ plug assay. The ability of lupulone to inhibit angiogenesis when administered to mice in the drinking water was tested. The lupulone-treated group (n=9) received lupulone (0.01%) plus 0.001% ascorbic acid (to prevent oxidation) in drinking water every day, starting 3 days before Matrigel injection and until the end of the experiment (3 weeks). The control group (n=9) identically received water containing the excipient (0.1% ethanol and 0.001% ascorbic acid). On average, each group consumed an amount of about 4 ml of water per day and body weight gain was identical in the different groups. The growth factor bFGF (500 ng) plus standard heparin (100 µg/ml) were included in the Matrigel plugs. These compounds induced new vessel generation which was quantified by measuring the haemoglobin content of the plugs. As shown in Figure 5, the angiogenic process was reduced by 50% in mice consuming 0.01% lupulone (20 mg/kg/day) over a period of 3 weeks.

Discussion

In the present paper we show that lupulone, a hop-derived bitter acid, can be considered as a new inhibitor of angiogenesis. The compound is able *in vitro* to inhibit endothelial cell basal and bFGF-promoted angiogenesis by approximately 50% at concentrations as low as 5 µg/ml. Lupulone reduced transmembrane cell migration stimulated by the extracellular matrix component fibronectin and dose-dependently hindered bi-dimensional capillary-like tube formation on Matrigel gels. In addition, lupulone administered daily in the drinking water to mice significantly inhibited new vessel formation in a Matrigel plug assay.

Angiogenesis represents an essential factor in tumorigenesis, both in primary tumor development and in metastatic spreading (17, 18). The physiological process of angiogenesis is the result of intricate steps regulated by the fine tuned balance between agonists and antagonists (19). Disturbance of this balance, due to pathologic conditions (such as cancer) leads to an 'angiogenic switch' which most often is a predictive sign of tumor progression.

Recently, some beer components (xanthohumol, humulone), especially those extracted from hop cones, have emerged as potential chemopreventive agents with angiostatic properties (3, 4, 10, 20-23). The present study demonstrates that another hop constituent (lupulone) already known for its antibiotic and sedating effects (24, 25) exhibit significant angiopreventive properties.

Hop acids are divided into α -acid (humulone) and β -acid (lupulone). They are present in hop cones at up to

25% by dry weight and have important bacteriostatic properties (26). Besides their important antibiotic properties these compounds have many biological activities related to cancer prevention. Humulone and lupulone have radical-scavenging and lipid peroxidation inhibitory activities (11). Surprisingly most studies have focused on the biological effects of humulone and very few data concerning the biological properties of lupulone are available (21). It was reported that humulone significantly prevented *in vivo* angiogenesis in chick embryo chorioallantoic membranes in a dose-dependent manner. It also inhibited *in vitro* capillary tube formation and proliferation of rodent vascular endothelial cells and diminished the production of the pro-angiogenic VEGF by these endothelial cells and by tumor cells (3).

We herein report that lupulone induced a dose-dependent inhibition of human endothelial cell proliferation. Furthermore, the compound inhibited cell proliferation induced by the angiogenic growth factor bFGF. In this model, VEGF exhibited only moderate effects since, compared to bFGF this growth factor remains a poor mitogen, at least for human endothelial cells *in vitro* (27). Transmembrane migration toward FN was also inhibited by 60% by lupulone at a concentration of 20 µg/ml. In a capillarogenic assay lupulone hindered significantly capillary-like tube formation by almost 60% at 5 µg/ml. Thus *in vitro*, lupulone is able to significantly inhibit various steps leading to the 'angiogenic switch' *i.e.* endothelial proliferation, migration and tube-like formation. Furthermore, lupulone administered to mice in the drinking water for three weeks was able to inhibit growth factor induced angiogenesis in a Matrigel plug assay. The angiogenic process was diminished by 50% in mice receiving 0.01% lupulone (20 mg/kg) daily in their drinking water. Lupulone-treated mice showed similar body weight gain and food consumption as controls over the whole experimental period. Furthermore, a dose of 150 mg/kg has been reported to be the no-observed-adverse-effect level (NOAEL) for hop acids in dogs, indicating that these substances have a wide safety margin (28). The amount of lupulone consumed daily per mice (20 mg/kg) would correspond to a daily amount of 60 mg/m² in man, a dose which might be compatible with its use in humans.

In conclusion, although pro-apoptotic mechanisms have been put forward for the chemopreventive effects of lupulone, according to experiments performed on colon-derived metastatic cells (12), our data show that inhibition of an angiogenic process by lupulone may also be involved and deserves to be further investigated.

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