

# Mechanisms of Cellular Uptake, Intracellular Transportation, and Degradation of CIGB-300, a Tat-Conjugated Peptide, in Tumor Cell Lines

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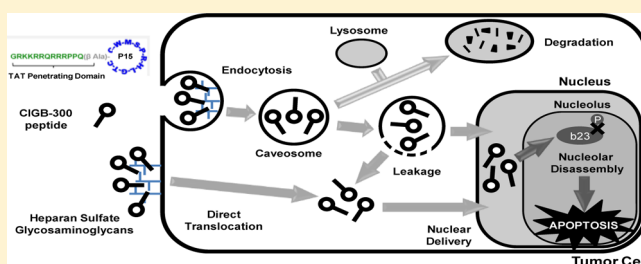
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## Supporting Information

**ABSTRACT:** CIGB-300 is a cyclic synthetic peptide that induces apoptosis in malignant cells, elicits antitumor activity in cancer animal models, and shows tumor reduction signs when assayed in first-in-human phase I trial in patients with cervical tumors. CIGB-300 impairs phosphorylation by casein kinase 2 through targeting the substrate's phosphoacceptor domain. CIGB-300 was linked to the cell penetrating peptide Tat to facilitate the delivery into cells. Previously, we showed that CIGB-300 had a differential antiproliferative behavior in different tumor cell lines. In this work, we studied differential antiproliferative behavior in terms of cellular uptake, intracellular transportation, and degradation in tumor cell lines with dissimilar sensitivity to CIGB-300. The internalization of CIGB-300 was studied in different malignant cell lines. We found that the cell membrane heparan sulfate proteoglycans act as main receptors for extracellular CIGB-300 uptake. The most sensitive tumor cell lines showed higher intracellular incorporation of CIGB-300 in comparison to less sensitive cell lines. Furthermore, CIGB-300 uptake is time- and concentration-dependent in all studied cell lines. It was shown that CIGB-300 has the ability to penetrate cells mainly by direct membrane translocation. However, a minor proportion of the peptide uses an energy-dependent endocytic pathway mechanism to gain access into cells. CIGB-300 is internalized and transported into cells preferentially by caveolae-mediated endocytosis. Lysosomes are involved in CIGB-300 degradation; highly sensitive cell lines showed degradation at earlier times compared to low sensitive cells. Altogether, our data suggests a mechanism of internalization, vesicular transportation, and degradation for CIGB-300 in tumor cells.

**KEYWORDS:** CIGB-300, TAT, CPP, cancer, CK2



## INTRODUCTION

Two key features of cancer cells are growth deregulation and apoptosis escape.<sup>1</sup> The mechanism of oncogenesis is associated with a high number of changes in the cells biology. The casein kinase 2 enzyme (CK2) is a highly conserved serine/threonine protein kinase with more than 300 intracellular protein substrates in eukaryotic cells that displays a highly conserved acidic phosphorylation domain.<sup>2,3</sup> CK2 is constitutively active and is frequently overexpressed in tumor cells, which sustains the neoplastic transformation process through regulation of the proliferative, survival, and stress pathways.<sup>4,5</sup>

CIGB-300 is an anticancer peptide that inhibits CK2-mediated phosphorylation by direct binding to the phosphoacceptor site on their substrates, opposing the common small molecule inhibitors that target the enzyme per se.<sup>6</sup> CIGB-300, previously termed P15-Tat, is formed by a phosphorylation inhibitor domain (P15) fused through a  $\beta$ -Ala spacer to the cell penetrating peptide (CPP) Tat (Tat<sub>48–60</sub>), derived from the protein Tat of human immunodeficiency virus-type 1 (HIV-1). P15 peptide was isolated from a random cyclic peptide phage

library using the conserved acidic phosphoacceptor domain of E7 oncogenic protein of HPV-16 as substrate.<sup>6</sup> The antiproliferative effect of CIGB-300 was examined in several tumor cell lines; notably, a differential antiproliferative effect was observed clearly differentiating two types of populations. The populations were called “high-sensitive”, having IC<sub>50</sub> values below 100  $\mu$ M, and “low-sensitive”, which presented IC<sub>50</sub> concentrations greater than or equal to 100  $\mu$ M. In addition, our group has previously demonstrated the in vivo efficacy of CIGB-300 in cancer mouse models and also showed tumor reduction signs when assayed in first-in-human clinical patients with cervical malignancies.<sup>7,8</sup> Interestingly, our previous findings suggest that the multifunctional nucleolar protein B23/nucleophosmin (NPM) might be the most important nuclear target for CIGB-300. In this model, the peptide

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interacts with NPM at the nucleolus, impairing its CK2-mediated phosphorylation and inducing a nucleolar disassembly leading to apoptosis in tumor cells.<sup>9,10</sup> Nevertheless, the internalization, trafficking, and degradation mechanisms of CIGB-300 in tumor cells are unknown.

In spite of the high number of biological applications using Tat peptide, the mechanism of internalization is not well understood and the underlying mechanism remains under active debate. Not long ago, it was presumed that the cellular uptake of cationic Tat peptide is an energy- and receptor-independent mechanism where it is directly transported through the plasmatic membrane.<sup>11,12</sup>

On the other hand, there has been evidence indicating that the internalization of the Tat protein occurs by endocytic pathways and depends on the presence of cell surface heparan sulfate receptors.<sup>11,13</sup> Interestingly, practically all different cell types studied demonstrated an efficient uptake of Tat fusion proteins.<sup>14,15</sup> The cellular uptake process of Tat peptides is a useful tool to design appropriate strategies for delivering drugs into tumor cells.<sup>16</sup>

Cells have several intracellular pathways for protein degradation, but the major route is the enzymatic digestion in lysosomes. This digestive process is dynamic and involves different stages of lysosomal maturation together with the digestion of both exogenous proteins and digestion of endogenous proteins and cellular organelles.<sup>17</sup> When taking into account the great number of articles published during the last 20 years in the field of cell penetrating peptides (CPPs), it is surprising that so few have analyzed the intracellular degradation mechanism of these peptides.<sup>18–20</sup>

In the present study, we aimed to characterize the mechanisms involved in the different antiproliferative responses of CIGB-300 in tumor cells. For this purpose, we investigated the mechanisms of cellular uptake, intracellular transportation, and the degradation pathway for the CIGB-300 in low- and high-sensitive tumor cell lines.

## ■ EXPERIMENTAL SECTION

**Cell Lines and Cultures.** Mycoplasma-free cell lines derived from human adenocarcinoma from non-small-cell lung cancer (H-125, ATCC CRL-5801; A549, ATCC CCL185), cervix adenocarcinoma (HeLa, ATCC CCL-2), epithelial prostate cancer (PC-3, ATCC CRL1435), promyelocytic leukemia (HL-60, ATCC CCL-240), and the murine Lewis lung carcinoma (3LL, also known as LLC, ATCC CRL-1642) were originally obtained from American Type Culture Collection (Rockville, MD, USA). All cell lines were grown in a 5% CO<sub>2</sub> humidified atmosphere at 37 °C, in Roswell Park Memorial Institute medium 1640, Dulbecco's modified Eagle's medium, or Ham's F-12 nutrient mixture medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (SFB; Internegocios, Mercedes, Argentina) and gentamicin (Fabra, Buenos Aires, Argentina). Cells were passaged by trypsinization with trypsin/EDTA 0.05/0.02% (Gibco, Invitrogen, USA) in phosphate buffered saline (PBS) every three to 4 days.

**Materials.** The peptide CIGB-300 and CIGB-300-F conjugated with carboxyfluorescein were synthesized as previously described.<sup>10</sup> The antibodies and reagents used were the following: rabbit polyclonal anti-early endosomal antigen 1 (EEA1) and anti-caveolin 1 (CAV-1) (Abcam, United Kingdom); goat rhodamine-labeled monoclonal anti-rabbit IgG, LysoTracker red DND-99 (Molecular Probes, Invitrogen, USA); HS sodium salt [average molecular mass of 16500 Da]

(Applichem, Darmstadt, Germany); heparinase III, rotenone, sodium azide amiloride, and nystatin (Sigma, USA); Vectashield mounting medium with DAPI (Vector, USA).

**Cell Viability Experiment.** The cytotoxicity of the peptide was monitored by a MTT assay according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). Peptide concentrations ranging from 0 to 200  $\mu$ M were added to  $1 \times 10^4$  cells per well in complete medium for 72 h at 37 °C, 5% CO<sub>2</sub>. Subsequently, 20  $\mu$ L of 5 mg/mL MTT solution in PBS was added to each well for 2 h. After removal of the medium, 100  $\mu$ L of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a Synergy HT plate reader (Bio-Tek Instruments, USA). Each condition was assayed in sextuplicate in two different experiments, and SD was determined. IC<sub>50</sub> values were obtained from individual growth curves.

**Metabolic and Endocytosis Inhibitor Assays.** For assays at 4 °C, cells were maintained in a custom-built cooling chamber for 30 min before peptide incubation and throughout the experiments. To induce ATP depletion, cells were incubated for 30 min with 10 mM sodium azide or 2  $\mu$ M rotenone in normal culture medium prior to the addition of the peptides. Cells were incubated with CIGB-300-F at a concentration of 50  $\mu$ M for 10, 60, and 240 min. For inhibition of macropinocytosis, samples were pretreated with amiloride 5 mM; for disruption of caveolae endocytosis, cells were pretreated with 25  $\mu$ g/mL nystatin. Amiloride and nystatin were dissolved in DMSO. The final concentration of DMSO was less than 0.5%. Control cells were treated with the same concentrations of DMSO in each experiment. The internalized peptides were visualized by fluorescence microscopy or quantified by flow cytometry (see below).

**Cell Treatment with Heparinase III and Soluble Heparin.** Enzymatic treatment with heparinase III was performed as described.<sup>21</sup> Cells were incubated overnight with heparinase III in PBS containing 0.1% SFB, 0.2% gelatin, and 0.1% glucose at 37 °C in a CO<sub>2</sub> incubator. Cells were washed 3 times with PBS and incubated in complete medium in the presence of 50  $\mu$ M CIGB-300-F for 60 min. In order to study the role of polyanionic cell-surface proteoglycans, the cells were coincubated with heparan sulfate (5  $\mu$ M, 10  $\mu$ M) and CIGB-300-F (50  $\mu$ M) in complete medium for 60 min at 37 °C. Peptide uptake was visualized as mentioned above.

**Flow Cytometry Analysis.** Cells were seeded at a density of 150,000 per well in 12-well plates (GBO, Germany) in 1 mL of serum-containing medium. One day later, the culture medium was replaced, CIGB-300-F (50  $\mu$ M) was added, and the plates were incubated during 10, 60, and 240 min. After incubation, cells were washed with PBS and trypsinized. The cells were resuspended in ice-cold PBS and then fixed with 4% paraformaldehyde in PBS, pH 7.4 (25 °C for 15 min). Samples were centrifuged and resuspended in 400  $\mu$ L of PBS. Fluorescence analysis was performed with a FACScalibur fluorescence-activated cell sorter (FACS, Becton Dickinson, USA). The fluorescent emission of 20,000 vital cells was acquired on a FL1 channel (520 nm, fluorescein). Vital cells were gated based on side scatter and forward scatter. To quantify the effects of various treatments on cellular uptake, the median of cell fluorescence distribution in treated cells was normalized to the median of cell fluorescence distribution in the untreated control. Each experiment was performed in triplicate and repeated at least twice. The mean values  $\pm$  SD of all analyzed samples are indicated in the figures. The

significance of the effects of treatments as compared with untreated controls was evaluated by the paired Student's *t* test at a 95% confidence level.

**Fluorescence Microscopy.** Fluorescence microscopy experiments were performed on a TE2000 inverted microscope from Nikon equipped with a mercury fluorescence lamp and a digital camera Sight DS-U2 (Nikon, Japan). Images acquired with a 100× objective were processed using NIS-Elements 3.0 software (Nikon, Japan). Briefly, cell suspensions of  $5 \times 10^5$  cells/mL were cultivated overnight on a circular coverslide in a 24-well plate at 37 °C and 5% CO<sub>2</sub>. The following day, the CIGB-300-F conjugate was added to the cell culture at a final concentration of 50 μM and was further incubated 10, 60, and 240 min. Afterward, cells were fixed with 4% paraformaldehyde, washed with PBS, and mounted with Vectashield with DAPI. Finally, fixed cells were analyzed by fluorescence microscopy. LysoTracker DND-99 red fluorophore was added during the last 2 h of incubation.

**Indirect Immunofluorescence.** Treated cells were fixed and washed as described above. After that, cells were permeabilized with 0.1% Triton X-100 and blocked by incubation with 3% SFB for 30 min, washed again, and incubated for 1 h at 37 °C with the following reagents: rabbit anti-EEA1 polyclonal antibody, or rabbit anti-CAV1 polyclonal antibody (diluted 1:50). Incubation with rhodamine-conjugated anti-rabbit IgG (diluted 1:400) was carried out at 37 °C for 1 h. Finally, cells were mounted and analyzed by microscopy. Extensive washing with PBS was performed after each step throughout the procedure. Both antibodies were bought from Abcam; anti-CAV1 reacts with mouse, rat, hamster, dog, human, and Chinese hamster; anti-EEA1 reacts with mouse, rat, hamster, cow, dog, human, *Xenopus laevis*, zebrafish, Rhesus monkey, and *Aplysia*.

## RESULTS

**Biological Effect of CIGB-300 in Tumor Cells.** We examined the biological effect of CIGB-300 in several tumor cell lines from different origins. The results (Table 1) showed a

**Table 1. Effect of CIGB-300 on Cell Proliferation in Different Tumor Cell Lines<sup>a</sup>**

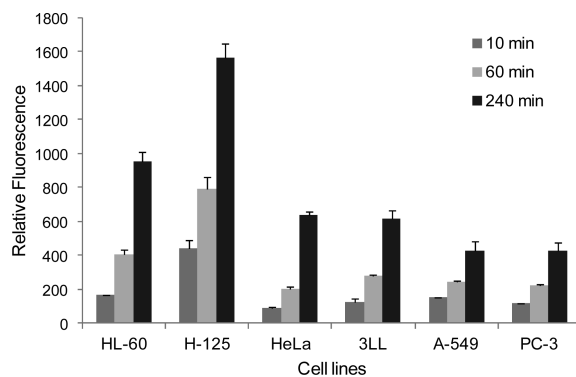
tumor cell line	origin	IC <sub>50</sub> (μmol/L) <sup>b</sup>
HL-60	human promyelocytic leukemia	30 ± 3
H-125	human non-small-cell lung cancer	68 ± 9
HeLa	human cervical carcinoma	132 ± 18
3LL	murine Lewis lung carcinoma	143 ± 4
A549	human lung carcinoma	171 ± 12
PC-3	human prostate cancer	201 ± 16

<sup>a</sup>CIGB-300 concentrations ranging from 0 to 200 μM were added to 5,000 cells per well and incubated for 72 h at 37 °C in 5% CO<sub>2</sub>. IC<sub>50</sub> values were calculated from the growth curves. <sup>b</sup>Mean ± SD of three independent experiments.

differential antiproliferative effect in the tumor cell lines evaluated. CIGB-300 exerts a wide antiproliferative effect in the micromolar range (30–200 μM) in tumor cells from lung, leukemia, cervix, and prostate. On one hand, the human cells derived from non-small-cell lung cancer and promyelocytic leukemia seemed to be highly sensitive to CIGB-300 with an IC<sub>50</sub> range of 30 to 100 μM. On the other hand, cervix adenocarcinoma, prostate cancer, lung adenocarcinoma, and murine Lewis lung carcinoma showed a low-sensitivity effect to

CIGB-300 (IC<sub>50</sub> range: 100–200 μM) in these experimental conditions.

**Kinetics of CIGB-300 Internalization.** Cancer cells were incubated with the fluorescein-labeled CIGB-300 (CIGB-300-F) for 10, 60, and 240 min, and the amount of internalized peptide was evaluated by flow cytometry. The cellular uptake of peptide was determined by the relative fluorescence intensity of the cells (Figure 1). We found that highly sensitive cell lines (H-125, HL-60) incorporated a greater amount of the CIGB-300 compared to low-sensitive cells at all times evaluated.



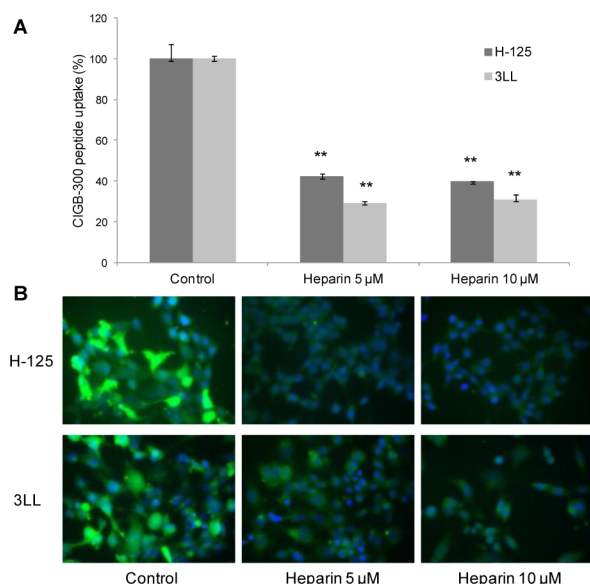
**Figure 1.** Intracellular uptake measurements of CIGB-300 in different tumor cell lines. Comparative flow cytometric assay of the cellular uptake kinetics of CIGB-300. Cell lines were incubated at 37 °C with a carboxyfluorescein-labeled peptide at a concentration of 50 μM for 10, 30, and 240 min. Bars represent relative fluorescence intensity (mean), and the error bars represent the standard deviation.

To simplify and better understand the results, we decided to choose one representative cell line from each population: H-125 as high-sensitive and 3LL as low-sensitive.

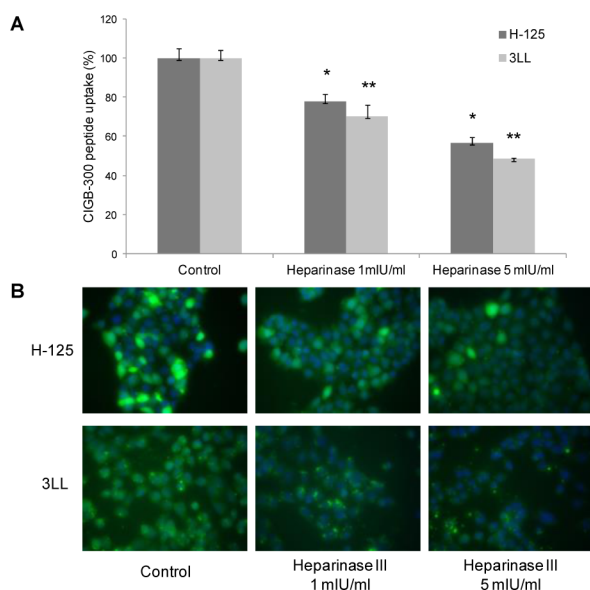
**Heparan Sulfate (HS) Receptor Involved in CIGB-300 Internalization.** Evidence suggests that Tat uptake depends on the presence of HS receptors.<sup>22–24</sup> First we studied the effect of soluble heparin on CIGB-300 internalization. Heparin is a close structural homologue of the HS glycosaminoglycans (GAGs), a major constituent of cell surface and matrix proteoglycans,<sup>25</sup> thus suggesting that heparin could compete with cell surface HS proteoglycans for binding to CIGB-300. In order to evaluate this hypothesis, cells were preincubated for 30 min with heparin in PBS and then incubated with CIGB-300-F for 60 min. Internalization of fluorescent peptide was analyzed both by flow cytometry (Figure 2A) and by fluorescence microscopy (Figure 2B). Heparin almost completely prevented CIGB-300-F entry in both H-125 and 3LL tumor cell lines. These two experiments demonstrated an inhibition of peptide internalization by heparin. In addition, we studied CIGB-300 internalization after removal of cell surface HS GAGs by heparinase III (Figure 3). Cells were treated with heparinase III, incubated with CIGB-300-F peptide, and analyzed 1 h later by flow cytometry and fluorescence microscopy (Figures 3A and 3B respectively). Cell treatment with an enzyme mostly active on HS<sup>23</sup> impaired CIGB-300 internalization in a dose-dependent manner in both tumor cell lines.

**Uptake Kinetics of CIGB-300 upon Shutting off Endocytic Pathways.** To further characterize the uptake mechanism, we studied the effects of low temperature and ATP depletion on CIGB-300 uptake in H-125 (high-sensitive) and 3LL (low-sensitive) tumor cell lines. The cells were incubated with fluorescein CIGB-300-F for 10, 60, and 240 min, and the





**Figure 2.** Inhibition of the internalization of CIGB-300-F (50  $\mu$ M) by treatment of H-125 and 3LL cells with soluble heparin. Cells were pretreated with sodium heparin (5, 10  $\mu$ M) for 30 min, and then fluorescein-labeled CIGB-300 was added to the medium. After 60 min of incubation, the cells were washed and fixed, and cellular uptake was analyzed with FACS (A) or fluorescence microscopy (B). Cells pretreated with PBS were used as control. \*\* $p \leq 0.01$  vs control (internalization at 37  $^{\circ}$ C).



**Figure 3.** The effect of enzymatic digestion of cell surface HS proteoglycans on CIGB-300 uptake. H-125 and 3LL cells were incubated with heparinase III (1, 5 mIU/mL) overnight, and then incubated with 50  $\mu$ M CIGB-300-F for 60 min. This short incubation time was chosen to minimize synthesis of novel proteoglycans. After this period cells were extensively washed and analyzed by FACS (A) and fluorescence microscopy (B). Cells pretreated with PBS were used as control. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$  vs control (internalization at 37  $^{\circ}$ C).

amount of internalized peptide was evaluated using FACS and fluorescence microscopy (Figures 4A and 4B respectively). As shown in Figure 4, CIGB-300 uptake was greatly inhibited at 4  $^{\circ}$ C in both of the tested cell lines. When cells were preincubated with sodium azide and rotenone to deplete the

cellular ATP pool, a smaller but still significant inhibition of CIGB-300 uptake was observed.

**Effect of Endocytosis Inhibitors on the CIGB-300 Uptake.** Then we determined whether cellular uptake of CIGB-300 occurs through a specific endocytic pathway or not. Removal of cholesterol from the plasmatic membrane disrupts several lipid raft-mediated endocytic pathways, including caveolae and macropinocytosis.<sup>26</sup> We pretreated tumor cells for 30 min with nystatin to sequester cholesterol from the plasmatic membrane, or amiloride, a specific inhibitor of  $\text{Na}^+/\text{H}^+$  exchange required for macropinocytosis. Cells were incubated with CIGB-300-F for 60 min, and the amount of internalized peptide was evaluated using FACS. As shown in Figure 5, CIGB-300 uptake was significantly inhibited with nystatin while a small inhibition of CIGB-300 internalization was observed with the amiloride treatment.

#### Evaluation of Intracellular Trafficking of CIGB-300.

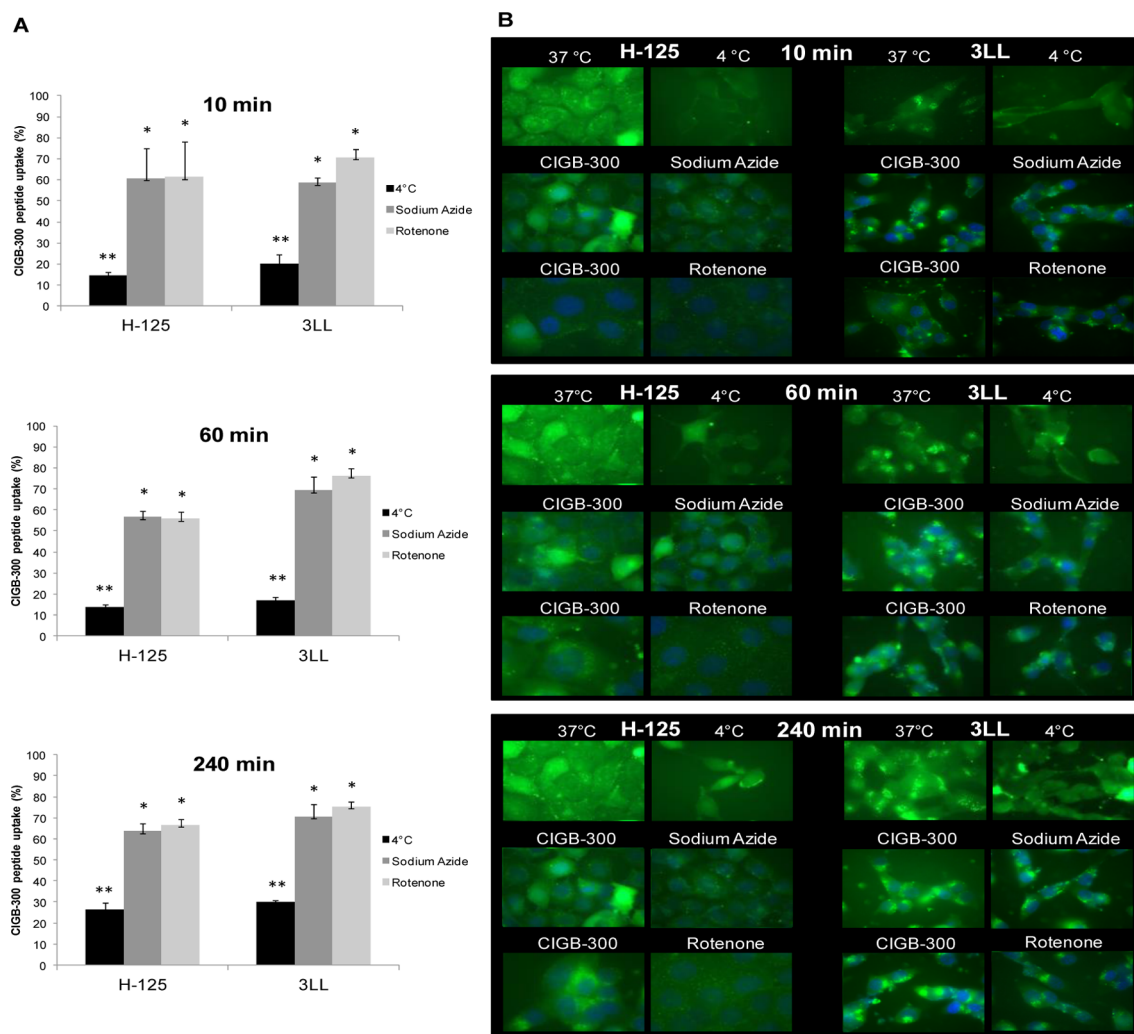
Moreover, we corroborated the endocytic pathway involved in CIGB-300 trafficking in tumor cell lines. We studied the colocalization of labeled CIGB-300-F with vesicular markers from clathrin and caveolae pathways such as EEA1 and CAV-1. Figure 6 shows the merged immunofluorescence photographs of CIGB-300-F and CAV-1 protein, suggesting that this peptide uses caveolae-mediated endocytosis. On the contrary, EEA1 protein did not colocalize with the CIGB-300, indicating that the clathrin pathway is not being used in this model (Supporting Information Figure S1)

**Internalized CIGB-300 Is Degraded in Acidic Compartments.** As endocytosed proteins pass from early to late endosomes and to lysosomes, the vesicular compartments undergo a progressive acidification. We took advantage of the fact that late endosomes and lysosomes show an acidic pH (4–6), therefore a pH-sensitive fluorophore was employed that emits light only at acidic compartments. We incubated H-125 and 3LL cells with 50  $\mu$ M CIGB-300-F for different time lapses (1, 4, 8, 12, and 24 h) followed by a 2 h incubation in the presence of LysoTracker. Localization of CIGB-300 in acidic compartments, which conceivably corresponds to late endosomes/lysosomes, was demonstrated by image merging (Figure 7). We found that colocalization of CIGB-300 and acidic vesicles occurred earlier in low-sensitive cell lines (8 h, 3LL), whereas colocalization was observed many hours later (24 h, H-125) in high-sensitive cell lines. In our Supporting Information, we added the colocalization of CIGB-300 and LysoTracker for the other studied cell lines (Supporting Information Figure S2).

## DISCUSSION

In this article, we described the internalization, trafficking, and degradation mechanisms of the proapoptotic peptide CIGB-300 in different tumor cell lines. CIGB-300 has been described as a cyclic peptide that impairs CK2 phosphorylation by targeting the phosphoacceptor site on its substrates rather than the enzyme per se.<sup>6</sup> In both previous preclinical<sup>7,27,28</sup> and preliminary clinical findings<sup>8,29</sup> we have described the antineoplastic effects of CIGB-300 in different tumor models.

The results found in this work evidenced that sensitivity toward CIGB-300 varies depending on tumor cell line, showing a varied antiproliferative effect (Table 1). To study this difference we separated the cell lines into two populations: a high-sensitive one ( $\text{IC}_{50}$  less than 100  $\mu$ M) and a low-sensitive one ( $\text{IC}_{50}$  higher than or equal to 100  $\mu$ M). Therefore, we focused on the characterization of CIGB-300 internalization to give an explanation for the observed differential responses in



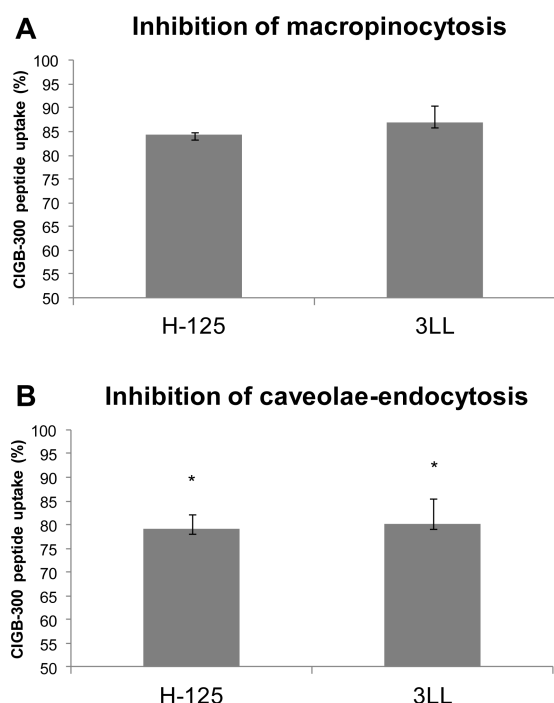
**Figure 4.** Cellular uptake of CIGB-300 is inhibited at 4 °C and by depletion of cellular ATP in tumor cells. Panel A, flow cytometry analysis. Panel B, immunofluorescence microscopy photographs. Lung cancer cells 3LL and H-125 were incubated for 10, 60, and 240 min in the presence of 50  $\mu$ M labeled CIGB-300 at 37 °C or at 4 °C or after depletion of the cellular ATP pool. Uptake is expressed as the median of cell fluorescence distribution (normalized to the cell fluorescence distribution median in untreated control cells at 37 °C). Means  $\pm$  SD are indicated. For depletion of cellular ATP pool, cells were preincubated for 30 min with sodium azide (10 mM) and rotenone (1  $\mu$ M). Significant values (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ).

both populations. Data from flow cytometry analysis revealed that CIGB-300 uptake in tumor cells is similar to that described for the Tat peptides in terms of kinetics and concentration.<sup>30,31</sup> The most relevant result that we found is that the rate and amount of internalized peptide correlates with the cellular sensitivity for CIGB-300. Highly sensitive cells (H-125, HL-60) showed a major incorporation of peptide in comparison to low-sensitive ones (3LL, A549, Hela, and PC3), probably due to differences in membrane composition.<sup>32,33</sup> Thus, the potential antitumor effect of CIGB-300 on highly sensitive cell lines derived from lung cancer and leukemia would be worthy of further studies.

Common internalization mechanisms, such as classical receptor- or transporter-mediated, might not be involved in CIGB-300 uptake. Accordingly, the inverse and retroinverse forms of Tat show the same pattern of internalization as the native peptide, invalidating the involvement of a determined receptor-based process.<sup>24,34</sup> The high cationic nature of Tat peptide and the many membrane anionic motifs such as HS, heparin, or phospholipids determine the first stage of the Tat internalization process through direct ionic interactions. The

HS proteoglycans receptors were demonstrated to be involved in the internalization of Tat peptide by internalization studies, which used CHO mutant cells, with partial or total inhibition of HS metabolism.<sup>13,34</sup>

Heparan sulfate proteoglycans (HSPGs) are macromolecules expressed on cell surfaces and in the extracellular matrix of most animal tissues. HSPGs are important for development and homeostasis in animals and are implicated in several pathological processes. The HSPGs functions are mainly exerted by the interaction between the HS side chains and diverse types of ligands, including various molecules such as cytokines, enzymes, and pathogens.<sup>35</sup> The literature shows that HS receptors are ubiquitous to the cell surface of both vertebrate and invertebrate species. Furthermore, these receptors are homologous in human, monkey, and rodent cells.<sup>36,37</sup> The role of HS in CIGB-300 uptake was tested in two different ways. In the first experiment, heparin was added to the cell medium, competing with the cellular acceptor for the CIGB-300. Under this condition, CIGB-300 uptake was strongly inhibited in high- and low-sensitive cell lines (Figure 3), indicating that exogenous heparin acts as a competitive

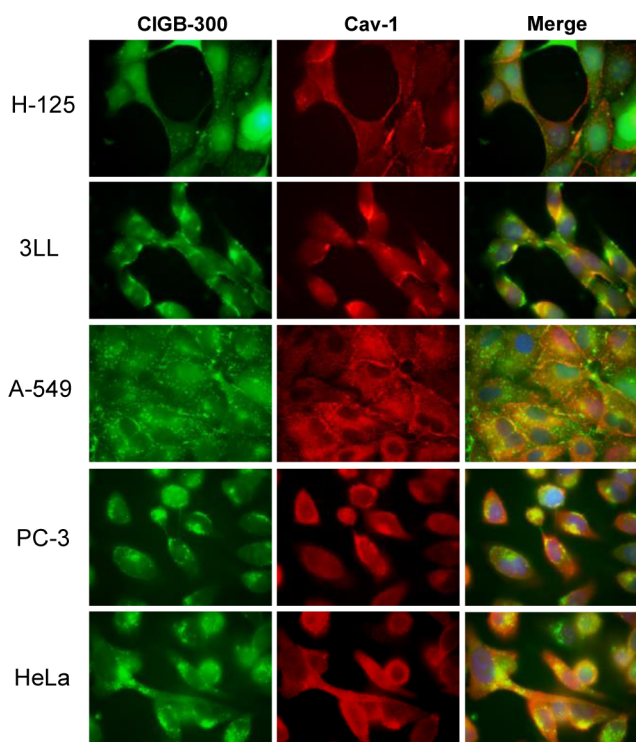


**Figure 5.** Effect of amiloride and nystatin in cellular uptake of CIGB-300. For inhibition of macropinocytosis and caveolae, samples were pretreated 30 min with amiloride (A) (5 mM) or nystatin (B) (25 µg/mL). Lung cancer cells H-125 and 3LL were incubated for 60 min in the presence of 50 µM CIGB-300-F at 37 °C. Uptake was measured by flow cytometry and is expressed as the median of cell fluorescence distribution (normalized to the cell fluorescence distribution median in untreated control cells). Control cells were treated with the same concentrations of DMSO in each experiment (less than 0.5%). Means  $\pm$  SD. Significant values (\* $p \leq 0.05$ ).

inhibitor for peptide uptake.<sup>24</sup> Likewise, the phosphate anionic groups of the fatty acids composing the plasmatic membrane could also be directly implicated in ionic interactions with guanidinium groups on the CIGB-300 molecule.<sup>25</sup>

In the second assay, membrane-bound HS were enzymatically removed prior to the administration of CIGB-300. Treatment with heparinase III, an enzyme that degrades HS chains, significantly reduced the amount of internalized CIGB-300 (Figure 4). These results indicate that CIGB-300 uses HS receptors for internalization. However, the uptake of CIGB-300 (a Tat conjugated peptide) might be also mediated by other pathways as suggested by the fact that the internalization of CIGB-300 is not completely inhibited in cells preincubated with heparin or after enzymatic treatment. The promiscuity of CPPs in terms of receptor use is not surprising given the highly cationic nature of the Tat peptide and the abundance of negatively charged moieties on the cell surface.<sup>24,38</sup>

The exact mechanisms by which CPPs gain access into the cells are not completely understood. It is admitted that the electrostatic interaction between CPPs and cell surface proteoglycans are involved in the primary stage of the cellular internalization process.<sup>39,40</sup> The interaction between CPPs and GAGs induces their clustering on the cell surface and activates intracellular signal pathways that influence the remodeling of the actin cytoskeleton, inducing entry through different routes of cellular internalization (direct translocation and endocytic process). Furthermore, different mechanism of membrane

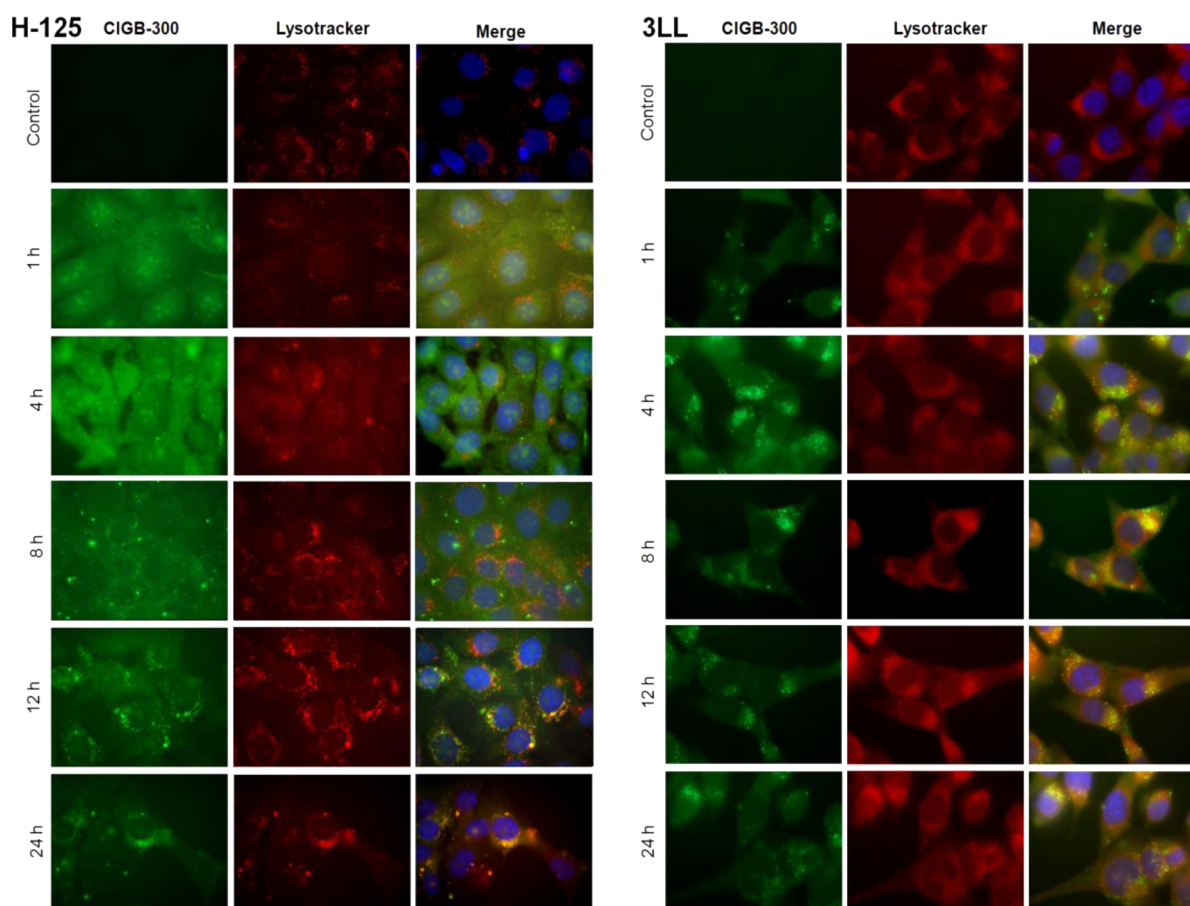


**Figure 6.** Caveolar vesicular trafficking of CIGB-300. Different tumor cell lines were incubated with CIGB-300-F (50 µM) for 60 min at 37 °C. Colocalization of CIGB-300 and caveolin-1 was detected by immunofluorescence (Merge).

translocation and endocytosis process may coincide at the same time for most CPPs.<sup>16,39</sup>

It was recently found that the main mechanism for traversing the extracellular membrane for Tat peptides is endocytosis.<sup>39,41,42</sup> General assumption is that temperatures above 4 °C and ATP are required for peptide internalization by endocytosis. However, our results indicate that CIGB-300 uptake was inhibited approximately 35% when the intracellular level of ATP was depleted using sodium azide or rotenone. The use of higher concentrations or longer incubation times for ATP inhibitors could be toxic for cells or may upregulate other internalization mechanisms. For that reason, we believe that there is not one single pathway exclusively employed by any CPP, but rather a predisposition for some mechanism. Several models were postulated to clarify CPPs' translocation through biological membranes. These models are based on the direct interaction of these peptides with phospholipids and other membrane components. First, Derossi et al. described the "inverted micelle model" in which the interaction of CPPs with biological membranes would lead to a perturbation of the lipid bilayer, involving the formation of inverted micelle structures. The CPPs would be trapped and surrounded by the hydrophilic environment of the micelle until their interaction with membrane components which caused the release of peptides into the cells.<sup>43</sup> Another proposed mechanism involves the formation of transient pores; here the interaction of CPPs in cellular membrane induces an insertion and oligomerization of the peptides in a "barrel stave" structure. The last model that explains direct translocation is the "carpet model", where the translocation of CPPs across membrane occurs due to a transient destabilization of the cellular membrane that is produced by a direct interaction between peptides and





**Figure 7.** Kinetic degradation of CIGB-300 in lysosomes. Cancer cell lines were incubated at different times (1, 4, 8, 12, 24 h) at 37 °C in the presence of fluorescein-labeled CIGB-300 (50  $\mu$ M). During the last 2 h of incubation, LysoTracker was added to the culture medium. Intracellular degradation of internalized CIGB-300-F in the course of time was monitored by colocalization with LysoTracker as visualized by immunofluorescence microscopy.

plasmatic membrane, causing the phospholipids reorganization.<sup>43</sup> The results obtained in this work support the idea that CIGB-300 could use a simultaneous translocation mechanism to penetrate the cellular membrane. The high concentrations of peptide used in this work could support this hypothesis.<sup>44</sup>

The complete obstruction of endocytosis was further confirmed when the temperature was decreased to 4 °C, and internalization of CIGB-300 was blocked. The reduction of peptide internalization at low temperature (Figure 4) could be due to the lack of energy dependent mechanisms and changes in the physical structure of the lipid bilayer.<sup>45</sup> At low temperature, growth and metabolism are clearly limited as a consequence of the decrease in several processes such as membrane fluidity, affinity of enzymes for their substrates, reaction rates, and thermal energy. Additionally, the low temperature could also increase the water viscosity.<sup>46</sup> In addition, Letoha and co-workers studied the effect of membrane fluidity on CPPs uptake. They used fibroblasts with different membrane fluidity and found low uptake of all the studied CPPs in the cell lines with quite rigid membrane fluidity and a high signal of incorporation in high fluidity membrane cells.<sup>47</sup>

The results found using ATP inhibitors and low temperature incubation demonstrate that internalization of CIGB-300 occurs by mechanisms both dependent on and independent of energy, and the decrease of plasmatic membrane fluidity further minimized the uptake of the peptide.

At least four morphologically different endocytic pathways have been characterized: macropinocytosis, clathrin-mediated, caveolae-mediated, and clathrin/caveolae-independent endocytosis. These pathways differs in the coating composition (if any), in the size of vesicles, and in the final destination of the internalized particles.<sup>48</sup> The cellular endocytic pathway determines the cell-compartment destination of the CPPs. Clathrin-mediated endocytosis involves a degradative pathway from early to late endosomes, and finally to lysosomes. On the other hand, in the caveolae-mediated route the vesicles are targeted to others organelles such as the Golgi apparatus or the endoplasmic reticulum.<sup>49</sup>

In concordance with previous reports,<sup>50–53</sup> we found in two different experiments that caveolae endocytosis was involved in CIGB-300 internalization. The uptake of CIGB-300 was significantly reduced with nystatin pretreatment (Figure 5), and the colocalization with cav-1 (Figure 6) confirmed caveolae mediated endocytosis in CIGB-300 uptake.

CIGB-300 did not colocalize with EEA1 (Figure S1 in the Supporting Information), suggesting that the clathrin pathway is not implicated in the endocytosis of this peptide. This result is in disagreement with previous publications.<sup>33,51</sup> Moreover, the inhibition of macropinocytosis by amiloride, which inhibits the  $\text{Na}^+/\text{H}^+$  exchange protein in the plasmatic membrane, does not show a significant decrease in CIGB-300 internalization, in contrast to other authors who consider macropinocytosis as the principal route for Tat peptides internalization.<sup>26,31,44</sup> This

could be due to the different physicochemical characteristic of compound CIGB-300 compared with native Tat protein.

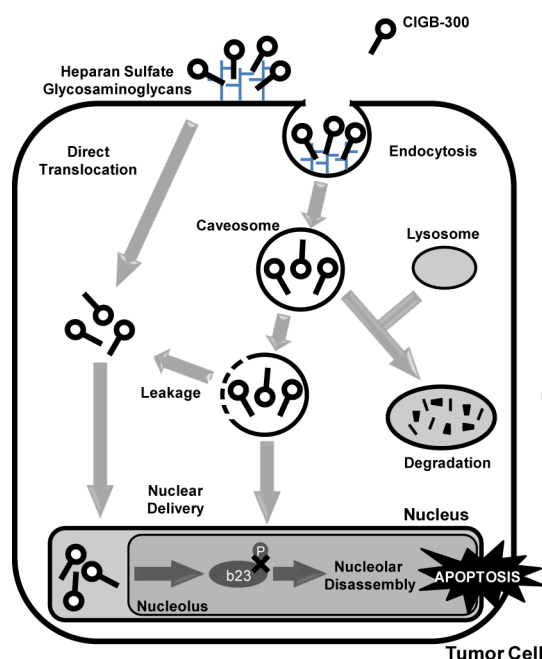
At the moment, we cannot discard the involvement of other endocytic mechanisms (like clathrin/caveolae-independent pathway) in CIGB-300 internalization. Furthermore, our data does not enable us to distinguish other pathways of cellular uptake; future studies will be necessary to elucidate other possible mechanisms involved in CIGB-300 internalization.

A remaining question is how is CIGB-300 released from HS and how does it escape from endocytic vesicles. HS located within the internalized vesicles is cleaved by heparanases, first in endosomes of neutral pH and then in acidic late endosomes.<sup>54</sup> The HS cleavage could decrease its anion valency, and this phenomenon could reduce affinity for CIGB-300. After that, CIGB-300 could escape to the cell cytoplasm by different mechanisms of endosomal escape as suggested by Erazo-Oliveras.<sup>55</sup>

It was assumed in the literature that caveosomes are nondegradative endosomal compartments with neutral pH, and their intracellular trafficking is slower than that of other noncaveolar pathways.<sup>53</sup> Nevertheless, a recent work details how caveolin trafficking using pH-sensitive fluorophores and did not support the existence of a neutral pH compartment as an essential intermediate in the trafficking of caveolae particles, suggesting that the caveolae pathway may use a lysosomal mechanism for degradation.<sup>56</sup>

Eukaryotic cells have several intracellular pathways for protein degradation, but the main and most nonselective protein degradation pathway takes place in the lysosomes. Lysosomes are responsible for the degradation of both exogenous proteins and some cytoplasmic proteins and cellular organelles.<sup>17,57</sup> For that reason, we studied the lysosomal degradation pathway. CIGB-300 colocalizes with active lysosomal vesicles in all studied cell lines, which would presumably indicate that this peptide was degraded at least in part by this pathway (Figure 7). Interestingly, we found that, in low-sensitive cells, the degradation of CIGB-300 begins at earlier times (8 h) than in highly sensitive cells (24 h), suggesting that this degradation rate could collaborate with the sensitivity of the cells toward CIGB-300. We did not study the additional cytosolic proteolytic mechanism that involves the proteasome, which degrades proteins flagged with ubiquitin molecules.<sup>17,57</sup> We consider this pathway as a possible route for degradation due to the fact that Tat peptide and CIGB-300 have a molecular signal for intracellular protein degradation, and consequently could also be degraded by the ubiquitin–proteasome system. However, the strong signal of CIGB-300 inside the active lysosomes found in our experiments makes us think that the place for degradation of CIGB-300 could occur in this organelle.

In summary, internalization of CIGB-300 is a multistep process, as represented in Figure 8. The result of this work showed that CIGB-300 is able to interact with HS proteoglycans at the cell membrane. Through this interaction, the peptide could enter the cell by energy-dependent and independent mechanisms of internalization. Most of CIGB-300 uses a direct translocation to penetrate the plasmatic membrane. Also, CIGB-300 could be internalized by a caveolae-mediated pathway in an energy-dependent mechanism. Once inside the endocytic vesicles, CIGB-300 could exit to the cell cytoplasm by different mechanisms of endosomal escape. In this model, CIGB-300 is degraded in acidic vesicles via lysosomes. Our previous data suggest that CIGB-300 is



**Figure 8.** Model of cellular uptake and intracellular trafficking of CIGB-300. The results of this work show that CIGB-300 is able to interact with HS proteoglycans in the cell membrane. Through this interaction, the peptide could enter the cell by energy-dependent and independent mechanisms of internalization. A great proportion of CIGB-300 uses direct translocation uptake for movement of peptide across the plasmatic membrane. On the other hand, CIGB-300 was internalized by caveolae-mediated pathway using an energy-dependent mechanism. Once in the endocytic vesicles CIGB-300 made its way into the cell cytoplasm by different mechanisms of endosomal escape, or otherwise inside late endosomes CIGB-300 can be degraded by the lysosomal pathway. Finally, CIGB-300 was transported from the cytoplasm to the nucleus by the nuclear localization signal of Tat. When the CIGB-300 reaches the nucleolus, it inhibits the CK2-mediated phosphorylation on the NPM with subsequent nucleolar disassembly and induction of apoptosis.

transported from the cytoplasm to the nucleus by the nuclear localization signal of Tat, and when the CIGB-300 reaches the nucleolus, it inhibits the CK2-mediated phosphorylation on the NPM with the subsequent nucleolar disassembly and induction of apoptosis.<sup>10,58</sup>

Altogether, the data provided here proposes a mechanism of internalization, trafficking, and degradation pathway for CIGB-300 that could explain the differential sensitivity to CIGB-300 observed in tumor cells. To better understand these mechanisms, more studies addressing the antitumoral effect of CIGB-300 in preclinical high- and low-sensitive animal tumor models derived from lung cancer and leukemia are needed. In conclusion, these results present a contribution in the understanding of the different mechanisms by which Tat-conjugated peptides deliver cargos into the cells, and how this mechanism can improve different therapeutic strategies.

## ■ ASSOCIATED CONTENT

### 🔍 Supporting Information

Immunofluorescence colocalization between CIGB-300/EEA1 and CIGB-300/LysoTracker for human studied cell lines. This material is available free of charge via the Internet at <http://pubs.acs.org>.



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### Notes

The authors declare no competing financial interest. Daniel Gomez, Daniel Alonso and Hernán Farina are members of the National Council for Scientific and Technical Research (CONICET, Argentina). Daniel Gomez is also a member of the Board of Directors of the National Cancer Institute of Argentina.

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