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Research Article

CIGB-300, a proapoptotic peptide, inhibits angiogenesis in vitro and in vivo☆

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ABSTRACT

We have previously demonstrated that a proapoptotic cyclic peptide CIGB-300, formerly known as P15-Tat delivered into the cells by the cell-penetrating peptide Tat, was able to abrogate the CK2-mediated phosphorylation and induce tumor regression when injected directly into solid tumors in mice or by systemic administration. In this work, we studied the role of CIGB-300 on the main events that take place in angiogenesis. At non-cytotoxic doses, CIGB-300 was able to inhibit adhesion, migration, and tubular network formation induced by human umbilical vein endothelial cells (HUVEC) growing upon Matrigel in vitro. Likewise, we evaluated the cellular penetration and localization into the HUVEC cells of CIGB-300. Our results confirmed a quick cellular penetration and a cytoplasmic accumulation in the early minutes of incubation and a translocation into the nuclei beginning at 12 h of treatment, with a strong presence in the perinuclear area. A microarray analysis was used to determine the genes affected by the treatment. We observed that CIGB-300 significantly decreased four genes strongly associated with tubulogenesis, growth, and differentiation of endothelial cells. The CIGB-300 was tested in vivo on chicken embryo chorioallantoic membranes (CAM), and a large number of newly formed blood vessels were significantly regressed. The results suggested that CIGB-300 has a potential as an antiangiogenic treatment. The mechanism of action may be associated with partial inhibition of VEGF and Notch pathways.

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Introduction

CK2 is a highly conserved protein serine/threonine kinase that is ubiquitously distributed in eukaryotes, constitutively active, and has been implicated in multiple cellular functions, as well as in tumorigenesis and transformation. Elevated CK2 activity has been associated with the malignant transformation of several tissues and is associated with aggressive tumor behavior [1]. Previously, we have

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described a peptide CIGB-300 (also named P15-Tat) targeting the acidic phosphorylation domain of the CK2 substrates. Different groups around the world have tried to manipulate this biochemical event by targeting the ATP-binding site of CK2 or its gene transcription using antisense oligonucleotides [2,3]. Otherwise, CIGB-300 peptide was developed following the innovative approach of targeting the phosphoacceptor site on the CK2 substrates rather than the enzyme per se. We demonstrated that CIGB-300 induced apoptosis in tumor cells and tumor regression when injected directly into solid tumors or by systemic administration in mice [4,5]. Preliminary studies showed a decrease in the vascularization of TC-1 tumors grown in C57BL6 treated daily for 5 days with 200 μL of 0.24 mmol/L CIGB-300 [4]. In order to see if this property may be part of their antitumor activity, we tested CIGB-300 in its capability to influence endothelial behavior. Here, we demonstrated that this peptide binder to the CK2 phosphoacceptor site could exhibit specific antiangiogenic properties on endothelial cells. We have focused in the exploration of CIGB-300 properties to inhibit proliferation, tube formation, and RNA pattern expression on endothelial cells (HUVEC). Likewise, we used the chicken chorioallantoic membrane (CAM) assay as a model in vivo to asses the CIGB-300 activity on angiogenesis. Other authors have studied the role of different CK2 inhibitors on the angiogenesis finding that CK2 is involved in endothelial cell proliferation, survival, migration, and tube formation [6]. Intraperitoneally administered CK2 inhibitors significantly reduced preretinal neovascularization in a mouse model of proliferative retinopathy [7]. Solid tumors require the growth of new blood vessels (angiogenesis) to grow. Tumor angiogenesis utilizes at least some of the angiogenic signaling pathways that are required during vascular development [8]. Tumor angiogenesis has become an important target for antitumor therapy, with most current therapies aimed at blocking the VEGF pathway. However, not all tumors are responsive to VEGF blockers, and some of them that are responsive initially may become resistant during the course of treatment; thus, there is a need to explore other angiogenesis signaling pathways [9].

In this paper, we have examined the antiangiogenic properties of CIGB-300 peptide in vitro and in vivo. The data suggest that CK2 is involved in angiogenic processes and CIGB-300 could act as promising antiangiogenic inhibitor.

Materials and methods

Peptide synthesis

The peptide CIGB-300 used in this work was synthesized as previously described [4].

Endothelial cells and culture conditions

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza Group Limited (Valais, Switzerland). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, HUVEC from passages 2 to 7 were used and cells were incubated in endothelial basal medium plus EGM SingleQuots® from Lonza Group Limited (Valais, Switzerland) including 0.1% human recombinant epidermal growth factor, 0.4% bovine brain extract, 0.1% hydrocortisone, and 0.1% GA-1000 (EGM) and 2% FBS.

Cell viability and proliferation assay

HUVEC cells (1×10^4 cells per well of sextupled 96-well plates) were incubated in endothelial basal medium plus EGM SingleQuots® from Lonza Group Limited (Valais, Switzerland). The treatments were added 24 h after seeding. Control cultures received medium with the inhibitor vehicle, PBS (DMSO in the case of ellagic acid). The number of cells was determined on days 1 and 3 using the MTS cell-proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Data are representative of five independent experiments.

Cytostatic activity of CIGB-300

The human endothelial cells HUVEC, in exponential growth phase, were removed from the flasks with 0.05% trypsin–0.02% EDTA solution. Cells were seeded in 24-wells per plate 2×10^5 in EGM medium. Cells were allowed to attach overnight. The next day, medium was removed and cells were incubated in fresh medium for 72 h, with increasing concentrations of CIGB-300. Six representative fields per well were photographed using phase contrast microscopy. Each experiment was carried out in triplicate. Growth inhibition was calculated from the formula: cytostasis (%) $= [1 - (A/B)] \times 100$ [10].

Intracellular kinetics of CIGB-300

Endothelial cells treated with biotin-CIGB-300 grown on glass coverslips were washed 3 times with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100/PBS for 1 min at room temperature. Cells were incubated with 50 mM NH_4Cl for 5 min, washed with PBS, and incubated in a solution of 3% bovine serum albumin (BSA) in PBS for 15 min. Cells were then exposed to FITC-streptavidin (Dako Cytomation) at 37 °C for 1 h. After 3 washes in PBS, using glass coverslips, cells were mounted in 80% glycerol containing 1 mg/mL of paraphenylenediamine in 0.2 mol/L of Tris–HCl buffer (pH 8.5) and analyzed using a Nikon TE2000 inverted fluorescent microscope (Nikon, Japan) and microphotographs were taken with a digital camera Nikon DS-5 M. Images acquired with a 100× objective were processed using NIS-Elements 3.0 software (Nikon, Japan).

Cell adhesion assay

Twenty-four-well culture plates were coated with Matrigel as previously described. HUVEC cells were subsequently harvested and treated with CIGB-300 or PBS. 5×10^4 cells were plated on pre-coated wells. Cells were left to adhere for 2 h at 37 °C, then the wells were washed with PBS for 3 times and subsequently fixed with 4% paraformaldehyde for 30 min. At last, cells were observed and photographed by using a phase contrast microscope (Nikon, Japan). Cells were counted and the results showed represent the mean of three independent experiments performed in duplicates. $^{**}P < 0.01$ (ANOVA followed by Dunnett's test).

Endothelial cell migration assay

The effect of CIGB-300 and ellagic acid on cell migratory activity was examined by scratch–wound assay. 5×10^5 cells were seeded on a 12-well plate and were cultured in EGM SingleQuots® basal

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medium until they were completely confluent. One linear scar was drawn in the monolayer by a yellow tip. Wound closure or cell migration was photographed when the scrape wound was made and at designated times after wounding. Microphotographs were taken with a digital camera Nikon DS-5M. Cells were counted between the initial and the remaining wound area and the wound closure was expressed as a percentage of migrating cells compared to the control. $^{**}P<0.01$ (ANOVA followed by Dunnett's test).

Endothelial cord formation assay

The capacity of endothelial cells to form a tubular network was evaluated in vitro, as reported [11]. Briefly, Matrigel from Becton Dickinson (Bedford, MA) was placed in 24-well tissue culture plates (150 μL/well) and allowed to gel at 37 °C for 30 min. HUVEC (2.5×10^4 cells per well) were seeded and cultured in the presence or absence of appropriate concentrations of CIGB-300 peptide or ellagic acid for 20 h. Endothelial cord formation was photographed at ×100 in an inverted microscope (Nikon, Japan), and the number of endothelial ramifications was quantified. Tube formation was documented using an inverted phase contrast microscope and pictures were captured with a Nikon digital camera and stored in a computer system.

Early apoptosis assessment assay

In early apoptosis assessment by Annexin V staining, cells treated or not with CIGB-300 peptide were incubated in a 24-well tissue culture plate with individual cover glasses for 24 h. We included a positive control group treated with paclitaxel 10 nM [12]. Subsequently, cells were washed twice with PBS and 500 μL of Binding Buffer 1X were added. Cells were incubated with Annexin V-FITC (Molecular Probes, Oregon, USA) according to the manufacturer's instructions for 15 min at room temperature in the dark; afterwards, cells were washed with Binding Buffer and fixed for 10 min with formalin 3% in PBS. Finally, the cover glasses were deposited onto slides, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame CA), and the stained cells were visualized using an inverted fluorescence microscope Eclipse TE2000 (Nikon, Japan).

Morphologic identification of apoptotic cells by fluorescence microscopy using acridine orange/ethidium bromide staining

Cells were seeded and treated as described for the Annexin V assay. Apoptosis was quantified with a fluorescent staining method that consists in the detection of condensed nuclei using Acridine orange (AO) and ethidium bromide (EB) with a fluorescence microscope. This staining method is able to identify alive or necrotic cells as well as discern between early and late apoptotic cells. AO/EB (Sigma, USA) at 10 μg/mL in PBS was added to cells for 1 min at room temperature. Staining medium was aspirated and replaced with 0.5 mL of PBS. Cells were photographed on a fluorescence microscope (TE2000; Nikon, Japan). Apoptotic morphological cells were counted in ten visual fields of two independent experiments. The percentage of apoptotic cells was calculated by adding the total number of apoptotic living (AL) and apoptotic dead (AD) cells, dividing it by the total number of cells and multiplying it by 100. The percentage of calculated apoptotic cells was as follows: apoptosis % = (VA + NVA) / total cell count × 100%, where VA is viable apoptotic cell and NVA is non-viable apoptotic cell [13].

Microarray expression analysis

Total RNA was isolated from HUVEC cells treated or not with 50 μM CIGB-300 using Total RNA Isolation Kit (SuperArray Bioscience, Frederick, MD). Using a TrueLabeling-AMP Linear RNA Amplification Kit (SuperArray Bioscience, Frederick, MD), the mRNA was reverse-transcribed to obtain cDNA and converted into biotin-labeled cRNA using biotin-16-UTP (Roche, Mannheim, Germany) by in vitro transcription. Prior to hybridization, the cRNA probes

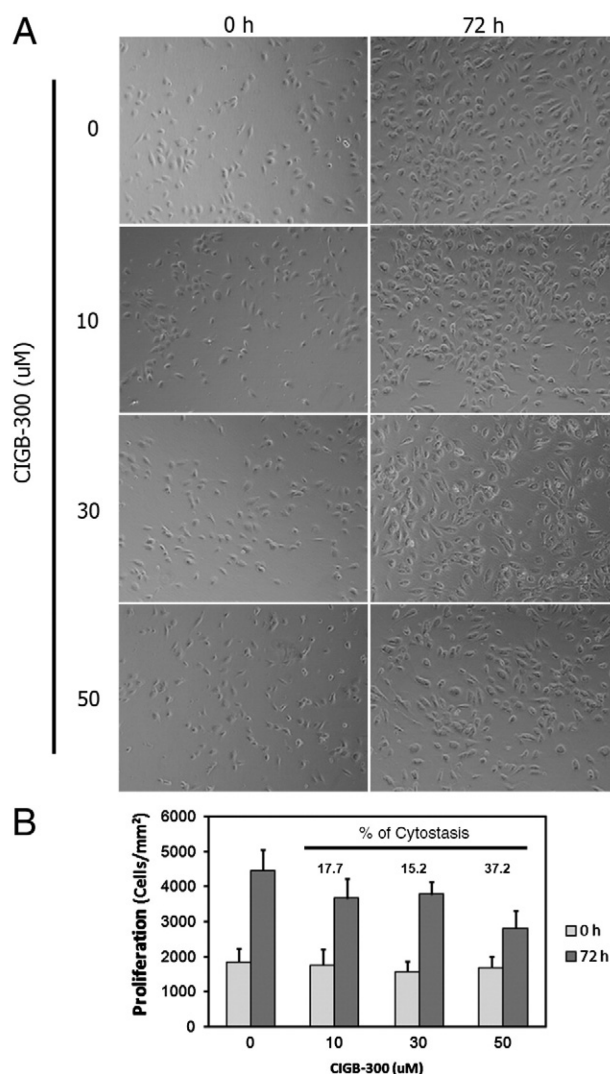

Fig. 1 – Cytostatic effect of CIGB-300 toward human venous endothelial cells. HUVEC cells were incubated in complete medium with increasing concentrations of CIGB-300.
A, Representative pictures of treatment were taken at 40× with a contrast phase microscope (Nikon). Photographic records were taken at the beginning (*t*, 0) and end (*t*, 72) of test.
B, Quantification of proliferation and cytostasis. Data were expressed as means ± SD, and values between bars represent % of cytostasis. Growth inhibition was calculated from the formula: cytostasis (%) = [1 − (A/B)] × 100 [10].

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were purified with the ArrayGrade cRNA Cleanup Kit (SuperArray Bioscience). The purified cRNA probes were then hybridized to pretreated Oligo GEArray Human Angiogenesis Microarray OHS-24 (SuperArray Bioscience), which covers 113 angiogenesis-related genes. Following several washing steps, array spots binding cRNA were detected using alkaline phosphatase-conjugated streptavidin and CDP-Star as a chemiluminescent substrate. Chemiluminescence was detected by exposing the array membranes to X-ray film. The image data were transformed into numerical data using GEArray Expression Analysis Suite software (SuperArray Bioscience). Using the same program and in order to obtain the significant changes induced by the CIGB-300 treatment, we processed 3 individual membranes in 3 independent experiments (3 control vs. 3 treated) and the changed genes were included when the significance of their variation was smaller than $P<0.05$. Data evaluation included background correction (subtraction of empty spots) and normalization by house-keeping genes [14].

Bioinformatic analysis

Proteins coding by genes down-regulated in HUVEC cells were analyzed with a network approach to reveal interactions with casein kinase 2. The protein–protein interaction network was constructed based on the information available in the Human Protein Reference Database (HPRD). This database also contains information about protein functions, posttranslational modifications, subcellular localization, and disease association of genes [15]. The network was visualized using Cytoscape version 2.6.0, an open-source software for analyzing molecular interaction networks and biological pathways [16].

CAM assay

Three-day-old fertilized eggs were incubated at 37 °C and 70%–80% humidity in a specific eggs chicken incubator (Yonar, Buenos Aires, Argentina). On day 4 of development, the air

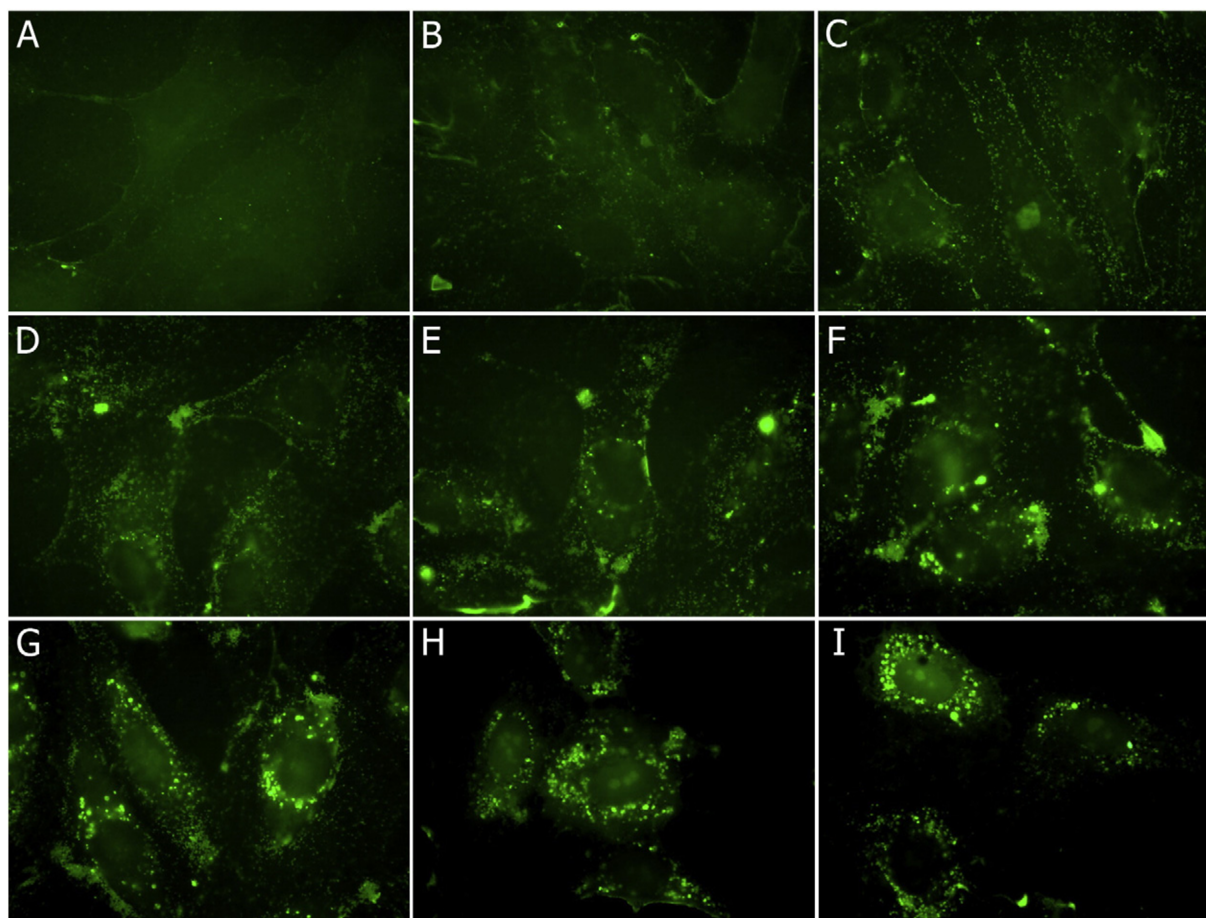

Fig. 2 – Intracellular localization of CIGB-300 peptide as a function of time by fluorescence microscopy in HUVEC cells. A rapid internalization of the peptide occurs in the first minutes. During all times evaluated, we see a strong cytoplasmic presence of CIGB-300 preferentially located in the perinuclear area. After twelve hours of incubation, a small mark in the cell nucleus can be observed. HUVEC cells were incubated with 50 µmol/L of CIGB-300 biotin for 1 min to 24 h, fixed, permeabilized, and incubated with the FITC-streptavidin-conjugated reagents (green). Image acquisitions were done using a Nikon TE2000 inverted fluorescent microscope (Nikon, Tokyo, Japan) and microphotographs were taken with a digital camera Nikon DS-5M. Images acquired with a 100× objective were processed using NIS-Elements 3.0 software (Nikon, Tokyo, Japan). The figure is composed of representative pictures taken from one of three independent experiments. a, 1 min; b, 5 min; c, 15 min; d, 30 min; e, 1 h; f, 2 h; g, 4 h; h,12 h; and i, 24 h.

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chamber was opened and a window of about 1 cm^2 was made into the egg. The window was sealed with cello tape and the eggs returned to the incubator until day 6 of development. The peptide was dissolved in distilled water and 20 µL of a solution of 3.2 mM was pipetted onto the Thermanox coverslips disks (Nunc, Naperville, IL). After air drying under laminar flow cabinet, the inverted disks were applied to the CAM of 6-day-old embryos and examined with a dissecting microscope (Nikon, Japan) after 5 days. The negative control was the vehicle, water. Experiments were repeated twice using a minimum of 10 eggs for each data point [17].

Results

Effects of CIGB-300 on cell viability of endothelial cells

The IC50 value for CIGB-300 peptide, evaluated by a 72-h and 24-h exposure to the compound, was 91 and 121 µM, respectively (data not shown). The cytostatic drug effect is shown in Fig. 1, demonstrating that doses between 10 and 50 µM of CIGB-300 peptide did not induce significant levels of cytostasis. Since further in vitro experiments were all performed treating HUVEC cells for up to 24 h, we used concentrations of CIGB-300 (10–50 µM) that were employed to achieve maximum levels of drug action but without evident signs of cytotoxicity and cytostasis.

In order to compare the response profile of CIGB-300 with other CK2 inhibitors, we evaluated the potential cytotoxic and anti-proliferative activities of ellagic acid, one of the most potent known inhibitors of CK2 [18]. We found an IC_{50} value of 20 and 53 µM for a 72- and 24-h exposure, respectively (data not shown). These results agree with those previously reported by Losso et al. [19].

Intracellular kinetics of CIGB-300. Cellular penetration and subcellular localization of proapoptotic peptide CIGB-300 in endothelial cells

To investigate the levels of cellular penetration and the subcellular localization of CIGB-300, we used a variant of CIGB-300 linked to biotin. We treated endothelial cells for different times from 1 min to 24 h. As shown in Fig. 2, we can see a rapid membrane interaction (1 min incubation) and internalization of CIGB-300 peptide into the endothelial cells. As early as 5 min after the treatment, we observed a clear cytoplasmatic localization pattern for CIGB-300. In all evaluated times, the cytoplasmatic label was very strong mainly in the perinuclear area. After 12 h of treatment, we observed also a small mark of CIGB-300 peptide within the nucleus of HUVEC cells. Similar results were found at lower doses (10 µM) (data not shown).

Adhesion assay on Matrigel

Adhesion of endothelial cells to the extracellular matrix is a prerequisite for new vessels to grow. We examined the effect of CIGB-300 peptide on HUVEC attachment to Matrigel. As shown in

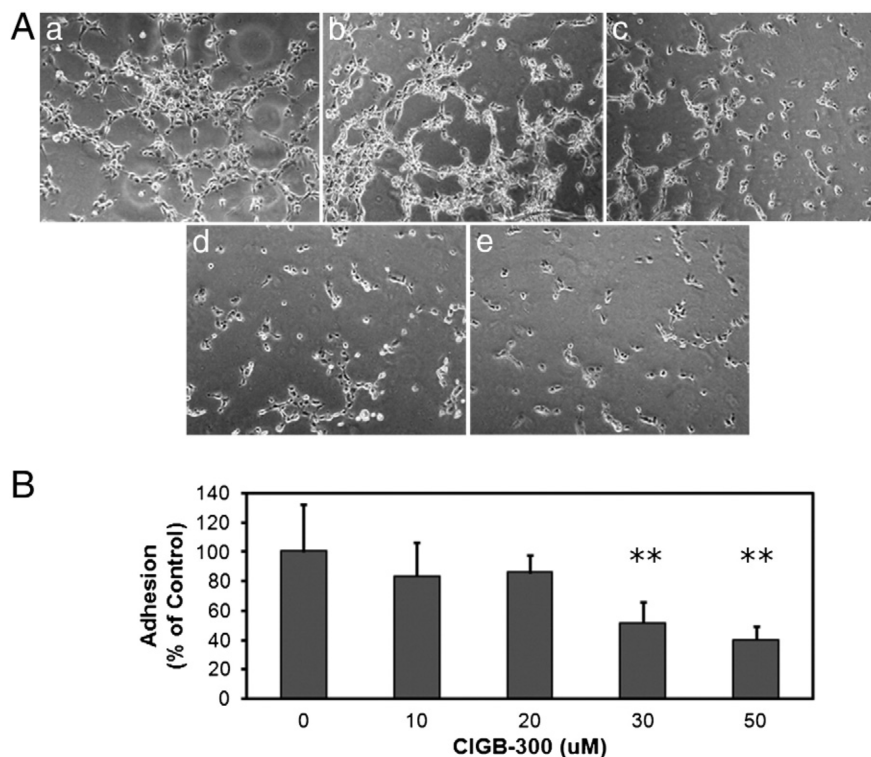

Fig. 3 – Effect of CIGB-300 on cell adhesion. HUVEC cells were added to Matrigel coated plates and incubated for 2 h in presence of CIGB-300. A, Representative photographs of HUVEC cells after the treatment with CIGB-300. B, Quantification of attached cells. The graph is expressed as a percentage of control (PBS treated cells) and represents the mean of three independent experiments and each experiment was done twice. Means without asterisk are not significantly different by ANOVA followed by Dunnett's test ($P < 0.01$).**

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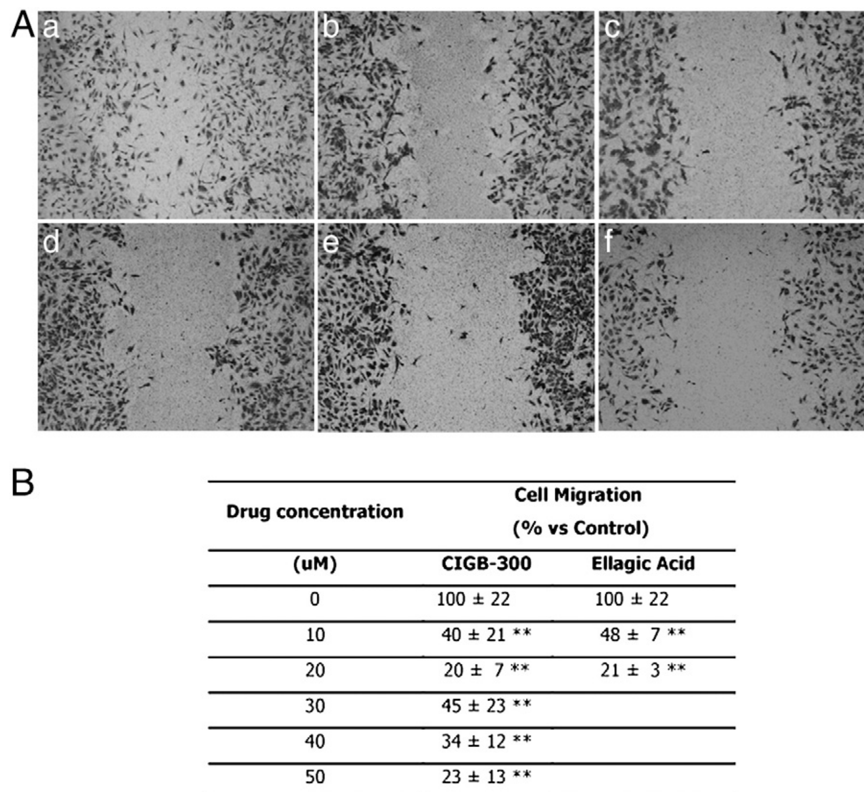

Drug concentration	Cell Migration (% vs Control)	
(uM)	CIGB-300	Ellagic Acid
0	100 ± 22	100 ± 22
10	40 ± 21 **	48 ± 7 **
20	20 ± 7 **	21 ± 3 **
30	45 ± 23 **	
40	34 ± 12 **	
50	23 ± 13 **	

Fig. 4 – Effect of CIGB-300 on endothelial cell migration. A, Confluent HUVEC monolayers were wounded, and fresh culture medium was added either in the absence or presence of increased concentration of peptide (10–50 μM). Photographs were taken at the beginning of the assay and after 20 h of incubation. a, Control; b, CIGB-300 10 μM; c, CIGB-300 20 μM; d, CIGB-300 30 μM; e, CIGB-300 40 μM; f, CIGB-300 50 μM. B, Quantification of migration assay. Ellagic acid was included as an example of a potent inhibitor of the enzyme CK2. Values represent means ± SD of at least 12 determination from three separate experiments ($P<0.01$ vs. control, ANOVA).**

Fig. 3, there was a significant decrease in adhesion of HUVEC cells after CIGB-300 treatment at doses of 30 and 50 μM.

CIGB-300 inhibits the migratory capability of HUVEC endothelial cell

Angiogenesis also involves the acquisition by endothelial cells of the capability to migrate through the extracellular matrix. The effect of CIGB-300 inhibition on cell migration was examined in a wound-healing assay. After the wounding of HUVEC monolayers with a pipette tip, we incubated the cells with CIGB-300 for 24 h. As shown in Fig. 4, CIGB-300 produced a significant inhibition of the migratory capability of HUVEC cells. Using another inhibitor of CK2, ellagic acid, we verified a similar profile of inhibition of endothelial cell migration to that found with the peptide CIGB-300.

Effects of CIGB-300 on the formation of capillary-like tube structures

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. In vitro endothelial cells plated on Matrigel align themselves forming cords, already evident a few hours after plating. In an attempt to elucidate the biological significance of the antiangiogenic effect of CIGB-300 on endothelial cells, we evaluated its capacity to inhibit the formation of capillary-like tube structures on HUVEC cells growing on Matrigel. Fig. 5 shows that CIGB-300 was able to completely inhibit the HUVEC alignment and cord formation. The minimal concentration of compound yielding a complete inhibition of endothelial morphogenesis on Matrigel was 20 μM. As in the cell migration assay, ellagic acid showed an inhibition profile very similar to the CIGB-300 peptide.

Effect of CIGB-300 on apoptosis in HUVEC cells

To discover the nature of the effect of CIGB-300 on HUVEC cells, we studied a few aspects of apoptosis: phosphatidylserine presentation to detect cells at an early stage of apoptosis staining with Annexin V; and nuclear fragmentation, cytoplasm thickening, and chromatin condensation staining with AO/EB. We included a treatment with paclitaxel 10 nM as positive control [12]. In most of the doses used in this work (20–80 μM), we found that the CIGB-300 treatment for 24 h did not induce an increase in the population of apoptotic cells. We only observed a significant increase on apoptosis using doses greater than 100 μM (Fig. 6A). Similar results were obtained using AO/EB technique (Fig. 6B). Apoptosis with longer incubation times showed the same profile (data not shown).

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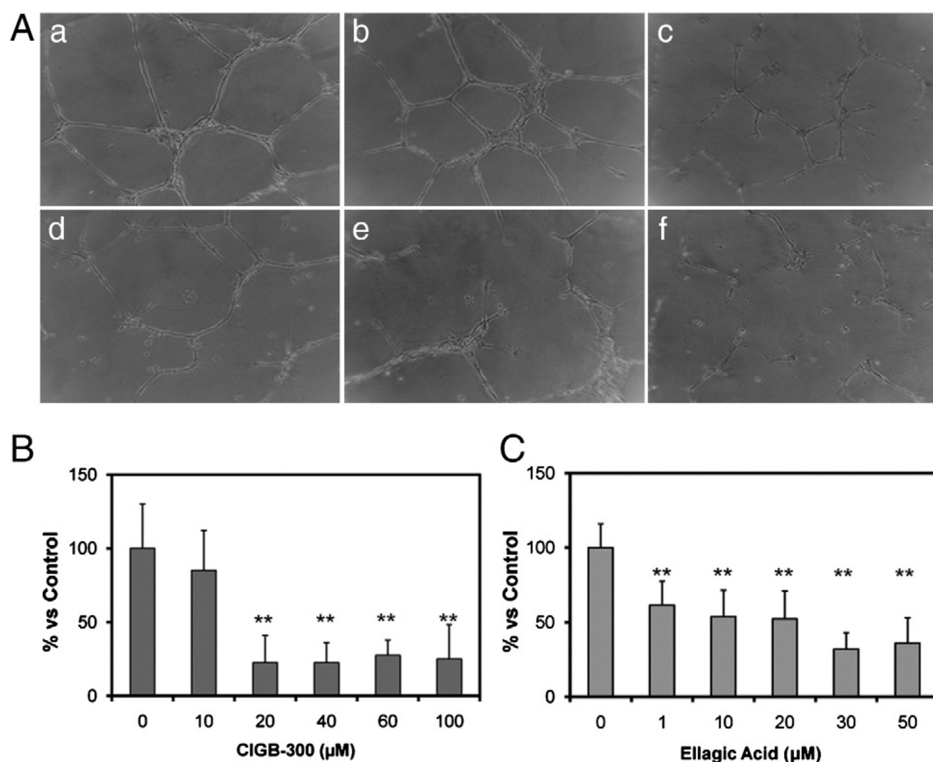

Fig. 5 – Effect of CIGB-300 on endothelial cell tube formation on Matrigel. A, Tube formation was assessed after 20 h incubation. The peptide was tested at a range varying between 10 and 100 μM. a,Control; b, CIGB-300 10 μM; c, CIGB-300 40 μM; d, CIGB-300 60 μM; e, CIGB-300 100 μM. The photographs were taken with an inverted microscope. Quantification of tube formation of HUVEC with (B) CIGB-300 and (C)ellagic acid. Control was normalized to 100%. Each value represents means ± SD from three independent experiments (*P* < 0.01 vs. control, ANOVA, Tukey test).**

Expression profile of angiogenesis-related genes in HUVEC cells treated with CIGB-300 peptide

To investigate the expression profile of angiogenesis related genes in HUVEC cells, treated or not for 20 h with CIGB-300, we performed microarray experiments with this cell population. Of the 112 genes covered by Oligo GEArray® Human Angiogenesis Microarray (OHS-024) related directly to angiogenesis pathways, only 4 were found to be significantly down-regulated with CIGB-300 treatment (GEArray Expression Analysis Suite software; GEArray Corporation, SA Biosciences). We did not find over expressed genes according to the chosen filter criteria. In particular, the under expressed genes included those promoting cell proliferation, cell movement, tubulogenesis, vascularization, and survival of endothelial cells (Table 1), as analyzed with a network approach using Cytoscape software. They were PIGF, ID: 5228 (placental growth factor); KDR, ID: 407170 (vascular endothelial growth factor receptor 2/VEGFR-2); NOTCH4, ID: 18132 (Notch homolog 4 (Drosophila)); and JAG1, ID: 182 (Jagged 1 (Alagille syndrome)).

CIGB-300 shows antiangiogenic activity in the chick embryo CAM

The capacity of CIGB-300 to interact directly with endothelial cells in vitro prompted us to evaluate the angiogenic potential of this peptide when delivered in vivo to a 6 day chick embryo CAM. Fig. 7 summarizes the evaluation of the in vivo inhibition of angiogenesis in the CAM assay by CIGB-300. The compound was exposed to the CAM via a Thermanox coverslip (Nalgene Nunc International, Naperville, IL) where a 20 μl from a 3.2 mM solution of CIGB-300 was added. At least 10 eggs were scored in 3 independent experiments. CIGB-300 markedly prevented blood vessel formation in the CAM system during 5-day exposure time period. The CIGB-300 produced a reduction in new vessel formation of 30% at 3.2 mM. The *P* values for the 3.2 mM was <0.05, when compared to control (paired *t* test).

Discussion

Protein kinase CK2 is a serine–threonine kinase frequently deregulated in human tumors. CK2 is essential for different cell functions like gene expression [20], cell growth [21], survival [22], chromatin remodeling [23], angiogenesis [7], and protection against apoptosis [24]. Furthermore, the potential of CK2 as a particularly suitable target for cancer treatment has been experimentally validated by different groups [25].

By screening a random cyclic peptide phage display library, we identified CIGB-300, a cyclic peptide which abrogates the CK2 phosphorylation by blocking recombinant substrates in vitro. Previously, we demonstrated that CIGB-300 produced a dose-dependent antiproliferative effect in a variety of tumor cell lines

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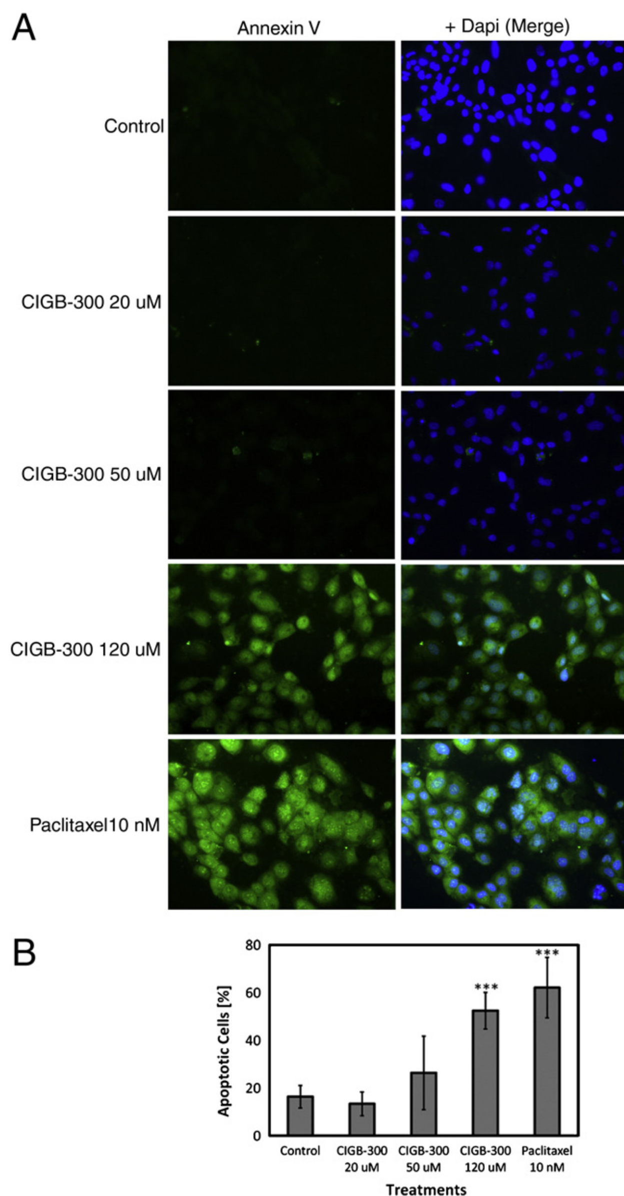

Fig. 6 – Effect of CIGB-300 on apoptosis. A, Effect of CIGB-300 on early apoptosis events in HUVEC cells. Image acquisitions were done using a Nikon TE2000 inverted fluorescent microscope (Nikon, Tokyo, Japan) and microphotographs were taken with a digital camera Nikon DS-5M. Images acquired with a 40× objective were processed using NIS-Elements 3.0 software (Nikon, Tokyo, Japan). B, Quantification of apoptotic cells in the AO/EB-stained cultures. Data are expressed as the percentage of total cells and are representative of 2 independent experiments. ****P* < 0.001 vs. control (Tukey–Kramer test, ANOVA).

and induced apoptosis as evidenced by rapid caspase activation. CIGB-300 elicited a significant antitumor effect, both by local and systemic administration, in murine syngenic tumors and human tumors xenografted in nude mice [4,5]. Likewise, we performed a first-in-man trial with CIGB 300 in patients with cervical malignancies. The peptide was found to be safe and well tolerated in the dose range studied; there were no adverse events related to the alteration of normal angiogenesis. Signs of clinical benefit were identified after CIGB-300 treatment as evidenced by significant decrease of the tumor lesion and histological examination [5,26].

Preliminary studies showed a decrease in the vascularization of TC-1 tumors grown in C57BL6 treated with CIGB-300 [4]. On the other hand, in tumor cells (HeLa and H-125), we have preliminary unpublished data obtained from genomic studies indicating that the CIGB-300 peptide can profoundly affect the expression of proangiogenic genes (data not shown). Here, we hypothesized that this peptide could exhibit specific antiangiogenic properties on endothelial cells.

Several authors previously reported the role of CK2 in angiogenesis. Kramerov et al. postulated that CK2 can modulate the signaling pathways involving receptor tyrosine kinases, receptors associated to G-protein, and extracellular matrix receptors, integrins, etc. [6,7]. CK2 may modulate Raf-ERK-S6K, p38 MAPK, and Akt pathways. This makes CK2 inhibitors good candidates for inhibiting angiogenesis [27].

In order to determine whether the CIGB-300 was able to inhibit angiogenesis, we begun analyzing the sensitivity of endothelial HUVEC cells to CIGB-300. This assay established the dose levels to treat the cells. We first identified the toxic dose range of CIGB-300 on proliferating endothelial cells. The IC50 was determined to be 91 μM for 72 h and 121 μM for 24 h. These cytotoxic doses, with few exceptions, are similar to those found on human tumor cells; however, the minimal dose of CIGB-300 to disturb the endothelial cell function is 2- to 10- fold lower than those affecting cancer cells (50–100 μM). Also, in order to avoid any growth inhibitory activity of the peptide on HUVEC cells, we confirmed that the doses used in this study (10–50 μM) did not show significant induction of cytostasis. Using the same scheme, we evaluated the sensitivity of HUVEC cells to different concentrations of ellagic acid, one of the most potent inhibitors of CK2, at 72 and 24 h. We found an IC_{50} value of 20 and 53 μM, respectively. We decided to include this drug in order to compare the pattern and levels of CIGB-300 peptide response to a well known CK2 inhibitor on migration and endothelial cord formation assay.

Previously, we reported a fast kinetics of peptide internalization and translocation into the nucleus and identified the multifunctional protein nucleophosmin/B23 as a major target for CIGB-300 [28]. Here, we found a preferential subcellular localization in cytoplasm for the CIGB-300 peptide in HUVEC cells. One reasonable explanation for the differential subcellular localization of CIGB-300 in endothelial vs. tumor cells could be based on the "opportunistic" entry mechanism described for the cell penetrating peptides (cpp). It means that one unique cpp can deliver a cargo by alternative mechanisms in different cell lines including endocitosis, transduction, and others which can influence the speed of delivering, subcellular localization, etc. [29]. Nonetheless, this particular finding is very interesting as many CK2 substrates exhibit a dichotomy in terms of subcellular localization with many of them working as shuttle between cytoplasm and nucleus (Ej. B23/nucleophosmin) [30,31]. We have no answer for this pattern of localization, and certainly more research is needed; however, we should emphasize the strong presence of the peptide inside the cell in every time evaluated (1 min–24 h).

Next, we tested the capability of CIGB-300 to inhibit cellular functions of HUVEC cells needed for angiogenesis using the Matrigel

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Table 1 – Selection and category of under expressed angiogenesis-related genes in human endothelial cells investigated by Oligo GEArray Human Angiogenesis Microarray OHS-24.

Position and description	Gene symbol	GenBank accession no.	Magnitude of change	Summary (category and cell functions)
61–Kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	NM_002253	2.32	Vascular endothelial growth factor (VEGF) is a major growth factor for endothelial cells. This gene encodes one of the two receptors of the VEGF. This receptor, known as kinase insert domain receptor, is a type III receptor tyrosine kinase. It functions as the main mediator of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis, and sprouting.
60–Jagged 1 (Alagille syndrome)	JAG1	NM_000214	2	The jagged 1 protein encoded by JAG1 is the human homolog of the Drosophilia jagged protein. Human jagged 1 is the ligand for the receptor notch 1. Mutations that alter the jagged 1 protein cause Alagille syndrome. Jagged 1 signaling through notch 1 has also been shown to play a role in hematopoiesis.
79–Placental growth factor, vascular endothelial growth factor-related protein	PIGF	NM_002632	3.28	Placenta growth factor (PlGF) is a key regulator of pathological angiogenesis and its overexpression has been linked to neoplastic progression. It was recently reported that both a high soluble fms-like tyrosine kinase 1 (sFlt1): placental growth factor (PlGF) ratio (sFlt1:PlGF ratio) and high soluble endoglin (sEng) levels are related to the later occurrence of preeclampsia. PIGF has been associated to monocites chemotaxis, migration, inhibition of apoptosis, and amplifying the VEGF signal.
69–Notch homolog 4 (Drosophila)	NOTCH4	NM_004557	1.81	This gene encodes a member of the Notch family. Members of this Type 1 transmembrane protein family share structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple, different domain types. Notch family members play a role in a variety of developmental processes by controlling cell fate decisions. The Notch signaling network is an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells. This protein functions as a receptor for membrane bound ligands and may play a role in vascular, renal, and hepatic development. NOTCH1 has been associated to cellular differentiation, vascular development, proliferation, and inhibition of apoptosis.

HUVEC cells pattern expression investigated by Oligo GEArray Human Angiogenesis Microarray OHS-24.

assay. In Matrigel, endothelial cells adhere and move on the extracellular matrix, forming capillary-like structures resembling blood vessels. At low concentrations (10 to 30 μM), CIGB-300 was able to significantly reduce adhesion, migration, and capillary like formation. We have not a definitive answer to the lack of a dose dependency in the observed effect on migration of HUVEC cells that is similar to the effect on capillary like formation. We may think that this effect could be due to the time of incubation with the drug in accordance with its subcellular location and access to the nucleus. In migration and capillary like formation assays, the incubation time was 20 h. For times of incubation under 24 h, the peptide is preferentially located in cytoplasm and perinuclear area; after 20 h, nuclear mark begins to intensify, in this context. The drug could have an effect on targets located in the cytoplasm that not necessarily should give a dose-dependent pattern due to the high number of substrates targeted by CK2. An interesting aspect to note here is that, by using another inhibitor of CK2 such as ellagic acid in assays like cell migration or endothelial cord formation, they showed a similar inhibition profile without dose dependency. Beyond the absence of dose dependence found with both inhibitors, an interesting fact that emerges from the comparison of the inhibition profile of the two drugs is that the peptide CIGB-300 maintains the same levels of ellagic acid inhibition at similar doses despite having a much higher IC_{50}.

Since our previous publications showed that CIGB-300 peptide induces apoptosis [32] and the current studies indicate that CIGB-300 peptide prevents angiogenesis, we analyzed whether the antiangiogenic effect was due to the killing of endothelial cells or an effect on the modulation of angiogenesis signaling. The results found here show that the doses, in which the peptide CIGB-300 inhibits angiogenesis in vitro, were unable to induce apoptosis. Inhibition of angiogenesis found in this study could be explained by another mechanism than cell death.

Therefore, with the aim of finding the mechanism by which CIGB-300 peptide could modulate angiogenesis, we used HUVEC cells in vitro to study the antiangiogenic potential of CIGB-300 by investigating their angiogenesis-related genes using a SuperArray platform that contains more than a hundred genes associated directly with angiogenesis. Using the GEArray Expression Analysis Suite 2.0 software from SABiosciences™, we found four genes significantly under expressed with CIGB-300 treatment. Those genes were the Notch homolog 4, NOTCH 4; the placental growth factor, PIGF; the Jagged 1 (Alagille syndrome), JAG1; and KDR, (vascular endothelial growth factor receptor 2/VEGFR-2). Using a trial version of Ingenuity software pathway analysis, Cytoscape, and different sources like Swissprot, Entrez Gene, Go, OMIN, PubMed, Kegg Biocarta, and String HPRD, we identified the more related pathways involving these genes. Notch signaling, nitric

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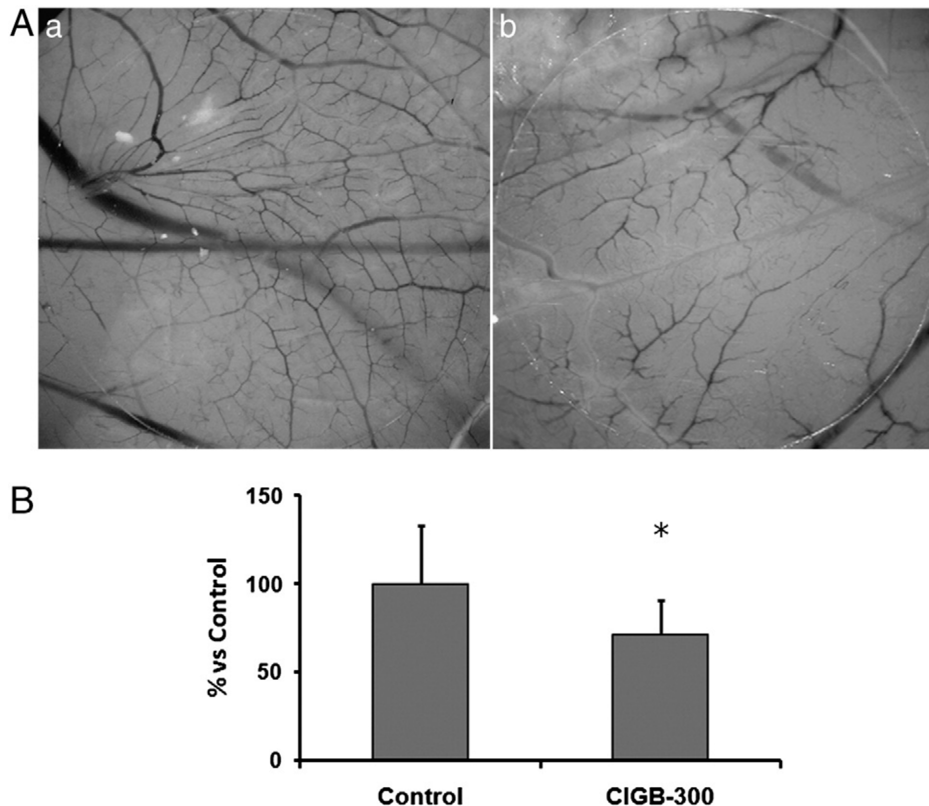

Fig. 7 – Effect of the CIGB-300 peptide on angiogenesis in the CAM assay. A, Photograph of CAMs overlying Thermanox coverslip. a, Control; b, CIGB-300 3.2 mM. B, Inhibition of new blood vessel formation in the chicken egg CAM test by 3.2 mM of CIGB-300. *$P<0.05$, ANOVA test.

oxide signaling in the cardiovascular system, and VEGF signaling were in this order the most important pathways that included three or four of those genes. In vivo, these pathways have been associated to different functions in endothelial cells, like migration, angiogenesis, organogenesis, development of vessels, differentiation, morphogenesis, and proliferation.

Angiogenesis is regulated by angiogenic factors through many signaling pathways. The VEGF pathway and Notch signaling are two of the most important mechanisms in the regulation of embryonic vascular development and tumor angiogenesis [33]. The VEGF pathway interplays at several levels with DLL4/Notch signaling in vasculature. VEGF induces DLL4/Notch signaling while DLL4/Notch signaling modulates the VEGF pathway. Combination therapy by blocking DLL4/Notch and VEGF pathways synergistically inhibits tumor growth in preclinical models. Thus, targeting the DLL4/Notch pathway, although still at an early stage, may lead to new therapies for clinical application [9].

Nowadays, the most current therapies are aimed at blocking the VEGF pathway. However, not all tumors are responsive to VEGF blockers, and some that are responsive initially may become resistant during the course of treatment; thus it is needed to explore other angiogenesis signaling pathways [9]. Keeping in mind that CIGB-300 was capable of inhibiting partially the expression of the genes NOTCH4 and VEGF, and that these genes are included in the main angiogenic pathways, this new peptide could be a strong candidate to be included in therapies directed to stop the angiogenesis and particularly in systems that are resistant to anti-VEGF therapies.

Using the Cytoscape program to build networks of cell signaling pathways, we conducted a search for interactions between CK2 and the genes that vary significantly with the treatment of CIGB-300. None of the four proteins encoded by the genes identified are CK2 substrates directly. Indirectly, CK2 phosphorylates four substrates that in turn interact with two proteins encoded by the identified genes. CK2 phosphorylates PSN2 (presenilin-2) which in turn interacts with NOTCH4 [34,35]. Moreover, CK2 phosphorylates HSP90A (heat shock protein HSP 90-alpha), CAV1 (caveolin-1), and CTNB1 (catenin beta-1) [36,37]. These proteins interact with KDR and modulate the signaling pathway mediated by the receptor [38]. These substrates of CK2 (PSN2, HSP90A, CAV1, CTNB1) may be targets of CIGB-300 in the cytoplasm of HUVEC cells. These interactions at least partly explain the reduction of expression found in the four genes. However, further experiments are needed to confirm the action over these two genes and to explain the way JAG1 and PIGF are modulated.

Finally, the antiangiogenic properties of CIGB-300 peptide were confirmed in vivo in the CAMs model, where the morphometric evaluation of the vascular density of the CAM at day 11 of incubation demonstrated that CIGB-300 exerted a discrete but significant antiangiogenic effect in the chick embryo similar to other CK2 inhibitors [7]. Recently, it was found that several key elements of angiogenic growth factor signaling cascades were substrates for CK2. Therefore, CK2 could be an important mediator of angiogenesis. Ljubimov described the effects of CK2 inhibitors on angiogenesis, finding an important reduction of angiogenesis using these inhibitors and postulated CK2 as a “master regulator”

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of many signaling pathways in angiogenesis, indicating that this enzyme may be a key component to target unwanted angiogenesis [6,7,29,39]. Because of the potential of this new drug in angiogenesis, it is essential to explore further into the mechanisms of action by identifying key molecular mediators and to validate the mechanism and its effectiveness in other in vitro and in vivo models.

Conclusions

In this paper, we showed that the CIGB-300 peptide is able to alter and inhibit the major angiogenic properties of HUVEC cells. The effects could be partly associated with the modulation of VEGF and NOTCH4-dependent pathways. The antiangiogenic properties of CIGB-300 peptide were confirmed in vivo in the CAMs model, where there was a significant decrease in vascularization in the CIGB-300-treated group.

Although additional studies will be needed to elucidate the molecular mechanism of the antiangiogenic activity of CIGB-300, data presented here suggest its potential in therapeutic applications for the treatment of angiogenesis-related malignancies.

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