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In vitro and *in vivo* antiangiogenic activity of carebastine: a plausible mechanism affecting airway remodelling

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ABSTRACT: Ebastine is a well-known selective, second-generation histamine H1-receptor antagonist, which is used for seasonal and perennial allergic rhinitis and chronic urticaria. Angiogenesis plays a crucial role in the development of airway inflammation and remodelling in allergic rhinitis and asthmatic patients in whom, indeed, the mucosa displays increased vascularity and overexpression of vascular endothelial growth factor (VEGF). Here we evaluated the antiangiogenic properties of carebastine, the active metabolite of ebastine.

The effects of carebastine were investigated *in vitro* on human umbilical vein endothelial cell (HUVEC) and human pulmonary artery endothelial cell (HPAEC) proliferation, migration and formation of capillary-like tubes, and *in vivo* in the chick embryo chorioallantoic membrane (CAM) assay. Moreover, the effect of carebastine on phosphorylation of the cell VEGF receptor foetal liver kinase-1 or receptor 2 (Flk-1/VEGFR-2) and Akt was evaluated by Western blotting.

Carebastine inhibited VEGF-induced HUVEC and HPAEC proliferation, migration and angiogenesis *in vitro* in a dose-dependent manner. Cell proliferation was inhibited by 42% and 64% in HUVECs and 62% and 75% in HPAECs upon a 48 h and 72 h exposure to carebastine 20 μM ($P < 0.03$ or better) and even more to 30 μM. Cell migration was inhibited by 37% and 70% in HUVECs ($P < 0.03$ or better) and by 60% and 78% in HPAECs ($P < 0.01$ or better) in the presence of 10 μM and 30 μM, respectively. Carebastine 20 μM exerted a significant lowering (-70% to -86%, $P < 0.01$) of topological parameters of the capillary network produced *in vitro* by both endothelial cell (EC) lines on a basement membrane extract. Carebastine 30 μM and 50 μM inhibited 2- and 3-fold respectively ($P < 0.001$) the VEGF-induced angiogenesis *in vivo* in the CAM assay. Finally, both EC lines exposed to carebastine 10 μM and 20 μM gave a 4- to 6-fold reduction ($P < 0.01$ or better) of both VEGF- and histamine H1-receptor-induced VEGFR-2 and Akt phosphorylation.

Overall data provide the first evidence on the antiangiogenic activity of ebastine, and suggest its potential use as an antiangiogenic molecule besides its antihistamine activity for the treatment of allergic diseases in which angiogenesis takes place.

Keywords: Allergic diseases; antiangiogenesis; asthma; carebastine; ebastine; endothelial cells.

INTRODUCTION

Histamine H1-receptor antagonists are commonly used for symptomatic relief in the treatment of hypersensitive reactions, such as allergic rhinitis, rhinoconjunctivitis and urticaria. The ability of antihistamines to inhibit histamine release by mast cells is well known, whereas their additional properties which contribute to their clinical efficacy are not completely understood. It has been reported that some antihistamines regulate the expression and/or release of cytokines, chemokines, adhesion molecules, and of other inflammatory mediators and, consequently, that they inhibit the recruitment of inflammatory cells [1].

The anti-inflammatory effect of H1-receptor antagonists may involve a receptor-dependent mechanism through the inhibition of secretion of NF-κB-dependent cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and hepatocyte growth factor (HGF), or the inhibition of adhesion molecules [2-4]. Others described a receptor-independent mechanisms involving the release of pre-formed mediators, such as histamine and eosinophil proteins, as well as production of eicosanoid and oxygen free radicals [5-7].

Ebastine (4-diphenylmethoxy-1 [3-(4-terbutyl-benzoyl)-propyl] piperidine) is a second generation, selective and potent H1-receptor antagonist with no anticholinergic or sedative effects, used for seasonal and perennial allergic rhinitis and chronic urticaria. After oral administration, it is rapidly converted into its active metabolite carebastine. It inhibits the secretion of interleukin-4 (IL-4), IL-5, IL-6, and tumor necrosis factor alpha (TNF-α) by inflammatory cells [8].

Recent findings indicate that angiogenesis (new vessel formation) is involved in both development of airway inflammation and pathophysiology of airway remodelling in the course of allergic rhinitis and asthma [9,10]. In fact, major structural and functional changes of the airway microcirculation include angiogenesis, increased blood flow and microvascular permeability, and

oedema formation in the airway wall [11]. Increased expression of angiogenic molecules, such as VEGF, FGF-2 and angiogenin and their receptors have been correlated with disease severity and accelerated decline of lung function [12-20]. Others showed that bronchoalveolar lavage (BAL) fluid from asthmatic patients exhibited enhanced *in vitro* angiogenic activity and increased FGF-2 [21] and VEGF levels [22]; the BAL angiogenic activity was blocked by an anti-VEGF antibody [22]. Also, VEGF levels in the BAL fluid of asthmatic patients were increased in proportion to the vessel number in the bronchial mucosa which, indeed, showed intense VEGF, VEGFR-1 and VEGFR-2 staining [23].

The direct contribution of VEGF in the allergic response in asthma has been proposed by Lee et al. [24] who generated lung-targeted $VEGF_{165}$ transgenic mice, and evaluated the role of VEGF in antigen-induced T_H2-mediated inflammation. Overexpression of VEGF induced angiogenesis, oedema, vascular remodelling, leukocyte infiltration in the lung, collagen deposition, overexpression of IL-13 (which promotes the collagen deposition into the basement membrane), increase of mucus production, and smooth muscle cell hyperplasia.

Here we have investigated the antiangiogenic activity of carebastine by using *in vitro* and *in vivo* assays. The former include human umbilical vein endothelial cell (HUVEC) and human pulmonary artery endothelial cell (HPAEC) proliferation, migration and formation of capillary-like tubes; the latter is the chick embryo chorioallantoic membrane (CAM) assay. Moreover, the effect of carebastine on cell VEGF receptor-2 (VEGFR-2) and Akt phosphorylation was evaluated by Western blotting.

MATERIALS AND METHODS

Reagents

Carebastine was kindly supplied by Almirall (Barcelona, Spain). Recombinant human $VEGF_{165}$, histamine and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Milwaukee, WI). Foetal Bovine Serum (FBS) was from Gibco® (Invitrogen Corporation, Carlsbad,

CA), anti-human VEGF receptor-2 (VEGFR-2/KDR) neutralizing monoclonal antibody (mAb) from R&D Systems Inc. (Minneapolis, MN), mepyramine (inhibitor of histamine H1-receptor) from Sigma Chemical Co. (St. Louis, MO), antibodies against VEGFR-2, Akt and phospho-Akt (Ser473) from Cell Signaling Technology (Danvers, MA), against phospho-VEGFR-2 (Y1054) from Abcam (Cambridge, UK), against β-actin, and goat IgG horseradish peroxidase-conjugated from Santa Cruz Biotechnology (Santa Cruz, CA), against mouse and rabbit IgG horseradish peroxidase-conjugated from Bio-Rad (Bio-Rad, Hercules, CA), MTT Cell Proliferation Assay from American Type Culture Collection (Manassas, VA).

Cell culture and *in vitro* functional assays

HUVECs were purchased from American Type Culture Collection (ATCC) (Manassas, VA), and HPAECs were from Lonza (Walkersville, MD). Both cell lines were cultured in endothelial cell (EC) growth EBM-2® medium supplemented with SingleQuots® (Lonza) and 10% FBS (complete medium) in a 5% CO_2 atmosphere at 37°C.

HUVEC and HPAEC proliferation was evaluated as previously described [25]: 5 x 10^4 cells/well in 96-well plates were cultured for 24, 48 and 72 h in starvation serum-free medium (SFM - negative control) or complete medium admixed with $VEGF_{165}$ 10 ng/mL (positive control) or supplemented with carebastine 5 to 30 μM in n. 5 independent experiments performed in triplicate. Cells were counted by the MTT assay, and expressed as mean percentage of control ± 1 standard deviation (SD). The EC number was also estimated by a slightly modified [25] crystal violet colorimetric method [26]. No aspecific cytotoxic effects (nuclear pyknosis, cytoplasmic vescicles and granules, round cell shape, cell detachment from supports) were seen in both HUVECs and HPAECs with carebastine even at 30 μM.

The chemotaxis assay was done in Boyden microchambers as previously described [27] towards SFM alone (negative control) or admixed with $VEGF_{165}$ 10 ng/mL alone (positive control)

or together with the carebastine doses in n. 5 independent experiments performed in triplicate. Migrated cells after 4 h were counted on x1,000 five oil-immersion fields/membrane, and given as mean percentage ± 1 SD.

Angiogenesis *in vitro* assay

HUVECs and HPAECs (1 x 10^5) were plated in 24-well plates precoated with CULTREX® Basement Membrane Extract (400 µL/well; Trevigen Inc., Gaithersburg, MD) in 1 mL/well of SFM alone (negative control) or in complete medium supplemented with $VEGF_{165}$ 10 ng/ml alone (positive control) or together with carebastine doses in n. 5 independent experiments performed in duplicate, and incubated in a 5% CO_2 atmosphere at 37°C. After a 18 h-incubation, tube and mesh formation were examined under a reverted, phase-contrast microscope (Leitz DM IRB, LEICA Microsystems GmbH, Wetzlar, Germany). Then, the skeletonization of the mesh was followed by measurement of its topological parameters, i.e. *i*) number of "areas", *ii*) "vessel length", and *iii*) number of "branching points" with a computed image analysis as previously described [28], and given as mean ± 1 SD of percent inhibition *vs.* the positive control. Representative fields were photographed using a Leica D-Lux3 digital camera (LEICA Camera AG, Solms, Germany).

CAM assay

This was performed on fertilized White Leghorn chicken eggs incubated at 37°C at constant humidity as previously described [29]. On the incubation day 3 a square window was opened in the shell and 2 to 3 ml of albumen were removed to detach the CAM. At day 8, the CAMs were implanted with 1 mm^3 sterilized gelatin sponges (Gelfoam Upjohn, Kalamazoo, MI) loaded with 1 µl of medium alone (negative control) or added with $VEGF_{165}$ 250 ng alone (positive control) or combined with 30 µM or 50 µM carebastine in n. 5 independent experiments for each dose and control performed in duplicate. No death of the embryo was seen even with 50 µM. The angiogenic

response was evaluated at day 12 as the number of vessels converging toward the sponge at x50, and photographed *in ovo* under an Olympus stereomicroscope (Olympus, Rozzano, Italy).

Western Blotting

HUVECs and HPAECs were grown in 6-well plate dishes in SFM containing 0.1% bovine serum albumin for 18-20 h. Then cells were exposed to $VEGF_{165}$ 10 ng/ml alone or combined with histamine 30 μM, followed by carebastine 10 and 20 μM, and lysed with a buffer containing Tris-HCl 25 mM, NaCl 150 mM, EDTA 1 mM, Triton X-100 1%, SDS 0.1%, NaF 1 mM, $Na_3VO_4$1 mM, and Protease Inhibitor Cocktail (Sigma-Aldrich). Total protein concentrations were determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad). Aliquots (30 μg protein) of the cell lysate were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using pre-cast 4-12% NuPAGE® Novex® Bis-Tris Gels (Invitrogen Corp.) under reducing conditions, electrotransferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences Inc., Boston, MA), incubated with the primary and secondary antibodies. Then bands were visualized by enhanced chemiluminescence (SuperSignal® West Pico substrate, Thermo Scientific Inc., Waltham, MA) using the Gel Logic 1500 Imaging System (Eastman Kodak Co., Rochester, NY), and their intensity quantified with the Kodak Molecular Imaging Software, and expressed as arbitrary optical density (OD) units $\pm$ 1 SD of n. 5 independent experiments.

For inhibition experiments, ECs were treated with a neutralizing anti-VEGFR-2 mAb 5 μg/ml for 24 h, then sequentially exposed to histamine 30 μM for 10 min and carebastine 10 μM for 1 h. In separate experiments, ECs were treated with mepyramine (inhibitor of histamine H1-receptor) 20 μM for 1 h, then sequentially exposed to $VEGF_{165}$ 10 ng/ml for 10 min and carebastine as above. The dose of mepyramine was chosen on the basis of a molar excess *vs.* carebastine. Cells were lysed and processed for Western blotting.

RESULTS

Carebastine inhibits *in vitro* HUVEC and HPAEC proliferation, chemotaxis and angiogenesis

The HUVEC and HPAEC proliferative response to $VEGF_{165}$ was significantly reduced by carebastine in a dose-dependent fashion, being maximal after a 72 h exposure to 30 μM in both HUVECs (-65%) and HPAECs (-80%; Fig. 1A, B). A significant lowering of cell proliferation was also achieved with 20 μM after both 48 h (HUVECs -42%, HPAECs -62%, $P < 0.03$ or better; 2-way ANOVA test) and 72 h (HUVECs -64%, HPAECs -75%; $P < 0.01$ or better) with an IC_{50} of 28.7 ± 6.9 μM and 16.5 ± 4.7 μM, respectively. These results were obtained with the MTT method and confirmed with the crystal violet colorimetric method (Supplementary Fig. 1). Carebastine exerted an inhibitory effect on the $VEGF_{165}$-induced HUVEC and HPAEC migration in the chemotaxis assay (Fig. 1 C, D). The cell migration was respectively inhibited by 12% and 45%, 37% ($P < 0.03$; 1-way ANOVA test) and 60% ($P < 0.01$), 46% and 68% ($P < 0.01$), 70% and 78% in the presence of 5, 10, 20 and 30 μM.

Carebastine significantly inhibited the HUVEC and HPAEC angiogenesis *in vitro* on the basement membrane extract. As shown in Fig. 2, after a 18-h incubation $VEGF_{165}$-exposed HUVECs produced a closely knit capillary network with thin, branching, and anastomosing tubes linked through numerous junctions: mesh area counts were 31.2 ± 7, length 6814 ± 618 μm, branching point counts 37 ± 5. Exposure to carebastine at 1 μM and even more to 20 μM gave rise in a dose-dependent manner to a poorly organized plexus with few straight and disorganized tubes with scarce junctions: mesh areas 19.6 ± 4.2 (-37%) and 6.3 ± 2.8 (-79%) respectively; vessel length 4225 ± 313 (-38%) and 1135 ± 126 μm (-83%); branching points 24 ± 4 (-37%) and 11 ± 3 (-70%) ($P < 0.01$ or better; Student's *t* test for paired data). Overlapping changes were observed in HPAEC capillary network following exposure to 20 μM: -82% of mesh areas, -86% of vessel length, and -75% of branching points (not shown).

Carebastine inhibits angiogenesis *in vivo* in the CAM assay

CAMs treated with sponges loaded with $VEGF_{165}$ (positive control) were surrounded by allantoic vessels as newly-formed capillaries converging radially toward the sponge in a "spoked-wheel" pattern (number of vessels = 27 ± 3, Fig. 3B). No vascular response was detectable around the sponges loaded with vehicle alone (number of vessels = 6 ± 2, Fig. 3A). When carebastine 30 μM and 50 μM (Fig. 3C) was added to $VEGF_{165}$, a significant reduction of the angiogenic response was found (number of vessels = 14 ± 2 and 8 ± 2 respectively; $P < 0.01$ or better; Student's *t* test for paired data).

Carebastine inhibits VEGF-induced phosphorylation of VEGFR-2 and of Akt

In vivo experiments provided evidence that carebastine exerts an inhibitory effect on the VEGF-induced angiogenic response. To determine whether carebastine was able to inhibit VEGFR-2 and Akt phosphorylation, Western blotting experiments were performed in $VEGF_{165}$-treated HUVECs. As shown in Fig. 4A, carebastine 10 μM blocked markedly the VEGF-dependent phosphorylation of VEGFR-2 (Y1054) 30 min after treatment, and the inhibitory effect was still detectable after a 24-h exposure to the drug. Moreover, the inhibition of receptor phosphorylation was correlated with a marked decreased in the ability of VEGF to induce Akt phosphorylation under the same experimental conditions. As shown in Fig. 4B and 4C, carebastine 10 and 20 μM inhibited significantly the VEGF-induced phosphorylation of both VEGFR-2 and Akt in a dose-dependent manner, and consequently blocked the HUVEC survival.

Carebastine antagonizes histamine signalling pathway mediated by histamine H1-receptor in HUVECs and HPAECs

To verify whether histamine H1-receptor was involved in the activation of VEGFR-2 and Akt, we investigated the effect of histamine treatment on protein phosphorylation. Western blotting

analysis showed that stimulation of both ECs with $VEGF_{165}$ followed by histamine produced rapid (after a 10-min exposure) and sizeable increase in the phosphorylation of VEGFR-2 and Akt. When carebastine 10 and 20 μM was added, it significantly blocked the histamine-stimulated phosphorylation of both VEGFR-2 and Akt in HUVECs (Figs. 5A,C,E) and HPAECs (Figs. 5B,D,F), demonstrating that inhibition of the VEGFR-2 and Akt pathways by carebastine in both EC types is mediated by the blocking of the histamine H_1-receptor activity.

To investigate whether carebastine was able to inhibit VEGFR-2 phosphorylation via a simultaneous interference with histamine H1-receptor and VEGFR-2, we performed inhibition experiments with a neutralizing anti-VEGFR-2 mAb and, separately, with mepyramine, an inhibitor of histamine H1-receptor. As shown in Fig. 6, the blockade of VEGFR-2 produced marked inhibition of VEGFR-2 phosphorylation in histamine-stimulated HUVECs and HPAECs. Also, a slight inhibition was observed in $VEGF_{165}$-stimulated cells blocked in the histamine H1-receptor with mepyramine and exposed to carebastine. Data suggest that the direct inhibitory activity of carebastine is mediated mostly by the histamine H1-receptor and, to a lesser extent, by VEGFR-2.

DISCUSSION

Enhanced angiogenesis is involved in many aspects of tissue inflammation and remodelling, and contributes to abnormal airway function including increased mucosa reactivity to agents in allergic rhinitis and fixed airflow obstruction with progressive decline of lung function in asthma [9, 10]. The complex network between allergic inflammation and angiogenesis and the limited benefit of the classical therapeutic approaches of airway remodelling in inflammatory airway diseases, such as allergic rhinitis and asthma, have led us to consider the use of angiogenesis inhibitors to control inflammation, angiogenesis, and remodelling in allergic disorders.

Herein, we have demonstrated for the first time that ebastine, a well known selective and potent H1-receptor antagonist widely used for seasonal and perennial allergic rhinitis and chronic urticaria, exerts a potent antiangiogenic activity as demonstrated by *in vitro* and *in vivo* assays.

Experiments performed by using the *in vivo* CAM assay have provided evidence that carebastine (the active metabolite) exerts an inhibitory effect on the VEGF-induced angiogenic response. These results have been confirmed by Western blotting, indicating that the inhibition of VEGFR-2 phosphorylation by carebastine interferes with the signalling pathways triggered by VEGF. Moreover, the inhibition of receptor phosphorylation was correlated with a marked decreased in the ability of VEGF to induce Akt activation under the same experimental conditions. Finally, carebastine was also able to significantly block histamine-stimulated phophorylation of both VEGFR-2 and the downstream Akt, suggesting that the inhibition of VEGFR-2 and Akt pathways is mediated through the blocking of histamine H1-receptor activity. In separate inhibition experiments of VEGFR-2 and histamine H1-receptor, we found that carebastine inhibited VEGFR-2 phosphorylation mostly via interference with histamine H1-receptor. It thus may be well that signallings downstream the histamine H1-receptor and VEGFR-2 are closely interrelated, perhaps via Akt. Also, we found that carebastine inhibited, albeit to a lesser extent, the VEGFR-2 phosphorylation after the blockade of histamine H1-receptor. Thus, an interference of carebastine with VEGFR-2, perhaps via aspecific mechanisms, can be hypothesized. Overall data suggest that the antiangiogenic activity of carebastine is mediated through both a histamine H1-receptor-dependent and -independent mechanism, though the former is prevalent.

In addition, it is conceivable to hypothesize that the histamine H1-receptor signalling affected by carebastine activates protein kinase C (PKC) instead of PKA, since a PKC activator (phorbol-12-myristate-13-acetate) enhances H1-receptor activation, whereas an activator of PKA does not [30]. Furthermore, histamine-induced activation of H1-receptor is fully suppressed by the PKC inhibitor Ro-31-8220 [31]. Down-regulation of NF-κB may be also involved in the mechanism of action of carebastine. In fact, low concentrations of H1-receptor antagonists (cetirizine and azelastine) have been demonstrated to down-regulate NF-κB expression in parallel to inhibition of pro-inflammatory cytokines [7].

VEGF seems to be the most important factor involved in angiogenic processes taking place in allergic rhinitis [9] and asthma [10], and plays an important role in the disease development and persistence. In fact, both VEGF and expression of its receptor VEGFR-2 are increased in the nasal mucosa of patients with allergic rhinitis when compared with the non-allergic controls [12]. Moreover, in the bronchial mucosa of asthmatic patients the number of VEGF-positive cells is significantly increased and correlated to the vessel counts and vascular area, and the expression of VEGFR-1 and VEGFR-2 is inversely correlated with the airway function [16- 20, 24]. VEGF levels in BAL were increased in asthma in direct proportion to vascularity [19,20]. Also, through its permeability activity VEGF contributes to oedema since favours microvascular leakage. VEGF is secreted by several inflammatory cells, such as macrophages, mast cells and eosinophils which intervene in the composition of the inflammatory infiltrate in allergic rhinitis and asthma [12, 32-35]. It has been demonstrated [36] that airway smooth muscle cells from patients with mild or moderate asthma, but not from healthy (control) subjects, promote angiogenesis *in vitro.* This pro-angiogenic capacity resides in elevated VEGF release and suggests that airway smooth muscle cells regulate airway neovascularization in asthma.

In view of these findings, new clinical strategies aiming to downregulate VEGF and its receptors and signallings might be of benefit in the treatment of allergic diseases. Tentatively, our data providing the first evidence of the antiangiogenic activity of ebastine suggest its potential use as an antiangiogenic molecule besides its antihistamine activity in the treatment of allergic diseases in which angiogenesis occurs.

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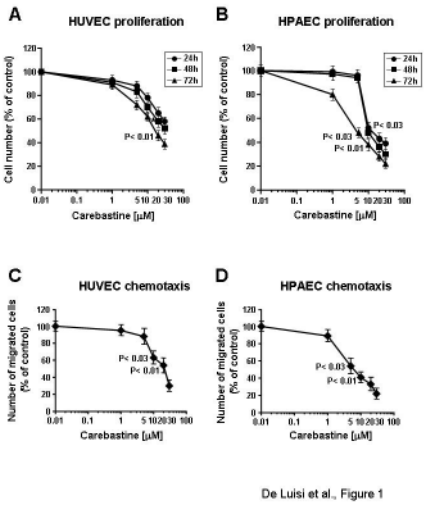
LEGENDS

Figure 1. **Effect of carebastine on proliferation and migration of endothelial cells.** Effects of different carebastine doses on $VEGF_{165}$-induced cell proliferation of HUVECs (A) and HPAECs (B) at 24, 48 and 72 h. Data are expressed as the mean percentage of control $\pm$ 1 SD of n. 5 independent experiments performed in triplicate. Effects of the same doses on $VEGF_{165}$-induced chemotaxis of HUVECs (C) and HPAECs (D). Both cell types were plated on a collagen-coated polycarbonate membrane in a Boyden microchamber assay, and exposed to $VEGF_{165}$ and carebastine at the indicated doses for 4 h. Data are expressed as mean percentage $\pm$ 1 SD of migrated cells of n. 5 independent experiments performed in triplicate. Significance by 2-way ANOVA test. Asterisc = $P < 0.03$.



De Luisi et al., Figure 1

Figure 2. **Effect of carebastine on *in vitro* angiogenesis**. HUVECs were seeded on CULTREX® in serum-free medium (SFM), in SFM supplemented with $VEGF_{165}$ alone or together with the carebastine doses. Tube formation was observed periodically under a phase contrast microscope and pictures were taken after a 18-h incubation. Original magnifications: x50. Dots in line: mean

percentage of inhibition of the indicated topological parameters ± 1 SD in n. 5 independent experiments performed in duplicate. Pictures refer to a representative experiment.

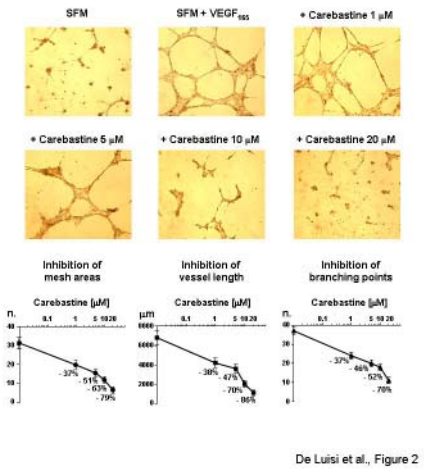


Figure 3. **Effect of carebastine on *in vivo* angiogenesis (CAM assay)**. CAM were implanted with sponges loaded with SFM alone (A) or with $VEGF_{165}$ (B) or with $VEGF_{165}$ and carebastine 50 µM (C). VEGF induces a strong angiogenic response in the form of newly-formed capillary vessels, whereas no vascular response was detectable around the sponges loaded with SFM. Carebastine inhibits the $VEGF_{165}$-induced angiogenic response. Representative pictures of n. 5 independent experiments performed in duplicate for the carebastine dose and controls. Original magnifications: A-C, x50.

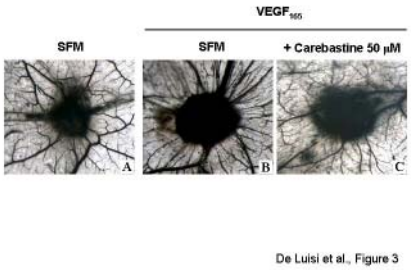


Figure 4. **Western blotting of the effect of carebastine on phosphorylation of VEGFR-2 and Akt.** (A) Time-course of the phosphorylation of VEGFR-2 (pVEGFR-2) and of Akt (pAkt) in response to carebastine. HUVECs were synchronized in SFM for 18-20 h, then treated with $VEGF_{165}$ and carebastine 10 μM for 24 h. Samples were subsequently analyzed on a separate blot using antibodies to unphosphorylated (total) VEGFR-2 and Akt to confirm equal loading in each lane. (B) Dose-dependence of the inhibitory effect on VEGFR-2 and Akt phosphorylation by carebastine 10 μM and 20 μM. Lanes shown are representative of n. 5 independent experiments. (C) Densitometric analysis performed with the Kodak Molecular Imaging Software, and expressed and averaged ± 1 SD (columns and bars) as arbitrary optical density (OD) units. Significance by 1-way ANOVA test.

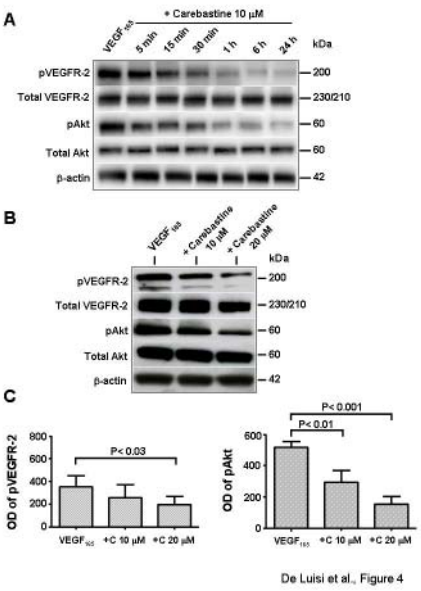


Figure 5. Western blotting of the effect of carebastine on VEGFR-2 and Akt phosphorylation mediated by histamine H1-receptor. (A), (B) Increased VEGFR-2 and Akt phosphorylation induced by histamine 30 μM, and inhibition of histamine H1-induced phosporylation by carebastine 10 μM and 20 μM in HUVECs (A) and HPAECs (B). Cells were synchronized in SFM for 18-20 h, then treated with $VEGF_{165}$ and histamine 30 μM alone or added with carebastine 10 μM or 20 μM. Samples were subsequently analyzed on a separate blot using antibodies to unphosphorylated (total) VEGFR-2 and Akt to confirm equal loading on each lane. Pictures shown are representative of n. 5 independent experiments. (C) to (F) Densitometric analysis performed with the Kodak Molecular Imaging Software, and expressed and averaged ± 1 SD (columns and bars) as arbitrary optical density (OD) units. Significance by 1-way ANOVA test.

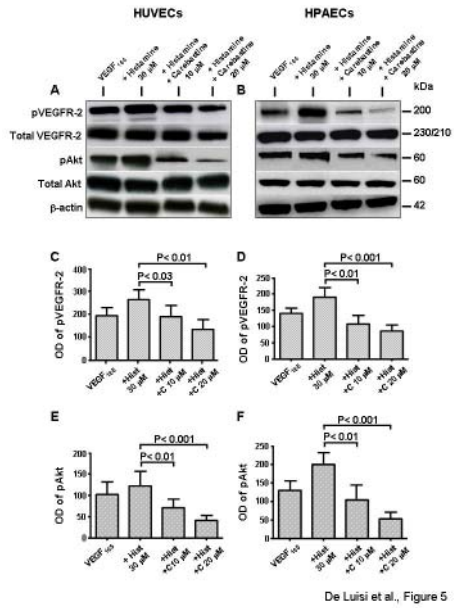
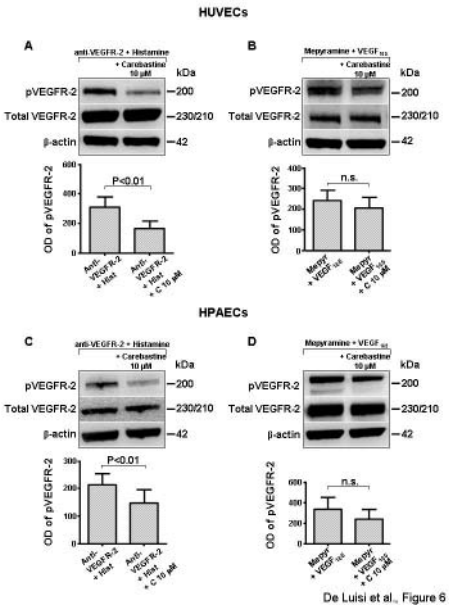


Figure 6. Separate blockade in HUVECs and HPAECs of VEGFR-2 and histamine H1-receptor by a neutralizing anti-VEGFR-2 antibody and mepyramine, respectively. Notice that the highest inhibition of VEGFR-2 phosphorylation (pVEGFR-2) in both HUVECs (A) and HPAECs (C) is given by interference of carebastine with the histamine H1-receptor; in contrast (B) (D), the interference with VEGFR-2 produces minimal inhibitory effects. Pictures shown are representative of n. 5 independent experiments. Densitometric analysis performed with the Kodak Molecular Imaging Software, and expressed and averaged ± 1 SD (columns and bars) as arbitrary optical density (OD) units. Significance by Student's *t* test.



De Luisi et al., Figure 6