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CIGB-300, an anti-CK2 peptide, inhibits angiogenesis, tumor cell invasion and metastasis in lung cancer models

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Highlight

- We examined the effect of CIGB-300, an anti-CK2 peptide, in lung tumor development.
- In vitro, CIGB-300 inhibited lung tumor cell adhesion, migration and invasion.
- CIGB-300 elicited an anti-angiogenic response in vivo.
- CIGB-300 systemic administration reduced lung tumor cell dissemination and colonization.

Abstract

Objectives: Casein kinase 2 (CK2) is overexpressed in several types of cancer. It has more than 300 substrates mainly involved in DNA reparation and replication, chromatin remodeling and cellular growth. In recent years CK2 became an interesting target for anticancer drug development. CIGB-300 is a peptidic inhibitor of CK2 activity, designed to bind to the phospho-acceptor domain of CK2 substrates, impairing the correct phosphorylation by the enzyme. The aim of this work was to explore the antitumor effects of this inhibitor in preclinical lung cancer models.

Materials and methods: Human H125 and murine 3LL Lewis lung carcinoma cell lines were used to evaluate the effect of CIGB-300 treatment *in vitro*. For this purpose, adhesion, migration and invasion capabilities of cancer cells were tested. Proteolytic activity of tumor cell-secreted uPA and MMP after CIGB-300 incubation was also analyzed.

In vivo anticancer efficacy of the peptide was evaluated using experimental and spontaneous lung colonization assays in C57BL/6 mice. Finally, in order to test the effect

of CIGB-300 on tumor cell-induced angiogenesis, a modified Matrigel plug assay was conducted.

Results and Conclusion: We demonstrate that treatment with low micromolar concentrations of CIGB-300 caused a drastic reduction of adhesion, migration and invasion of lung cancer cells. Reduced invasiveness after CIGB-300 incubation was associated with decreased proteolytic activity of tumor cell-conditioned medium.

In vivo, intravenous administration of CIGB-300 (10 mg/kg) markly decreased lung colonization and metastasis development of 3LL cells. Interestingly, after 5 days of systemic treatment with CIGB-300, tumor cell-driven neovascularization was significantly reduced in comparison to control group. Altogether our data suggest an important role of CK2 in lung tumor development, suggesting a potential use of CIGB-300 as a novel therapeutic agent against lung cancer.

Keywords: CK2; CIGB-300; lung cancer; metastasis; angiogenesis

1. Introduction

CK2 (formerly known as casein kinase 2) is a serine-threonine protein kinase that is constitutively and ubiquitously expressed in eukaryotic cells. Phosphorylation of the holoenzyme is not required for its activation; in fact, CK2 is always active in its tetrameric form. CK2 is typically composed of four subunits: two α or α' catalytic subunits (two α, α' or combined), and two non-catalytic regulatory β subunits. With more than 300 substrates, this kinase is implicated in a vast number of cellular functions. Given its role in cell proliferation, differentiation and apoptosis regulation, CK2 is implicated in malignant transformation [**1**], and its overexpression is thought to be the cause (not the consequence) of this event. The overexpression of CK2 confers tumor cells the ability to evade apoptosis through the activation of pro-survival signaling pathways [**2**]. Recently, Vilmont et al [**3**] reported a novel CK2-dependent apoptosis evasion mechanism by modulation of Fas associated death domain protein (FADD) localization. When FADD is phosphorylated by CK, this protein remains in the nucleus, impairing the association with other pro-apoptotic components in the cytosol and, consequently, apoptosis. Nevertheless, this is not the only advantage conferred to cancer cells. CK2 is also involved in the development of multidrug resistant phenotype, the activation of onco-kinome chaperones, and tumor angiogenesis [**4,5**]. Given these characteristics, is not surprising to find that CK2 is overexpressed in several types of cancer, including kidney, breast, head and neck, prostate and lung. Particularly in lung cancer, the abnormal overexpression of CK2α and CK2α' subunits is a common feature in squamous cell carcinoma and adenocarcinoma. CK2α' transcript is also overexpressed in large cell carcinoma (LCLC) and small cell lung cancer (SCLC). Additionally, regulatory subunit CK2β was found to be overexpressed in adenocarcinoma and LCLC [**6**].

The above-described characteristics position CK2 as an interesting target for new antitumoral drugs. In this regard, novel anti-CK2 peptide CIGB-300 has been the subject of multiple studies conducted by our group. Interestingly, this peptidic inhibitor was designed

and developed using an original approach. In general, the most common CK2 inhibitors were conceived as ATP competitive inhibitors. CX4945, Quinalizarin, TDB, Quercetin, Emodin, TBB and IQA are some examples of compounds designed with this strategy [**5,7,8,9**]. Instead of targeting the ATP binding site, CIGB-300 was developed as a "substrate inhibitor". This cyclic peptide (conformed by 11 aminoacids and a cell penetrating domain derived from HIV-1 Tat protein) targets the highly conserved phospho-acceptor domain of CK2 substrates, thus impairing the correct phosphorylation by the enzyme. E7 protein from human papiloma virus was chosen as a target for the screening process, based on the fact that this protein is phosphorylated by CK2 [**10**,**11**].

In previous studies the antitumoral response elicited by CIGB-300 was documented. Intravenous (i.v.) and intraperitoneal (i.p.) administration of CIGB-300 peptide was able to significantly reduce primary tumor growth using heterotopic cervix and lung cancer models [**12**]. Both *in vitro* and *in vivo*, CIGB-300 also displayed an important anti-angiogenic and pro-apoptotic effect on tumor cells. These responses were associated with the modulation of the VEGF-Notch pathway and the inhibition of B23/nucleophosmin phosphorylation leading to nucleolar disassembly, respectively [**13,14,15**]. A phase 2 clinical trial is currently ongoing with the aim of evaluating the effect of CIGB-300 on patients with stage IB2/II cervical cancer (ClinicalTrials.gov Identifier: NCT01639625). After the completion of several phase 1 clinical trials, safety and tolerance of intratumoral administration of the drug was demonstrated. Preliminary evidence of drug effectiveness was also observed [**16**,**17,18**].

Taking into account previous preclinical results in lung cancer models, and the fact that lung cancer is one of the main tumor types in which CK2 is overexpressed, our focus in this work is to study in depth the effect of CK2 inhibition using CIGB-300 as a modulator of key features of lung cancer cell biology.

As we expected, CIGB-300 reduced the adhesion, migration and invasion capability of human H125 and murine 3LL lung cancer cells**.** *In vivo* studies using the syngeneic 3LL lung cancer model in C57BL/6 mice also showed a robust anticancer activity against lung

colonization and metastases, and tumor cell-driven neovascularization. In summary, in this work we present evidence that supports the potential of CIGB-300 as an antitumoral agent for lung cancer treatment, showing how CK2 inhibition impairs the progression of this disease.

2. Materials and methods

2.1 Peptide Synthesis

CIGB-300 was synthesized as previously described [**19**]

2.2 Cells and cell culture conditions

The non-small cell lung cancer (NSCLC) cell line NCI-H125 (H125) and Lewis lung carcinoma cell line 3LL were obtained from ATCC. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. H125 cells were cultured in RPMI 1640 (Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). 3LL cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS.

2.3 Tumor cell spreading and adhesion assays

For all *in vitro* studies using CIGB-300, cells were treated at concentrations lower than their reported IC_{50} (68 μM for H125 and 143 μM for 3LL) [**20**]. In order to study the effect of CIGB-300 treatment in the dynamics of cell spreading, H125 cells were plated in 24 well plates and immediately treated at a concentration of 60 μM. Two and three hours after incubation, non-adherent cells were washed with Phosphate Buffered Saline (PBS) and four pictures of each condition were taken under phase contrast microscope. Then, spread cells were quantified and percentage of cells in spreading relative to total number was calculated.

For cell adhesion assay, H125 and 3LL cells were seeded at a density of $5x10^4$ cells/well in 24 well plate in the presence of CIGB-300 (concentrations ranging from 10 to 50 µM).To mimic the tumor cell normal environment *in vitro*, the plates were coated with Matrigel, a protein matrix rich in adherence factors. After 2 hours, non-adherent cells were washed with PBS and the remaining cells were fixed with 4% paraformaldehyde in PBS during 20 min at room temperature. Adherent cells were counted using a phase contrast inverted microscope (Nikon TE 2000).

2.4 Preparation of conditioned media (CM)

Tumor cell-secreted urokinase plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP2) activities were evaluated in CM. Briefly, subconfluent cell monolayers growing in 35-mm plastic Petri dishes were extensively washed with PBS. Then 1 ml serum-free medium, containing different doses of CIGB-300 (20 and 50 µM), was added and the incubation continued for 12 hours. CM were individually harvested, the remaining monolayers were lysed with 1% Triton-X100-PBS, and cell protein content was determined (Bio-Rad Protein Assay). CM samples were centrifuged (600 x g, 10 min), aliquoted and stored at -80ºC. Samples were used only once after thawing.

2.5 Quantification of uPA activity by radial caseinolysis

To determine uPA activity, a radial caseinolysis assay was used. Briefly, 4 mm wells were punched in the plasminogen-rich casein-agarose gels and 10 µl of CM were seeded. Gels were incubated for 24 h at 37°C in a humidified atmosphere. The diameter of lytic zones was measured, and the areas of degradation were referenced to a standard curve of purified urokinase (0.1 - 50 IU/ml) and normalized to the original cell culture protein content.

2.6 Zymography for MMPs

MMPs enzymatic activities were determined on substrate-impregnated gels. Briefly, conditioned media were collected and run on 9% SDS polyacrylamide slab gels containing 1 mg/ml of gelatin under non-reducing conditions. After electrophoresis, gels were washed for 30 min using in 2.5% Triton X-100 and subsequently incubated for 48 h at 37°C in a buffer containing 0.25 M Tris-HCl pH 7.4, 1 M NaCl, and 25 mM $CaCl_2$. After incubation, gels were fixed and stained with 0.5% Coomassie Brilliant Blue G-250 in methanol / acetic acid / H_2O (30:10:60). Gelatinolytic bands were measured using a digital densitometer. Data were expressed as arbitrary units and normalized to the values observed in control cells.

2.7 Wound migration assay

H125 and 3LL cells were seeded at a density of $1x10^6$ cells/well in a 12 well plate. When cells reached 95% confluence, wounds were made in the cell monolayer. Plates were incubated during 20 h in presence of CIGB-300 (concentrations ranging from 10 to 50 µM). 1 h prior to assay finalization, control wounds were made. After that, cells were washed with PBS, fixed with 10% formalin and stained with 5% toluidine blue. Photographs were taken using a camera connected to the inverted microscope (Nikon, NIS elements software), and wound invasion was quantified using Image J software.

2.8 Transwell invasion assay

$4x10^5$ cells in 200 µL of medium without FBS were plated in the upper chamber of 8 micron Transwells (Costar Inc), coated with 150 uL 0.1% Matrigel. The cells were incubated in the presence of CIGB-300 during 20 h, in concentrations ranging from 10 to 50 µM. Complete medium (with FBS as chemoattractant) was placed in the lower chamber.

After the incubation period, cells that remained in the upper chamber were removed using a cotton swab. Cells adhered in the transwell lower chamber were washed with PBS, fix and stained with 0.1% crystal violet, 20% methanol in PBS for 20 min. Cells that invaded

and migrated to the lower chamber were photographed using a camera coupled to a phase contrast inverted microscope for quantification.

2.9 Animals

8 weeks old C57BL/6 mice (specific pathogen free) were purchased from UNLP (Universidad Nacional de La Plata, Buenos Aires, Argentina), and kept 5–8 mice per cage in our animal house facility at the National University of Quilmes. Food and water was provided ad libitum and general health status of the animals was monitored daily. All protocols were approved by the National University of Quilmes Institutional Animal Care Committee.

2.10 Lung colonization assay

To study CIGB-300 effect in 3LL lung colonization, $2.5x10^4$ cells were injected into the lateral tail vein of C57BL/6 mice. During the next 5 days, mice were treated intravenously with 10 mg/kg CIGB-300 in PBS. Control animals were injected i.v. with the vehicle (PBS). On day 21, lungs were excised and fixed in Bouin's solution and lung nodules were counted under a dissecting microscope.

In additional experiments, CIGB-300 dose was reduced to 2 mg/kg or injected from day 15 to 20 maintaining the initial dose of 10 mg/kg i.v.

2.11 Spontaneous dissemination assay

To study CIGB-300 effect in the dissemination and lung colonization of 3LL tumor cells, 4 x 10^5 cells were injected subcutaneously in the right flank of C57BL/6 mice. Animals were treated daily with a 10 mg/kg i.v. dose of CIGB-300, five days after tumor cell injection, and five consecutive days after day 15 post-inoculation. On day 33 animals were sacrificed, lungs were excised and fixed in Bouin's solution and spontaneous lung tumor nodules were counted.

2.12 In vivo angiogenesis assay

To determine the anti-angiogenic potential of CIGB-300 in vivo, a modified Matrigel plug assay was conducted. Matrigel was injected subcutaneously, mixed with 1 x 10^6 3LL cells in 100 µl of DMEM, and heparin (50 U/ml) (Final volume: 0.6 ml). Mice were treated daily with CIGB-300 (10 mg/kg i.v.), or PBS, for five consecutive days after plug generation. On day 7, animals were sacrificed and plugs were recovered and scanned at high resolution. The extent of vascularization was assessed by the amount of hemoglobin detected in the implants using the Drabkin method (Sigma Aldrich). The absolute hemoglobin content in the plugs was calculated using a rat hemoglobin calibration curve.

2.13 Statistical analyses

All statistical analyses were performed using the PRISM 6, version 6.01 (GraphPad Software Inc, La Jolla, CA, USA). Tukey's multiple comparisons test was used after ANOVA analysis between 3 or more experimental groups. Statistical analyses between 2 experimental groups were conducted using unpaired t test or Mann-Whitney test, depending on parametric or nonparametric data distribution, respectively. Differences were considered statistically significant at a level of $p<0.05$. Data were presented as mean ± SD, or scatter dot blot with mean or median, depending on parametric or nonparametric data distribution, respectively.

3. Results

3.1 Effect of CIGB-300 on tumor cell spreading and adhesion

Cell adhesion is one of the first events during tumor colonization, and it is crucial to establish contact with the extracellular matrix and transduce external signals to start the proliferation process. As shown in figure 1a, *in vitro* treatment with CIGB-300 at concentrations of 40 and 50 µM was able to reduce up to ~60% of H125 and 3LL cell

adhesion in Matrigel matrix-coated wells. In H125 cells, adhesion inhibition was associated with altered cell spreading dynamics. After 2 or 3 h of incubation with a sub-IC_{50} concentration of CIGB-300, cancer cell spreading was retarded by ~40% in comparison to vehicle-treated cells (Figure 1b). Given the small size and particular morphology of 3LL cells, and the technical limitations of optical microscopy, cell spreading analysis could not be performed in this tumor cell line.

3.2 CIGB-300 inhibits uPA and MMP2 activity, cell migration and invasion

Enhancement of secretion and proteolytic activation of uPA and MMPs in cancer tissues represents one of the critical steps in tumor invasion, angiogenesis and metastatic spread [**21,22**]. In lung cancer, uPA was reported to be overexpressed in non-small cell lung cancer patients [**23,24**] and MMP-2 activity was found to correlate with invasiveness and disease progression in preclinical models [**25,26**].

In that regard, we first tested whether uPA and MMP2 activity was modulated by CIGB-300 treatment. As shown in figure 2, both in H125 and 3LL cancer cells, uPA and MMP2 enzymatic activities were significantly inhibited after incubation with 20 or 50 µM of CIGB-300. Particularly in 3LL cells, uPA activity was reduced up to ~40% and MMP2 proteolytic capacity was dramatically abolished compared to control group.

Next we evaluated whether modulation of proteolytic activity of tumor cell-secreted proteases correlates with changes in cell migration or invasion. Wound migration assays were conducted to determine if incubation of tumor cells with CIGB-300 modulates migration. After 20 h of peptide treatment, H125 and 3LL lung cancer cell migration was significantly inhibited, causing a ~60% decrease in cell motility at the higher concentrations tested (Figure 3). Particularly in H125 cells, CIGB-300 at concentrations lower than 30 µM, had no effect on cell migration.

We also studied CIGB-300 effect in cell invasion, using the Matrigel-coated transwell invasion assay. In agreement with previously described results, a significant decrease in the invasive capacity of CIGB-300- treated lung cancer cells was observed in nearly all

concentrations tested. As shown in figure 4, *in vitro* invasiveness of H125 and 3LL cells was reduced by ~70-80% after 20 h treatment with 50 µM of CIGB-300.

3.3 CIGB-300 treatment reduces 3LL lung colonization

The previous results showed an inhibitory effect of CIGB-300 on *in vitro* lung cancer cell migration and invasion. The next step was to evaluate the effect of CIGB-300 in tumor cell experimental dissemination and lung colonization *in vivo.* For this purpose, 3LL cells were injected in the lateral tail vein of C57BL/6 mice, and 21 days later mice were sacrificed and lung nodules were counted. During five consecutive days, at the beginning of the experiment, mice were treated with the peptide, at a concentration of 10 mg/kg. Interestingly, CIGB-300 treatment significantly inhibited the development of pulmonary nodules, compared to control group, reducing nearly ~30% the number of macroscopic lung lesions (Figure 5a). Alternative dosing schedules were also evaluated, where dosing was reduced to 2 mg/kg or mice were treated for five consecutive days immediately previous to sacrifice with 10 mg/kg. In these experimental settings, there were no differences between treated and control groups (data not shown).

Additionally, a spontaneous dissemination protocol from primary tumor to lung was carried out. For this purpose, primary heterotopic lung tumors were generated by subcutaneous injection of 3LL cells in the right flank of C57BL/6 mice, and after 33 days, 3LL tumor-bearing animals were sacrificed and necropsied for lung nodule assessment. CIGB-300 was administered in two rounds of five daily 10 mg/kg i.v. doses. As shown in figure 5b, CIGB-300 systemic treatment severely impaired pulmonary colonization by spontaneously disseminated 3LL cells, causing a ~50% reduction in the number of lung nodules.

3.4 CIGB-300 inhibits lung cancer cell-induced angiogenesis

The antiangiogenic potential of CIGB-300 *in vivo* was determined using a modified Matrigel plug assay. In order to generate the plug, a mix of Matrigel, 3LL cells and heparin was injected in the subcutis of C57BL/6 mice mice. After 7 days of angiogenesis induction,

CIGB-300 treatment (10 mg/kg i.v. daily doses during 5 days) was able to significantly inhibit the tumor cell-driven neovascularization process, reflected in a ~40% reduced content of hemoglobin in plugs recovered from treated mice (Figure 6).

4. Discussion

The potential of CIGB-300 as an antitumoral drug was widely studied since this peptide was first developed. There is a vast amount of evidence available describing the anticancer efficacy of this CK2 inhibitor and its capacity to modulate key features of tumor biology [**11-15,19,27,28**]. Importantly, these results led to different clinical trials with patients with cervical cancer [**16**]. However, evidence about the effect of CIGB-300 treatment in lung cancer is scarce. In a previous preclinical study we showed that intratumoral and systemic administration of CIGB-300 in mice bearing heterotopic lung tumors caused a marked decrease in primary tumor growth [**12**]. In this work, we analyzed for the first time the effects of CIGB-300 systemic administration on the growth of lung cancer cells lodged in the lungs of immunocompetent mice. With that purpose we used the Lewis lung carcinoma 3LL cell line and two different approaches to study the *in vivo* effects. First, an experimental lung colonization assay was executed, in which 3LL cells were injected in the lateral tail vein. In this case there was a significant reduction in the number of tumor nodules per lung reflecting an important role of CK2 in the process of cell implantation and adaptation for the proper growth in pulmonary tissue. Additionally, a spontaneous dissemination assay was carried out, where cells were first injected in the subcutis of mice in order to generate heterotopic lung primary tumors. Once again, a significant decrease in lung colonization and tumor formation was observed. The results obtained in the latter assay gives a wider view of the panorama, because in this experimental setting the cells must succeed in the completion of a larger number of steps before they successfully colonize the lung, including growth in the primary site, invasion

and intravasation into circulation, extravasation and conditioning of the pulmonary microenvironment, among others.

The results obtained *in vitro* using both human H125 and murine 3LL lung cancer models, are in agreement with *in vivo* findings. Given the large amount of CK2 substrates (in 2003 Meggio and Pinna described more than 300 [**29**]), is not surprising that CK2 inhibition affects a vast number of cellular functions. In the present study we focused on key features of tumor cell biology and events that markly define cancer aggressiveness. The adhesion process is one of the crucial events that tumor cells must accomplish to anchor to the target tissue and grow successfully. To investigate if CIGB-300 is implicated in the modulation of this process, cell spreading and adhesion dynamics were assessed. As we expected, CIGB-300 diminished the adhesive capability in both cell lines tested. Interestingly, CIGB-300 treatment also retarded the spreading process, an important step to trigger the main proliferative signaling pathways.

The effect of CIGB-300 treatment in migration, invasion and tumor cell-secreted proteases was also analyzed. CK2 inhibition by CIGB-300 was associated with a marked decrease in lung cancer cell motility and invasiveness, and this phenomenon correlated with an inhibition of uPA and MMP2 proteolytic activity. These proteases have a key role in the invasion process, since tumor cells need to degrade extracellular matrix and stroma, not only to invade surrounding tissue, but also to gain access to the blood circulation and disseminate to distant organs. In summary, the results obtained confirm that treatment with CIGB-300 provokes a switch in malignant cells to a less invasive phenotype, and consequently, a less aggressive one.

In a previous publication we established for the first time concrete evidence of the antiangiogenic potential of CIGB-300, using the endothelial morphogenesis assay and the chick chorioallantoic membrane assay. These findings were supported by the fact that CIGB-300 treatment inhibited Notch signaling pathway and VEGF expression in endothelial cells [**13**]. Nevertheless, aberrant angiogenesis promoted by tumor cells is a complex process difficult to mimic with *in vitro* or animal tumor cell-free angiogenesis

models. This limitation led us to work with a lung cancer cell-driven angiogenesis model in syngeneic mice. As it was previously described in the results section, CIGB-300 elicited a robust antiangiogenic effect, reducing the aberrant vascular response to 3LL cancer cells injected in C57BL/6 mice. During tumor-associated neovascularization, destruction of the basement membrane is a crucial step which requires the activation of several proteolytic enzymes, such as plasminogen activators or metalloproteinases. Besides regulating Notch/VEGF pathways on endothelial cells, CIGB-300 may impair angiogenesis by directly modulating secretion and activity of different proteases involved in tumor progression. Altogether, the data provided in this paper shed light in the understanding of the cellular processes affected by CIGB-300 treatment in lung tumor biology. This peptide showed to be effective in the modulation of important cellular processes that determine tumor aggressiveness, including invasion and angiogenesis. The decrease observed in lung colonization and dissemination by tumor cells is a reflection of the modulation described. The above mentioned findings highlight the potential of this peptide as a novel therapeutic approach in the treatment of lung cancer. In this regard, further clinical testing aiming to evaluate the tolerance and effectiveness of systemic administration of CIGB-300 in lung cancer patients is warranted.

5. Conclusions

In this paper we showed that CIGB-300, a novel peptidic CK2 inhibitor, reduces human and murine lung cancer aggressiveness by reducing tumor cell adhesion, migration and invasion. As revealed by animal protocols conducted in this study, systemic administration of the peptide was capable of reducing tumor cell dissemination and colonization in the lung. Additionally, treatment with CIGB-300 was also able to limit the vascular response in vivo using a lung cancer-induced angiogenesis model.

Although additional studies are warranted in order to fully understand the role of CK2 in lung cancer development and progression, here we report important preclinical evidence supporting further development of CIGB-300 as a novel therapeutic agent with potential application in this type of malignancy.

Conflict of interest statement

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Figure 1. CIGB-300 treatment inhibits lung cancer cell adhesion and spreading. A. Cell adhesion assay. H125 and 3LL cells were seeded in Matrigel-coated plates and treated with CIGB-300 for one hour. Substrate-attached tumor cells were counted under a phase contrast microscope. Six representative fields per condition were analyzed. Data are presented as mean ± SD and are representative of three independent experiments. ***p<0.001 ANOVA, Tukey's multiple comparison test. **B**. Tumor cell spreading assay. H125 cells were seeded and treated with CIGB-300 and, after 2 and 3 hours, four representative fields were photographed under phase contrast microscope to allow cell spreading quantification. Data are presented as mean ± SD and are representative of three independent experiments. ***p<0.001 ANOVA, Tukey's multiple comparison test.

Figure 2. CIGB-300 treatment reduces tumor cell-secreted uPA and MMP2 proteolytic activity. Radial caseinolysis (upper left and right) and zymography (lower left and right) assays were performed in order to measure the effect of CIGB-300 treatment in H125 (left) and 3LL (right)-secreted uPA and MMP2 enzimatic activities. Data are presented as mean ± SD, and are representative of three independent experiments. *p<0.05, **p<0.01 ANOVA, Tukey's multiple comparison test.

Figure 3. CIGB-300 treatment impairs lung cancer cell migration. Confluent monolayers of H125 (left) or 3LL (right) cancer cells were wounded and CIGB-300 was added at different concentrations during 20 hours. Wound closure was measured in six photographed representative fields, and control group was taken as 100%. Data are presented as mean ± SD, and are representative of three independent experiments. **p<0.01, ***p<0.001 ANOVA, Tukey's multiple comparison test.

Figure 4. CIGB-300 negatively modulates invasiveness of lung cancer cells. H125 cells (left) and 3LL (right) cells were seeded in the upper chamber of Matrigel-coated transwells. CIGB-300 was added at different concentrations, and FBS was used as chemoattractant. After 20 h, cells in the lower chamber were stained and counted under an inverted microscope. Representative pictures of each condition are shown on top. Data are presented as mean ± SD, and are representative of five transwells per condition. *p<0.05, ***p<0.001 ANOVA, Tukey's multiple comparison test.

Figure 5. CIGB-300 reduces tumor cell dissemination and lung colonization. A. Experimental lung colonization assay. 3LL cells were injected in the lateral tail vein of mice. Mice were systemically treated with CIGB-300 at a 10 mg/kg i.v. dose, during five consecutive days, and sacrificed at day 21. The number of lung nodules per animal is represented in the graph. Data are expressed as scatter dot plot and mean (animals in control or CIGB-300 experimental groups n=36 or 37, respectively). **p<0.01, Unpaired t test. **B.** Spontaneous cancer cell dissemination model. To study cell dissemination from primary tumor to pulmonary tissue, heterotopic tumors were generated by injecting 3LL cells in the subcutis of syngeneic mice. The treatment consisted in two rounds of five consecutive systemic administrations of CIGB-300 at a 10 mg/kg i.v. doses. Mice were sacrificed on day 33 and lung macroscopic nodules were counted. Data are expressed as scatter dot plot and medians (animals in control or CIGB-300 experimental groups n=9 or 8, respectively). **p<0.01 Mann-Whitney test.

Figure 6. CIGB-300 systemic administration elicits an antiangiogenic effect in lung cancer cell-driven neovascularization. Matrigel mixed with 3LL cells was injected subcutaneously in the flank of mice. CIGB-300 was administered immediately after cell inoculation for five consecutive days at a 10 mg/kg i.v. dose. At day 7, mice were sacrificed and the hemoglobin content of each plug was quantified. Representative pictures of each group are shown below the graphs. Data are presented as mean ± SD (animals in control or CIGB-300 experimental groups n=8 or 5, respectively). *p<0.05, Unpaired t test.

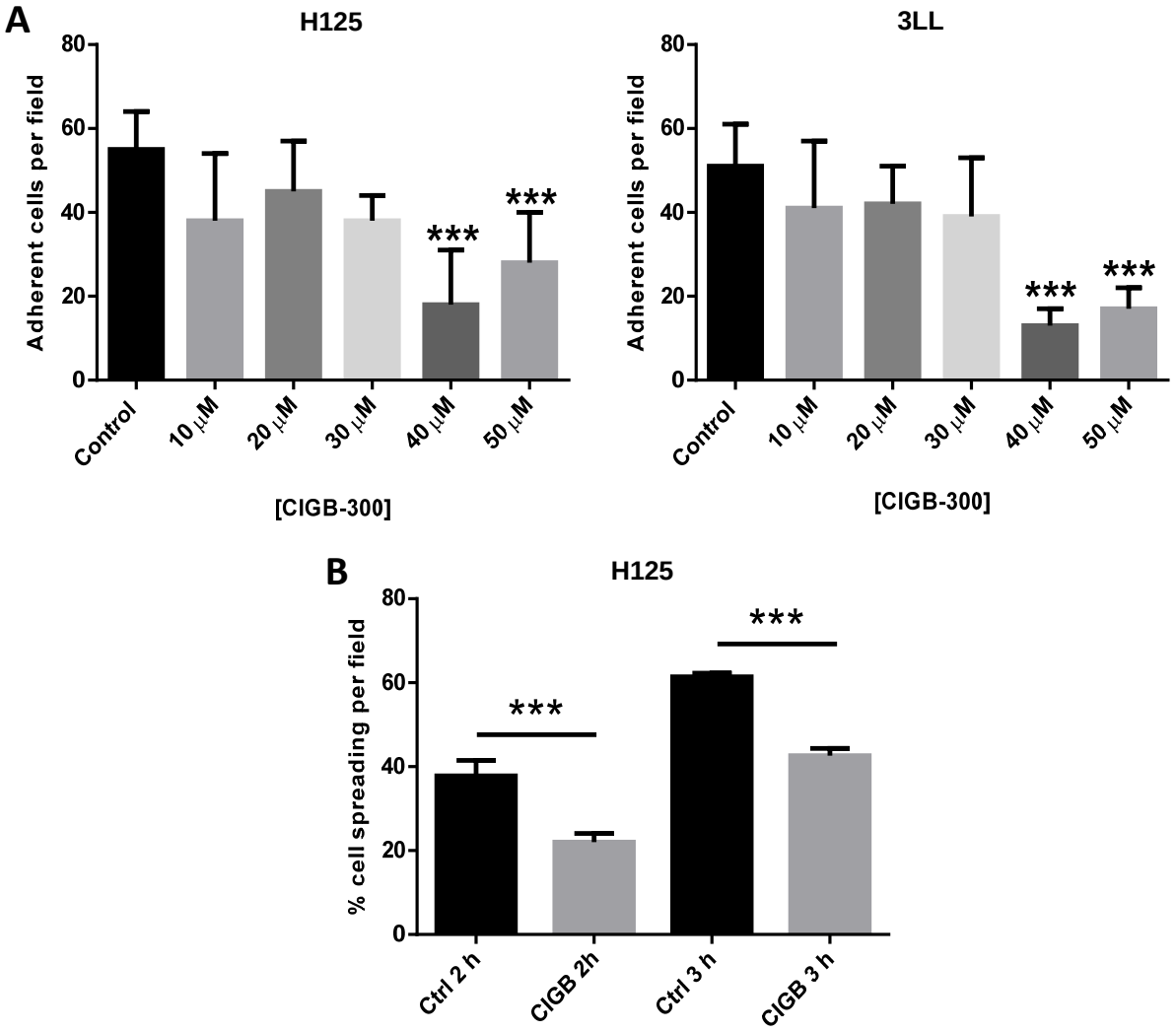

FIG 1

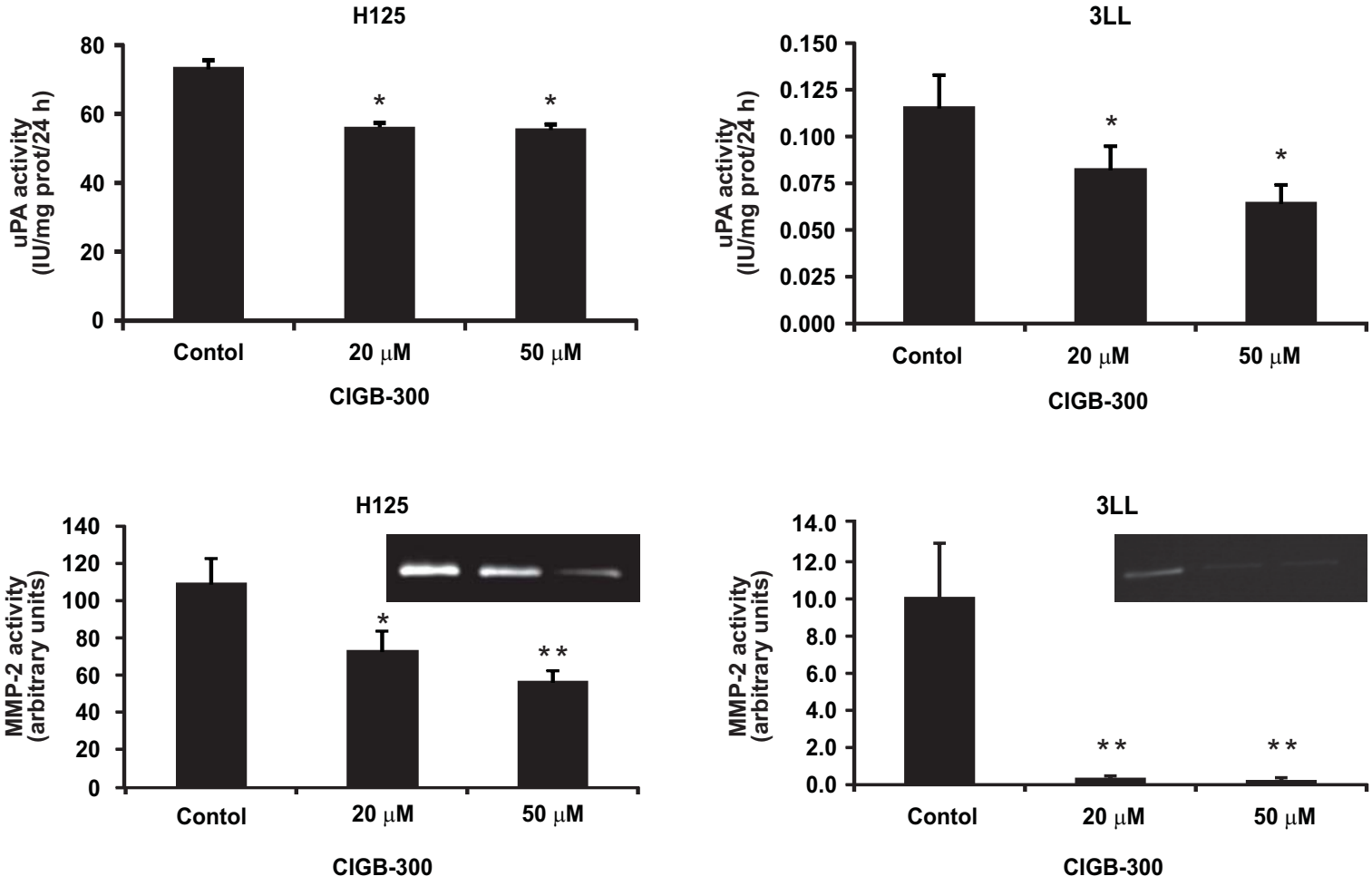

FIG 2

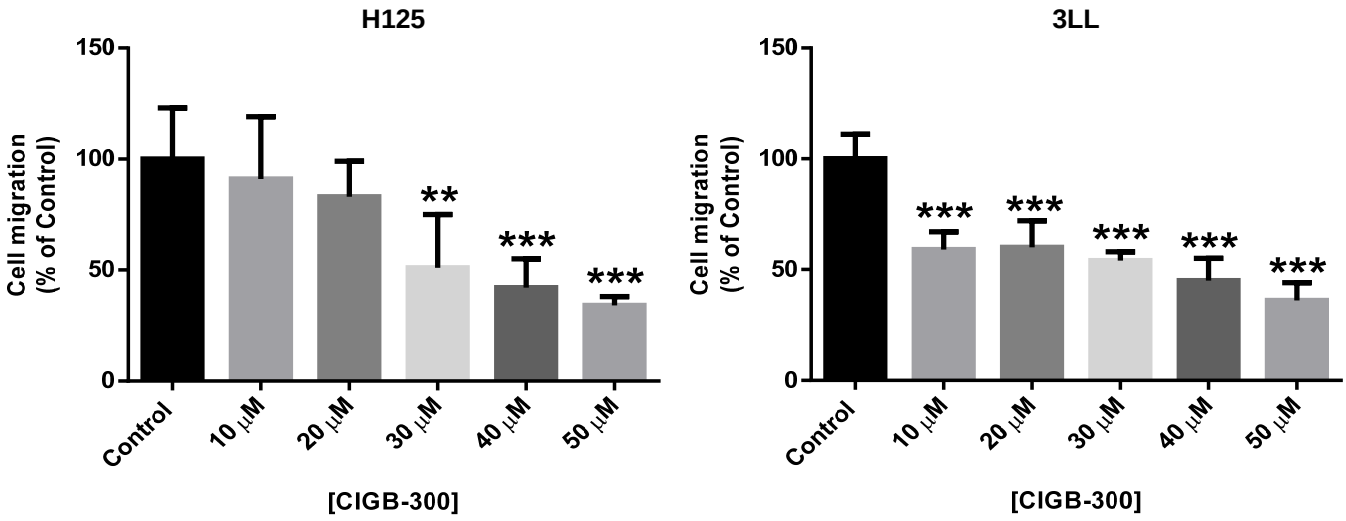

FIG 3

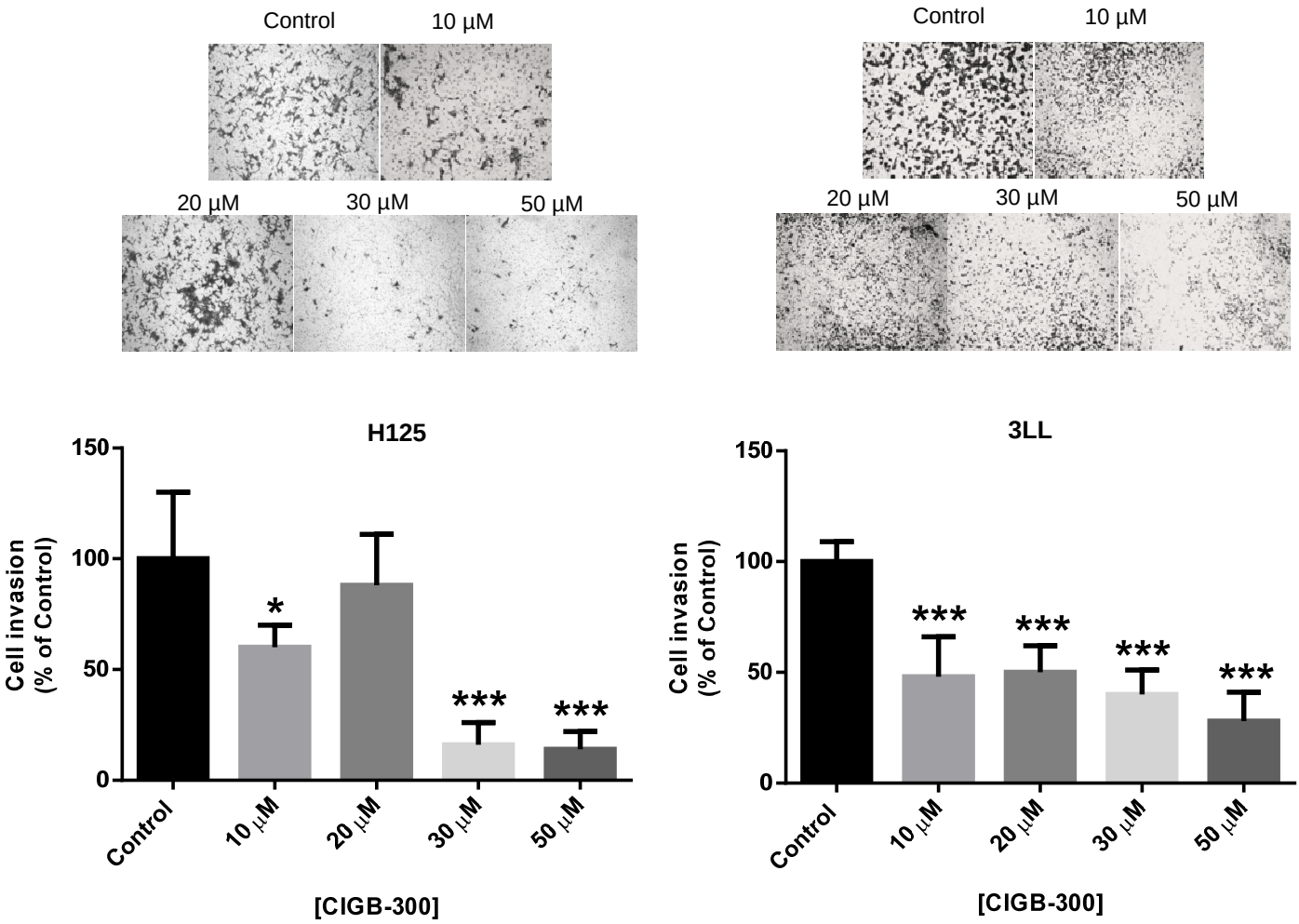

FIG 4

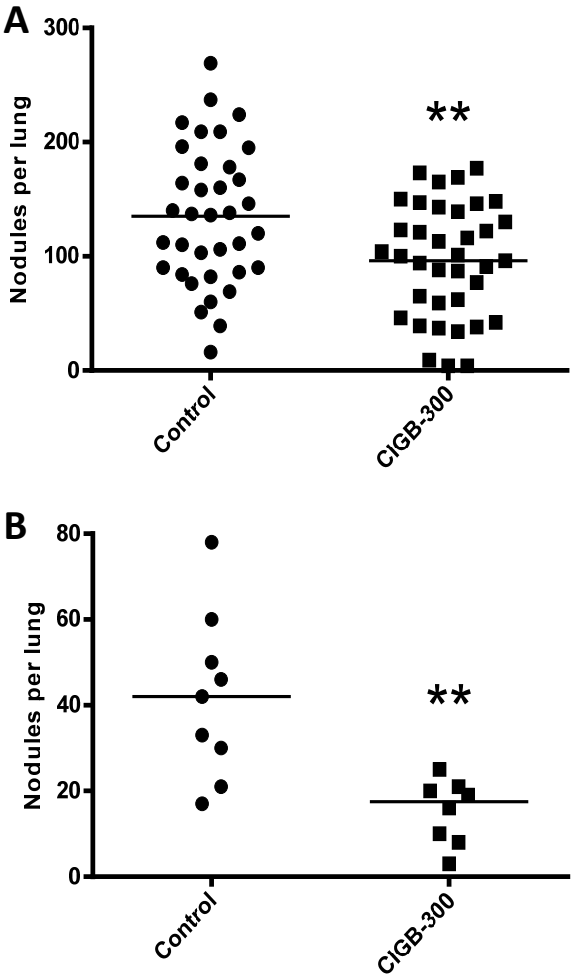

FIG 5

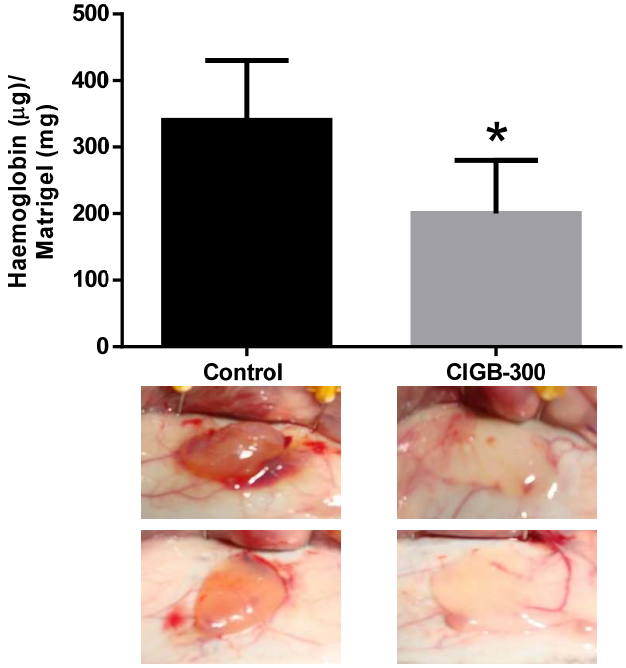

FIG 6