Title: Synergic effects of anticancer peptide CIGB-552 and Cisplatin in lung cancer models

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Abstract

Non-small cell lung cancer constitutes one the most frequent and lethal forms of the disease. The antitumor peptide CIGB-552 is a new targeted anticancer therapy which molecular mechanism is associated with the inhibition of the transcription factor NF-kB, mediated by COMMD1 protein stabilization. However, its pharmacological potential in combination with chemotherapy is unknown. In this study, we examined the antiproliferative capacity of CIGB-552 in combination with chemotherapeutic agents in the non-small cell lung cancer cell line NCI-H460 and we confirmed drug interactions in vivo, in a mouse model of TC-1 lung cancer. We focus our research in the combination of CIGB-552 and the antineoplastic agent Cisplatin (CDDP) in a concomitant treatment. Our results demonstrate a clear synergic effect between 37.5 μ M of CIGB-552 and 5 μ M of CDDP under concomitant scheme, on proliferation inhibition, cell cycle arrest, apoptosis induction and oxidative stress response. The effect of CIGB-552 (1 mg/kg) and CDDP (0.4 mg/kg) administrated as a combined therapy was demonstrated in vivo in the TC-1 murine model where the combination achieved an effective antitumor response, without any deterioration signs or side effects. These findings demonstrate the efficacy of the concomitant combination of both drugs in preclinical studies and support the use of this therapy in clinical trials.

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ABSTRACT

Non-small cell lung cancer constitutes one the most frequent and lethal forms of the disease. The antitumor peptide CIGB-552 is a new targeted anticancer therapy which molecular mechanism is associated with the inhibition of the transcription factor NF-kB, mediated by COMMD1 protein stabilization. However, its pharmacological potential in combination with chemotherapy is unknown. In this study, we examined the antiproliferative capacity of CIGB-552 in combination with chemotherapeutic agents in the non-small cell lung cancer cell line NCI-H460 and we confirmed drug interactions *in vivo*, in a mouse model of TC-1 lung cancer. We focus our research in the combination of CIGB-552 and the antineoplastic agent Cisplatin (CDDP) in a concomitant treatment. Our results demonstrate a clear synergic effect between 37.5 μ M of CIGB-552 and 5 μ M of CDDP under concomitant scheme, on proliferation inhibition, cell cycle arrest, apoptosis induction and oxidative stress response. The effect of CIGB-552 (1 mg/kg) and CDDP (0.4 mg/kg) administrated as a combined therapy was demonstrated *in vivo* in the TC-1 murine model where the combination achieved an effective antitumor response, without any deterioration signs or side effects. These findings demonstrate the efficacy of the concomitant combination of both drugs in preclinical studies and support the use of this therapy in clinical trials.

Key words: NSCLC, antitumor, synergism, combined therapy, CIGB-552, CDDP

INTRODUCTION

Cancer represents one of the most challenging diseases in XXI century. Malignant transformation is a multistep process, where cancer cells gain properties like immune evasion, apoptosis resistance, insensitivity to antiproliferative signals, invasion, angiogenesis and metastasis. (Hanahan and Weinberg 2011). The website GLOBOCAN and the International Agency for Research on Cancer (IARC) estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018. In both sexes combined, lung cancer is the most commonly diagnosed (11.6% of the total cases) followed by female breast cancer and prostate cancer and is currently the leading cause of cancer death (18.4% of the total cancer deaths), closely followed in mortality by colorectal (9.2%), stomach (8.2%), and liver cancer (8.2%). (Bray, Ferlay et al. 2018, 2020). In Cuba, cancer is the second cause of death (24% of total deaths) just surpassed by heart and circulatory diseases (36,8 % of the total number of deaths) and lung cancer is still the one with more incidence and mortality among Cuban population. (2020).

Non-small cell lung cancer (NSCLC), represents the most frequent subtype of lung cancer with an 85% of incidence. The therapeutic strategies include surgery by early diagnostic, chemotherapy and radiotherapy in advanced cancer. The conventional chemotherapy protocols for NSCLC comprise 4 to 6 cycles of platinumbased doublet chemotherapy in first-line treatment and 6 cycles of docetaxel (or equivalent taxol drug) as a second-line regimen. Both regimens employ unspecific cytotoxic agents, which display numerous side effects (Wagner, Stollenwerk et al. 2020). The toxicity associated to chemotherapy affect the life quality of patients and nephrotoxicity, lymphopenia among other adverse reactions limit its long-term application in cancer therapy in addition to other negative impacts such as multidrug resistance (MDR), mutagenicity and teratogenicity (Bordoni 2008, Tiwari, Sodani et al. 2011). The combination of chemotherapy agents with other drugs that target specific tumor antigens or intracellular proteins such as monoclonal antibodies, peptides or small chemical inhibitors has demonstrated to be an efficient therapeutic strategy in NSCLC and other types of tumor (Li, Zhao et al. 2014, Achkar, Abdulrahman et al. 2018, Wagner, Stollenwerk et al. 2020) This attractive therapy achieves efficacy and decreases toxicity, reducing the doses of the antineoplastic agents without losing their effect. This can also contribute to attenuate MDR (Tallarida 2001, Bayat Mokhtari, Homayouni et al. 2017).

CIGB-552 is an antitumor peptide developed at the Center of Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. Vallespi and her research group have demonstrated its cell penetrating capacity and proliferation inhibition of human and murine cancer cells in vitro (Fernández Massó, Oliva Argüelles et al. 2013, Astrada, Fernández Massó et al. 2018). Likewise, CIGB-552 elicits tumor regression in vivo, in syngeneic and xenograft mice models (Vallespí, Pimentel et al. 2014) as well as the stabilization of the disease and therapy improvement in pet dogs with spontaneous cancer (Vallespi, Rodriguez et al. 2017). The molecular studies of this peptide have shown inhibitory effects on NF-kB pathway through the stabilization and accumulation of the Copper Metabolism Mur 1 Domain Containing Protein 1 (COMMD1). This effect induces the ubiquitination and degradation of the NF-kB subunit RelA. In addition, CIGB-552 induces oxidative stress in tumor cells by the inhibition of the enzyme Superoxide-dismutase 1 (SOD1), causing lipid and protein peroxidation (Fernández Massó, Oliva Argüelles et al. 2013). These findings complemented by additional evidence in proteomic studies (de Villavicencio-Díaz, Gómez et al. 2015) suggest that the combination of CIGB-552 and conventional chemotherapeutic drugs such as Cisplatin (CDDP) or Paclitaxel could be attractive in preclinical and clinical settings. Therefore, in this work, we evaluated for the first time the combination of CIGB-552 with these antineoplastic agents widely use in lung cancer therapy, in the human NSCLC cell line NCI-H460. We explored two combination settings: pre-treatment and concomitant in order to select the optimal combination scenery to use in clinical trials, based on the estimation of the combination index (CI) (Chou 2006). All the interactions were classified as synergistic, additive or antagonistic and their proliferation inhibition capacity was compared. The best combination was evaluated in terms of its effect on cell cycle progression, apoptosis induction and oxidative stress triggering. Furthermore, the potential benefit of the best combination and therapeutic scheme was corroborated in vivo in a mouse model of lung cancer.

MATERIALS AND METHODS

Reagents and Chemicals

All regents and chemical substances used in this study were purchase from Sigma-Aldrich. Culture media, fetal bovine serum (FBS), and cell culture material were obtained from Life Technologies (USA), GE Heal-thcare, and Greiner. All reagents for peptide synthesis were of synthesis grade. Reagents for chromatography were of high-performance liquid chromatography (HPLC) grade.

Antineoplastic drugs

The clinical grade chemotherapeutic drugs Cisplatin (CDDP), and Paclitaxel (Drug Research and Development Center, Havana, Cuba) were kindly provided by the Oncology Service of the National Institute of Oncology and Radiobiology (Havana, Cuba). Both were dissolved in buffered saline solution (PBS) for *in vitro* experiments and CDDP was also prepared in PBS for its administration *in vivo*.

CIGB-552

Peptide CIGB-552 was synthesized on a solid phase and purified by reverse-phase-HPLC to >95% purity on an acetonitrile/H₂O trifuoracetic acid gradient and confirmed by ion-spray mass spectrometry (Micromass, Manchester, UK). The synthesis of the peptide in solid phase was performed using the Fmoc/t-Bu chemistry. The linking is direct to the N-terminus of the peptide; there are no additional residues. Lyophilized peptide was reconstituted in phosphate buffered saline (PBS) for use *in vitro in vivo*.

Cell lines

NCI-H460 (human non-small cell lung carcinoma), MRC-5 cells (human embryonic lung fibroblasts) and TC-1 (murine lung epithelial cells transfected with VPH-16) were acquired from the ATCC and cultured in RPMI 1640 (for NCI-H460 and TC-1) and DMEM (for MRC-5), supplemented with Glutamax and 10% (v/v) FBS according to the recommendations of the supplier. Cells were maintained in a 5% CO₂ atmosphere at 37°C in incubator. Cells were cultured for no longer than 10–15 passages.

Antiproliferative assays

The effect of CIGB-552, CDDP and Paclitaxel on cell proliferation was then evaluated by a sulforhodamine B (SRB)-based assay according to the method described by the National Cancer Institute (Boyd 1997). NCI-H460 Cells were seeded at a density of 4×10^4 cells per well in a 96-well plate (Costar, USA). Then after 24 hours they were incubated with the products (10, 40 and 100 μ M of CIGB-552; 0.05, 5 and 50 μ M of CDDP and 0.2, 20 and 200 μ M of Paclitaxel) for 48 hours and the viability relative to untreated cells was measured by the SRB method. The percentage of cell proliferation inhibition was determined using CalcuSyn software (Version 2.1; Biosoft, Cambridge, UK). The assay was performed twice with three replicas for each concentration.

In vitro drug combination study

The drug combination study was carried out in 96-well plates seeded with NCI-H460 cells $(4x10^4 \text{ cells per well})$ and the cell viability was measured after 48 hours of treatment using the (SRB)-based assay as we described above. Cells were treated with different concentrations of CIGB-552 (300 - 9.37 μ M) and the antineoplastic drugs (500 - $5x10^{-3} \mu$ M of CDDP or 2 - $2x10^{-4} \mu$ M of Paclitaxel) according to a Latin square design (Chou 2006). The assay was performed under two different treatment schemes: concomitant (CIGB-552 and the chemotherapeutic agent were added at the same time) and pre-treatment (cells were pre-incubated with CIGB-552 for 5 hours, then it was eliminated from culture media and the cytostatic drug was added). The effect on cell proliferation was determined relative to untreated cells in two independent experiments. The results were analyzed with CalcuSyn software to determine the type of interaction (synergism, additivity or antagonism), according to the obtained combination index (CI) values. The software also calculated the dose reduction index (DRI) and the fraction affected (Fa) which is related with the magnitude of effect for each combination. The 2D interaction maps with color-coded surfaces were created using Matlab R R2012a software based on the CI and Fa values.

Cell cycle analysis

The cell cultures were incubated with CIGB-552 (37.5 μ M), CDDP (5 μ M) and their combination for 12 and 24 hours. Subsequently, the cells were collected by trypsinization, washed and fixed with ice-cold methanol/acetone (4:1) at 4@C for 1 hour. Cells were then stained by incubation for 20 min at 37@C with a solution of 100 mg/ml PI and 10 mg/ml of DNase-free RNase. Stained cells were analyzed on a Becton Dickinson FACSCalibur cytometer using the cell cycle analyzer from CellQuest software. Prior to fitting the DNA distribution to a diploid DNA content for cell cycle profiling, cellular debris and doublets were properly excluded by gating out in FL3-A vs. FL3-W two-parameter dot plots.

Western Blot of apoptosis related proteins

NCI-H460 cells were treated with CIGB-552 (37.5 μ M), CDDP (5 μ M) and both drugs combined, as well as 1 μ M of Staurosporin (STS) as positive control. Cell fractioning was performed as described previously (Thoms, Loveridge et al. 2010) using Lysis Buffer (Cell Signaling, USA) and protein inhibitor cocktail (Roche, USA), according to the instructions of the supplier. Proteins in whole cell extracts (30 μ g) were resolved by electrophoresis on 12.5% and 15% polyacrylamide gels (Bio-Rad technology, USA) and analyzed according to the western blotting technique previously described (Burnette 1981). Briefly, the proteins were transferred to a nitrocellulose membrane (pore size 0.45 mm) and incubated with the appropriate primary antibody (1:500-1:1000 dilution). After incubation with peroxidase-conjugated secondary antibody (1: 2000), protein specific bands were visualized using chemiluminescence reagents followed by exposure to standard X-ray films.

Annexin V/ Propidium iodide double staining

Cells in early and late stages of apoptosis were detected with an Annexin V-FITC apoptosis detection kit from Sigma (041M4083). NCI-H460 cells (1x10⁵ cells per well) were treated with CIGB-552 (37.5 μ M), CDDP (5 μ M) or the combination in 12-well plates (Costar, USA) and incubated for 48 hours prior to analysis. STS (1 μ M) was used as positive control of apoptosis induction. Then, cells were trysinazed, collected and resuspended in 1x binding buffer. To 100 μ L of cell suspension, 5 μ L of Annexin V-FITC and 10 μ L propidium iodide (PI) were added and incubated for 10 min at room temperature prior to analysis. Samples were analyzed (20,000 events) using the Becton Dickinson FACSCalibur instrument and CellQuest software. Cells that were positive for Annexin V-FITC alone (early apoptosis) and Annexin V-FITC and PI (late apoptosis) were counted.

Superoxide anion accumulation assays

The detection of superoxide anion formation as a measure of oxidative stress induction was determined as previously described (Wojtala, Bonora et al. 2014). NCI-H460 and MRC-5 cells were seeded at a density of 6×10^3 and 1×10^4 cells per well, respectively, in 96-well plates. After 24 hours they were treated with CIGB-552 (37.5 μ M), CDDP (5 μ M) and the combination for 1 hour. Hydrogen peroxide (H₂O₂) 2.5 μ M was used as positive control. Then cells were incubated with 10 μ M of Hydroethidine (HE) for 1 hour to visualize the superoxide anion formation. The fluorescence was detected and measured using confocal microscopy (Inverted microscope Olympus, Japan). The images acquired were analyzed with the software ImageJ 1.41 to quantify the fluorescence intensity as fold of control for all treatments. We processed images from two independent experiments.

To confirm the induction of oxidative stress in NCI-H460 cells, we perform a flow cytometry-based assay for HE detection in treated cells according to the method described by Walrand and colleagues (Walrand, Valeix et al. 2003). Briefly, we seeded 1 x 10⁶ cells per well and treated with the products alone and combined for 12 hours. Untreated cells and cells incubated with 2.5 μ M of Hydrogen peroxide (H₂O₂) for 12 hours were the negative and positive control, respectively. Cells were mechanically detached in cold and cell suspensions were obtained by centrifugation at 300 x g for 5 minutes at room temperature. Pellets were re-suspended in PBS 1X and incubated with HE (10 μ M) for 30 minutes at 37°C protected from light. Then cells were analyzed in the Becton Dickinson FACSCalibur cytometer and corresponding CellQuest software. A total of 2x10⁴ events were analyzed and HE positive cells were quantified. We established forward and side scatter gates from negative control cells to exclude debris and cellular aggregates.

In vivo drug interaction experiments

C57/BL6 female mice of 8 weeks old and 18-20 g (a total of 40 animals) were randomly divided into 4 groups of 10 animals each. Mice were injected with $5x10^4$ cells per animal of the murine tumor cell line TC-1 on the right flank, according to the procedures reported for this animal model (Tanaka, Delong et al. 2005). When tumors reached 70-90 mm^3 we start the administration of the products at the indicated doses. Group 1 received saline solution (PBS 1x) by subcutaneous route three times a week during three weeks (control group). Group 2 was subcutaneously administered with 1 mg/kg of CIGB-552, three times a week during three weeks. Group 3 received 0.4 mg/kg of CDDP by intraperitoneal route three times a week only during the first week of administration. Group 4 was administered with a combination of both drugs: CIGB-552 (1 mg/kg) subcutaneously three times a week during three weeks and CDDP (0.4 mg/kg) intraperitoneally three times a week during the first week. Animals were weighted once a week since the beginning of the experiment. Tumors were measured three times a week with a caliper and their volumes were calculated according to the following formula: volume = length x width $^2/2$. Survival rate was daily registered during the experiment and only when tumor volumes reached 2,000 mm³, the animals were sacrificed due to ethical considerations. Mice were maintained under pathogen-free conditions and all the procedures were performed in accordance with the recommendations for the proper use and care of laboratory animals at the Center for Genetic Engineering and Biotechnology (Havana, Cuba). This animal study complies with all the international requirements and is according the National Institutes of Health guide for care and use of laboratory animals.

Statistical analysis

The drug-drug interactions were determined and validated using the software CalcuSyn version 2.0, (1997, Biosoft, EUA). The percentages of cell proliferation inhibition, AV + AV/PI positive cells, HE fluorescence intensity (fold of control) and number of affected animals from *in vivo* experiments were compared between the combination and the individual treatments using one way ANOVA and Dunnet post test. The number of HE positive cells in percent determined using flow cytometry, as well as tumor volumes from *in vivo* experiments were compared between the combination and the individual treatments by unpaired T tests. The statistical significance of differences in survival rates was determined by log-rank test p^{*}<0,05. All statistical analysis were done using the software GraphPad Prism 7.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHAR-MACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACO-LOGY 2019/20 (Alexander et al., 2019).

RESULTS

CIGB-552 and cytostatic drugs are synergic in human lung cancer cells

First, we evaluated and compared the cytotoxicity of our anticancer peptide CIGB-552 in combination with cytostatic drugs commonly used in chemotherapy of lung cancer, such as CDDP and Paclitaxel, in the human lung cancer cell line NCI-H460. We selected this cell line based on previous results obtained in our lab that reveal a major cytotoxic potential in NCI-H460 cells in comparison to other human lung cancer cell lines (data not shown). In addition, the molecular mechanism of CIGB-552 has been fully characterized by us in NCI-H460 cells (Fernandez J et al., 2013). We have previously obtained and corroborated the cytotoxic effect of CIGB-552, CDDP and Paclitaxel in this cell line, with IC_{50} values of 44.6, 1.99 and 0.01 μ M, respectively (data not shown). Based on these evidences we decided to evaluate the antiproliferative capacity of the peptide and both cytostatic drugs separately and combined in NCI-H460 cells, testing different concentrations below and above the IC_{50} as it is recommended. All treatments were able to inhibit cell proliferation in a concentration dependent manner, but this effect was more potent combining the peptide with the cytostatic drugs, particularly at lower concentrations where the effects of both products is not overlapped (Figure 1A and B). To corroborate this possible synergic effect and to identify other pharmacological interactions, the antiproliferative capacity of CIGB-552 combined with CDDP and Paclitaxel in a wider range of concentrations was evaluated in the lung cancer cell line NCI-H460, by an *in vitro* drug combination assay. We treated the cells with CIGB-552 and both cytostatic drugs alone and in combination, using two different settings: concomitant and pre-treatment. The results were analyzed with the software Calcusyn to determine the significance or grade of combination (synergism, additivity and antagonism) based on two main parameters: combination index (CI) and dose reduction index (DRI) (Table 1).

In the concomitant setting, we observed antagonism between 300 μ M of CDDP and 150 μ M of CIGB-552; however, this concentration of the peptide interacted with 50 μ M of CDDP, producing an additive effect with 95% of cell proliferation inhibition. Interestingly, the synergism was observed in concentrations below 150 μ M of CIGB-552 and 50 μ M of CDDP where the cell growth inhibition is still 95% in the range of 37.5 μ M to 9.37 μ M of CIGB-552. The synergism is potent in 0.5 - 5 μ M of CDDP and 18.75 - 9.37 μ M of CIGB-552, with CI values between 0.7 and 0.1 (**Figure 1C**). Compared with the concomitant, pre-treatment analysis showed antagonic effect in maximal and minimal concentrations of both drugs, being potent in two zones: 500 μ M of CIGB-552, demonstrating that the surface of synergism is reduced under this treatment scheme. Therefore, the concomitant scenario is better to combine these drugs in NCI-H460 cells.

The combination of CIGB-552 with Paclitaxel showed a different behavior. In concomitant scheme we observed additivity between various concentrations of Paclitaxel and 150 - 75 μ M of CIGB-552. On the

other hand, the synergic effect was found in concentrations lower than 75 μM of CIGB-552 and 0.2 μM of Paclitaxel, with 90% of cell proliferation inhibition. Specifically, the range 0.002 - 0.0237 μM of Paclitaxel with the full range of CIGB-552 achieves a potent synergism (CI = 0.3 - 0.1) with 85% of cell death. As occurs with de combination of CIGB-552 and CDDP, the pre-treatment setting reduced the synergism surface to the area of 0.002 - 0.02 μM of Paclitaxel with concentrations of CIGB-552 under 150 μM (Figure 1C). In addition, the effect on cell growth inhibition was minor in comparison to the concomitant design.

These results show a clear synergic effect for the peptide with both chemotherapeutic drugs demonstrating a major surface of synergism under the concomitant combination respect to pre-treatment condition. Another aim of this analysis is to predict a reduction of toxicity using the DRI value, especially for drugs such as cytostatics due to their adverse effect during cancer treatment. The DRI of CDDP and Paclitaxel in combination with CIGB-552 is attractive and could be beneficial for chemotherapy, particularly at low concentrations (DRI > 1) (**Table 1**). According to this data, by using CIGB-552 as adjuvant treatment the doses of CDDP could be reduced from 7 to 11 times in a combined therapy maintaining a proliferation inhibition around 80-90%, whereas Paclitaxel doses could be reduced up to 13 times, but achieving a 60% of proliferation inhibition. As is suggested by these results, the combinations studied could help to reduce the doses of CDDP and Paclitaxel currently used in the clinical practice, keeping the efficacy and synergism observed.

From these experimental data we also conclude that CIGB-552 combined with CDDP exhibits a higher synergism index and a greater cell growth inhibition compared to Paclitaxel in several concentrations evaluated. Also, the synergism observed was better in surface and inhibition under concomitant treatment. For those reasons, we selected the combination of CIGB-552 and CDDP in concomitant scheme to continue the study. Interestingly, the major inhibition of cell growth and the higher synergism were obtained at concentrations below the IC₅₀ of CIGB-552 (37.5 μ M) and above the IC₅₀ of CDDP (5 μ M). According to this, we decided to evaluate the effect of both drugs on subsequent experiments at these particular concentrations.

The combination of CIGB-552 and CDDP affects cell cycle progression

CIGB-552 and CDDP inhibited the proliferation of NCI-H460 cells and both products combined reached a higher effect compared with their individual inhibition. According to this, we evaluate their capacity to interfere with cell cycle progression in NCI-H460 cells at 12 and 24 hours of exposure, assessing the DNA content and the cell cycle phases with Propidium Iodide (PI) measured by flow cytometry. CIGB-552, CDDP and their combination affected cell cycle progression of NCI-H460 cells in a time dependent manner (**Figure 2**). CIGB-552 induced cell cycle arrest at G2/M phase (20.95%) from 12 hours of treatment compared to control (untreated cells) and this effect was maintained after 24 hours of exposure (20.76%). On the other hand, CDDP strongly modified the cell cycle profile of NCI-H460 cells, inducing cell death (2.58% at 12 hours and up to 10.57% at 24 hours) and causing a clear cell cycle arrest at G2/M phase (47.82% and 53.40% at 12 and 24 hours, respectively). Interestingly, both drugs together induced a mixed cell cycle profile which results from the combination of their individual effects causing G2/M arrest (19.67% in 12 hours and 21.12% at 24 hours) and potentiating cell death, particularly at 12 h of incubation (**Figure 2A**).

CIGB-552/CDDP combination activates the apoptotic pathway

The combination of CIGB-552 and CDDP showed an increased capacity to inhibit the proliferation of NCI-H460 cells promoting cell death and causing perturbations in cell cycle progression, so we decided to explore the ability of both products combined to induce apoptosis in this cell line. For this purpose, we evaluated the effect of CIGB-552 and CDDP separately and combined on the activation of the apoptotic pathway. First, in order of explore at a molecular level the effect of the combination on apoptosis-related proteins we performed a western blot analysis in NCI-H460 cells, to study whether the cell death observed is related to the activation of Caspase 3 (effector) and Caspase 8 and Caspase 9 (initiators), as well as the cleavage of PARP, a well-known apoptosis marker. We treated the cells with CIGB-552, CDDP and the combination of both for 12 and 24 hours. We used 1 μ M of Staurosporin (STS) as positive control. In**Figure 3A**, we show how the Pro-Caspase 3 decreases in the combination treatment after 24 hours of exposure respect to CIGB-552 and CDDP, indicating the formation of active Caspase 3. At 12 hours, the cleavage of Caspase 8 was observed in STS treatment solely, but after 24 hours, we detected the active form of Caspase 8 (41/43 kDa) in all conditions evaluated, with a major accumulation in the combined treatment. In the case of Caspase 9, the band corresponding to the active form (37/35 kDa) was detected at 12 hours, and this band was more intense in the combination. However, after 24 hours of exposure we found no differences in the cleavage of this protein between CDDP and the combination. Regarding the cleavage of PARP protein as a general marker of apoptosis it was observed since 12 to 24 hours of exposure in all treatments and the band is particularly strong after 12 hours of incubation with CIGB-552/CDDP combination. Interestingly, after 24 hours of treatment with the combination, the cleavage of PARP was extensive and similar to STS. These results indicate the activation of Caspases 3, 8 and 9 as well as the cleavage of PARP by the combination of CIGB-552 and CDDP, demonstrating the stimulation of the apoptotic pathway.

Next, we decided to confirm apoptosis induction in NCI-H460 cells by the Annexin V/Propidium iodide (AV/PI) double staining, evaluated by flow cytometry. Cancer cells were incubated with CIGB-552 and CDDP in monotherapy and combination during 48 hours. We also used STS (1 μ M) as positive control. All treatments increased the population of cells stained with AV (early apoptosis) and AV/PI (late apoptosis) respect to non-treated cells. In particular, the detection of AV/PI positive cells was higher for the combined treatment in comparison to CIGB-552 and CDDP alone (**Figure 3B**). We graphed the percentage of AV + AV/PI positive cells for a better understanding. The effect of the combination reaches a 62.7 % of stained cells respect to 20.4 % and 34.5 % with CIGB-552 and CDDP, respectively (**Figure 3C**).

Altogether, these results demonstrate that the combination of CIGB-552 and CDDP have a negative effect on the survival of NCI-H460 lung cancer cells inducing apoptosis as the mechanism of cell death. The effects exerted by the combination are more potent compared to the drugs alone confirming the synergic interaction between them.

Synergic antitumor effect of CIGB-552/CDDP combination is mediated by Oxidative Stress activation

The molecular mechanism of CIGB-552 peptide in lung cancer cells is related with the induction of oxidative stress. CDDP in addition to its DNA intercalating activity also promotes the formation of Reactive Oxygen Species (ROS) and oxidative damage in tumor cells. For that reason, we expected a connection between the antitumor mechanism of the combination and the cell redox balance. According to this, we evaluated the formation of superoxide radical (O2*-) in NCI-H460 cells after exposure to the products in study. Cells were incubated with CIGB-552, CDDP and the combination during 1 hour and then they were stained with Hydroethidine (HE) to visualize the formation of superoxide anion. A 1 hour exposure to 2.5 µM of hydrogen peroxide (H_2O_2) was used as positive control for the experiment. Both, the treatment with CIGB-552 and the combination promoted the increase of fluorescence in treated cells, demonstrating the accumulation of O2^{*}-, in contrast to CDDP that showed a minor effect (Figure 4A). Comparing the mean fluorescence intensity of HE for the different treatments we can observe that CIGB-552 produced a 1.8 fold increase in comparison to control (untreated cells) and combined with CDDP the fold increase is up to 2.8, which is statistically different from their individual effects (Figure 4B). To evaluate the selectivity of ROS induction as antitumor mechanism we tested the same concentrations of CDDP and CIGB-552 alone and combined during 1 hour of exposure in human lung fibroblasts (MRC-5 cell line). As we expected the evaluated products do not stimulate $O2^*$ - accumulation in these cells (Figure 4C), suggesting that CIGB-552 and the combination induce this mechanism selectively in tumor cells in comparison to non-transformed cells from the same histologic localization. Then, in order to corroborate the ROS production in NCI-H460 cells in response to our products and to verify this effect after a prolonged exposure, we determined the percentage of HE positive cells after 12 hours of incubation with CIGB-552, CDDP and the combination of both, using flow cytometry. We used H_2O_2 (2.5 μ M) as positive control. The accumulation of O2*- was detected after 12 hours of incubation in cells treated with CIGB-552 (5% of positive cells) and interestingly in CDDP-treated cells in a similar extent (5.3 % of positive cells). Comparing this effect with the HE fluorescence obtained after 1 hour of exposure, we confirm our hypothesis that CIGB-552 induces oxidative stress as an early event

that is maintained, whereas CDDP activates ROS later in time; but in both cases this mechanism leads to apoptosis induction and cell death. In line with this, the combined treatment showed a significant increase in the percentage of HE positive cells (13.8%), demonstrating synergy in the induction of oxidative stress mediated by the products (**Figure 4D**).

There is an increase on superoxide radicals when cells are treated with CIGB-552 and this effect is stronger in combination with CDDP, as occurs with the other parameters included in our study. In addition, not CIGB-552 or the combination promote the generation of superoxide anion in normal cells. Thus, the combination could help to decrease the non-specific toxicity promoted by Cisplatin, keeping the increase of ROS as a selective mechanism of cytotoxicity in tumor cells.

CIGB-552 and CDDP are synergic in vivo in a mouse model of lung cancer

The combination of CIGB-552 and CDDP has shown synergistic effect in NCI-H460 cells inducing apoptosis and oxidative stress. However, the *in vivo* antitumor efficacy of both drugs combined in lung cancer animal models is still unknown. We have previously established the pharmacokinetic profile of CIGB-552 in BALB/c mice and validated its antitumor activity in syngeneic and xenograft mouse models of colon cancer (Vallespí, Pimentel et al. 2014). Subcutaneous administration of 0.72 or 1.44 mg/kg of CIGB-552 was able to inhibit tumor growth and improve survival rate without toxicity signs or body weight loss in comparison to a reference drug such as Oxaliplatin. In line with these previous results, we decided to explore the effects of the systemic administration of CIGB-552 and CDDP alone and as a combined therapy in a syngeneic mouse model of TC-1 lung cancer. First, we analyzed the antiproliferative capacity, in vitro drug interactions and the oxidative stress induction promoted by CIGB-552 and CDDP in the mouse tumor cell line TC-1 (murine lung epithelial cells transfected with VPH-16) under the same conditions of NCI-H460 cells. In this murine cell line, CIGB-552, CDDP and the combination inhibited cell proliferation, displaying synergism in a similar concentration range compared to NCI-H460 cells and increasing the accumulation of superoxide radicals (Supplementary Figure 1S). Then we generated the syngeneic mouse model of lung cancer by subcutaneous implantation of TC-1 cells in C57/BL6 mice, to evaluate the antitumor activity of CIGB-552/CDDP combination in vivo. We based on our previous results of CIGB-552 pharmacokinetics and antitumor efficacy in colon cancer models to select the dose and administration route, following the experimental design described in materials and methods (section 2).

The administration of CIGB-552 and CDDP on tumor-bearing mice led to a significant reduction of tumor growth compared with the group treated with saline solution (p < 0.05) (Figure 5A and B). In addition, mice treated with the combination exhibited significantly less tumor volume than those only administered with CIGB-552 or CDDP (p < 0.05). The treatment was also safe and tolerable for mice included in the study, as we could corroborate by monitoring the body weight and possible toxicity signs of treated animals. CIGB-552 and the combination practically did not affect the life quality, whereas animals administered with CDDP or saline solution (control group) presented obvious signs of physical deterioration such as piloerection, ulcers, fallen hind legs and bending. All treatments in general were tolerable maintaining a constant increase in body weight during the whole experiment (Figure 5C). However, the peptide and the combined treatment significantly decreased the presence of physical deterioration signs and the percentage of affected animals in comparison to CDDP administration (Table 2).

In line with the reduction observed for tumor volume in this model and the absence of physical deterioration signs, mice treated with the peptide CIGB-552 and the combination improved life quality and increased survival rate respect to CDDP or saline solution groups. The survival rate for all treatments was significantly different compared to PBS administration (p < 0.01) and the combination shows a tendency to a superior overall survival in comparison to individual treatments, marked by the fact that only the combined treatment group still have live animals at the end of the experiment (**Figure 5D**). The treatment with the combination was very effective in comparison to monotherapies, with a treated/control ratio (T/C) less than 15 (1.9), a tumor growth delay (TGD) of 9.5 days respect to control group and achieving a 98 % of tumor growth inhibition (**Table 3**). Thus, our results demonstrate that CIGB-552/CDDP combination scheme elicits its antitumor activity *in vivo*, with high tolerability and effectiveness.

DISCUSSION

The generation of new therapeutic strategies and alternative treatments has been a focus on cancer research. Drug combination in particular, is getting attention as an interesting approach with a great current impact in cancer therapy. The idea is to achieve a synergic or additive effect between the drugs in the combination, in order to potentiate their individual properties, reducing the doses but maintaining the pharmacological effect, which allows the reduction of tumour growth and metastatic potential, decreasing stem cell populations and inducing apoptosis, and at the same time reducing toxicity and MDR (Tallarida 2001, Li, Zhao et al. 2014, Bayat Mokhtari, Homayouni et al. 2017, Achkar, Abdulrahman et al. 2018).

In this work, we evaluated the pharmacological effects of the combination between our anticancer peptide CIGB-552 and classic antineoplastic agents currently employed in the clinics for lung cancer treatment, such as CDDP and Paclitaxel. We evaluated potential pharmacological interactions between CIGB-552 and both CDDP and Paclitaxel through an *in vitro* drug combination assay in the NSCLC cell line NCI-H460. We used two different combination schemes: concomitant (both drugs acting at the same time) and pre-treatment (preincubation with CIGB-552 and then add the other drug) similar to clinical schemes used for cancer patients.

This drug interaction study showed a clear synergic effect between CIGB-552 and both chemotherapeutic agents but the synergism and the antiproliferative capacity were higher with CDDP compared to Paclitaxel under the two treatment settings, and particularly under concomitant conditions, where the synergism surface and the fraction affected were greater. This indicate that co-administration of both drugs is essential to obtain a better synergistic effect and a greater inhibition of cell proliferation in non-small cell lung cancer lines. This study also revealed some additivity between CIGB-552 and CDDP at middle concentrations, which could potentiate their overall effect, although synergism was the predominant interaction observed. Antagonism was only present at higher concentrations in the concomitant condition, suggesting that these drugs combined are more effective at middle and lower concentrations. According to this, CDDP/CIGB-552 could help to reduce the doses of CDDP currently used in the clinic, improving the patient responses to this antineoplastic agent. Based on this result, we selected the combination of CIGB-552 and CDDP, under concomitant scheme, to further evaluate its antitumor properties and the synergism between both drugs.

Next, we investigated the capacity of the combination to modulate cell cycle progression and induce apoptosis in NCI-H460 cells. Our results demonstrated cell cycle arrest at G2/M phase and DNA fragmentation which is an indicative of cell death. On the other hand, we confirmed apoptosis induction in NCI-H460 cells in response to our products, which was increased by the combination. Cell death by apoptosis is one of the most important mechanisms that intrinsically controls malignant transformation. Thus, apoptosis induction in tumor cells is considered a key indicator of antitumor activity for new products/drugs and is also a desirable effect for drug combinations (Meng, Wang et al. 2015). It has been described that some chemotherapeutic agents such as CDDP, Topotecan and Gemcitabine are able to induce apoptosis in NCI-H460 cells and other NSCLC cell lines by a Caspase 8-dependent but death receptors and Caspase 9-independent pathway, with mitochondrial permeabilization and cytochrome c release as primary events (Ferreira, Span et al. 2000). In our study, the cleavage of PARP and Caspases 3, 8 and 9 confirmed apoptosis induction and suggested the activation of intrinsic and extrinsic pathways by both products but mainly by the combined treatment. Some authors have also showed that Cisplatin-acquired resistance in other types of tumor like malignant pleural mesothelioma is associated with a reduction in Caspase 8 activation and therefore apoptosis induced by CDDP depends mainly on Caspase 9 activity (Janson, Johansson et al. 2010) Our results demonstrated a preferential cleavage of Caspase 9 in cells treated with the combination, particularly at 12 hours of incubation, thus the action of CIGB-552 could help to overcome or decrease Cisplatin resistance in treated cells. Finally, we corroborate apoptosis induction in NCI-H460 cells by Annexin V/PI double staining, which revealed also a major percentage of apoptotic cells in response to the combined treatment in comparison to the individual drugs, confirming the synergic interaction between them.

Different authors have reported that the transcription factor NF-kB interferes with the mechanism of action

of antineoplastic drugs by induction of antiapoptotic genes. Thus, the use of NF-kB inhibitors or new drugs that target this molecular factor as adjuvant treatments, could help to improve chemotherapy (Morotti, Cilloni et al. 2006, Lagadec, Griessinger et al. 2008, Achkar, Abdulrahman et al. 2018). NF-kB activation has been detected in many types of cancer including small and non-small cell lung cancer and high expression of this nuclear factor is correlated with progressive cancer and poor prognosis (Chen, Li et al. 2011). NF-kB is induced in cancer cells in response to chemotherapeutic agents like CDDP, as a tumor escape mechanism. related with chemoresistance and insensitivity to chemotherapy (Galluzzi, Senovilla et al. 2012, Godwin, Baird et al. 2013). Therefore, there are many studies that demonstrate a synergistic activity combining an NF-kB inhibitor with antineoplastic drugs. For example, Wang et al. demonstrated that Gambogic acid (GA), a strong NF-kB inhibitor, synergically potentiates CDDP-induced apoptosis in NCI-H460 cells (Wang, Li et al. 2014). Gambogic acid has antineoplastic and antiangiogenic properties and is currently in phase II of clinical trials for NSCLC treatment (Wang, Deng et al. 2013). Likewise, Bortezomib, a proteasomal inhibitor that decreases NF-kB activation, enhanced the sensitization of bladder and cervical cancers to CDDP (Miyamoto, Nakagawa et al. 2013, Konac, Varol et al. 2015). More recently, the natural bioflavonoid Galangin (GG), which inhibits NF-kB activity through downregulation of p-STAT3 signaling pathway, has demonstrated to inhibit proliferation and enhance the apoptosis induced by CDDP in human resistant lung cancer cells (Yu, Gong et al. 2018).

According to this, we corroborate synergism in antiproliferative effect and apoptosis induction between CIGB-552 and CDDP in NCI-H460 cells. The molecular mechanism of CIGB-552 is based on the inhibition of NF-kB signaling pathway mediated by the stabilization and accumulation of the intracellular protein COMMD1. (Fernández Massó, Oliva Argüelles et al. 2013). Thus, this could be a mechanism that plays an important role in the synergic effects between both drugs and could contribute to decrease cisplatin resistance in NSCLC. In addition, COMMD1 has demonstrated strong anticancer and antimetastatic effects in different cancer models (Van De Sluis, Mao et al. 2010, Riera-Romo 2018). Furthermore, Fedoseienko et al. demonstrated that nuclear expression of COMMD1 sensitizes tumor cells derived from advanced ovarian cancer patients to platinum-based therapy. They suggest that COMMD1 modulate the G2/M checkpoint, controlling expression of genes involved in DNA repair and apoptosis (Fedoseienko, Wieringa et al. 2016). Then, is reasonable to think that COMMD1 is also playing a key role in the molecular mechanism that mediates CIGB-552 synergism with CDDP in NCI-H460 cells.

On the other hand, tumor cells have increased levels of ROS due to their own metabolism deregulations, and it contributes to tumor development and drug resistance. In line with this, the pharmacological manipulation of the redox status to sensitize cancer cells to chemotherapeutic agents is another attractive strategy to increase efficacy and avoid MDR. (Dayem, Choi et al. 2010, Ma, Yang et al. 2014). In this work, we demonstrated that the combination of CIGB-552 and CDDP increases intracellular levels of ROS at short or prolonged exposure in NCI-H460 cells and do not have effect on normal cells from the same localization, like MRC5 cells. These results suggest that selective induction of oxidative stress could be an additional mechanism by which CIGB-552/CDDP combination elicits its antitumor effects in NSCLC; an important advantage of the combination compared to CDDP monotherapy.

One of the mechanisms underlying CIGB-552 cytotoxicity in NCI-H460 cells is the COMMD1-mediated inhibition of SOD1 enzyme and the subsequent induction of oxidative stress (Fernandez Masso, Oliva Arguelles et al. 2013). CDDP also cause an unspecific production of high ROS levels, which constitutes one of the main reasons of its toxicity (Chirino and Pedraza-Chaverri 2009). Therefore, the combination with CIGB-552 could contribute to reduce the nephrotoxicity and lymphopenia induced by CDDP in cancer patients.

Our results showed that CGB-552 induces oxidative stress as an early event, probably by COMMD1 stabilization and COMMD1-dependent SOD1 inhibition, whereas CDDP triggers ROS accumulation later in time, as a secondary event, derived from its sequential enzymatic biotransformation. This suggests that the peptide specifically sensitizes tumor cells to CDDP through the modulation of the redox state and consequently, both drugs combined generate sustained oxidative stress that reinforces apoptosis induction and achieves a higher antiproliferative effect (**Figure 6**). Besides, both products are modulating the same cellular process but acting trough different pathways, which could also explain the synergic interaction between them. This idea has been supported by other authors, who point out oxidative stress modulation as an interesting strategy to eliminate cancer cells and sensitize them to chemotherapeutic treatments (Trachootham, Alexandre et al. 2009, Raj, Ide et al. 2011, Gorrini, Harris et al. 2013). For example, small molecules such as Resveratrol (trans-3, 4', 5-trihydroxystilbene) and Phenothiazines are able to sensitize human colon and lung cancer cells, respectively, to the action of different chemotherapeutic agents such as 5-Fluoracil (5-FU) to colon cancer and Bleomycin and CDDP to lung cancer, through the modulation of intracellular oxidative stress response (Santandreu, Valle et al. 2011, Zong, Haag et al. 2011). Another example is the natural compound Shikonin, which triggering intracellular oxidative stress in colon cancer cells but not in normal cells, potentiated CDDP-induced DNA damage, followed by increased activation of the mitochondrial apoptotic pathway. The use of antioxidants and ROS scavengers revealed that ROS are essential to the synergism observed (He, He et al. 2016).

The results obtained in vitro were corroborated in vivo in a mouse model of TC-1 lung cancer, a recognized animal model for NSCLC (Tanaka, Delong et al. 2005). Co-administration of CIGB-552 and CDDP lead to a significant inhibition of tumor growth, with increased overall survival in treated animals and increasing the life of quality in comparison to control mice or mice treated with the drugs separately. In addition, the combination with CIGB-552 was able to significantly decreases the signs of physical deterioration associated with CDDP administration. This confirms that both drugs are acting synergically to achieve a better antitumor response and is correlated with the behaviour observed in *in vitro* studies. Similar results were obtained by Wang et al. with GA, which sensitizes human lung cancer cells to CDDP in vitro, by NF-kB inhibition and ROS intracellular accumulation, and was also effective in vivo, in a A549 xenograft mice model, where the combined administration with CDDP significantly decreased tumor volumes of treated animals, without body weight loss or associated toxicity (Wang, Li et al. 2014). In the same way, Shikonin that eliminates human colon cancer cells and sensitizes them to CDDP-induced apoptosis through the selective induction of oxidative stress, was also able to inhibit tumor growth in a HCT116 xenograft model in nude mice (He, He et al. 2016). More recently, Hsu et al. demonstrated the high potential of another natural compound, Withaferin A (WA) in lung cancer in vitro and in vivo. As occurred with CIGB-552, WA is selectively cytotoxic to different human lung cancer cells including various NSCLC cell lines, inducing apoptosis and increasing the intracellular accumulation of ROS as its antitumor mechanism. In addition, it decreases lung tumorigenesis in vivo in a NSCLC model of H441-L2G bioluminescent cells implanted in nude mice. Similar to our results, WA and CDDP synergically inhibited NSCLC cell proliferation in a drug combination assay and WA enhanced CDDP cytotoxicity and antitumor activity in cell cultures and tumor spheroids (Hsu, Chang et al. 2019).

Taken together, all these findings demonstrate that targeting NF-kB activity and ROS response in tumor cells is an effective therapeutic strategy in NSCLC and other types of cancer, which can improve the response to different chemotherapeutic agents but particularly to CDDP, achieving synergistic effects and decreasing CDDP resistance. The combination of CIGB-552 and CDDP is able to modulate both molecular pathways, representing an important advantage in NSCLC treatment. Based on the presented evidence we propose a model in which CIGB-552 sensitizes lung cancer cells to CDDP through ROS accumulation and NF-kB inhibition, achieving synergism in apoptosis induction and reduction of tumor growth. This research is the first preclinical evidence about the combination of CIGB-552 as an adjuvant treatment in the clinics.

CONCLUSIONS

CIGB-552 is a new cancer targeted therapy that acts synergically with CDDP to inhibit proliferation and tumor growth of NSCLC *in vitro in vivo*. The combination of CIGB-552 with chemotherapeutic agents like CDDP is an attractive strategy to selectively induce ROS and apoptosis in lung cancer cells, improving the antitumor efficacy of CDDP but decreasing its associated toxicity. This work also shows scientific evidence that could help in the rational design of a combined treatment for lung cancer based on CIGB-552, to be tested in clinical trials.

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CONFLICT OF INTERESTS

Authors do not declare any conflict of interests associated to this work.

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TABLES

Table 1. Combination parameters under concomitant treatment for maximal, minimal and median concentrations of CIGB-552, CDDP and Paclitaxel in NCI-H460 cells.

[•] ΙΓΒ-522 (μM)	$\Delta \Delta \Pi$ (μM)
300	500
37.5	5
9.37	0.005
CI, combination index; Fa, fraction affected; DRI, dose reduction index.	CI, combination index; Fa, fraction affected; DRI

Table 2. Signs of physical deterioration 36 days post-implantation in the syngeneic mouse model of TC-1 lung cancer treated with CIGB-552, CDDP and combined therapy.

		Physical	Physical	Physical	Physical	Physical	Physical		
		deterio-	deterio-	deterio-	deterio-	deterio-	deterio-		
		ration	ration	ration	ration	ration	ration		
		after 36	after 36	after 36	after 36	after 36	after 36		
		days	days	days	days	days	\mathbf{days}		
		post-	post-	\mathbf{post} -	post-	\mathbf{post} -	post-		
		implantat	tiomplantat	;i o mplantat	tiomplantat	tiomplantat	iomplanta	tion	
		(Num-	(Num-	(Num-	(Num-	(Num-	(Num-		
		ber of	ber of	ber of	ber of	ber of	ber of		
		affected	affected	affected	affected	affected	affected		
Experimen Ex perimentali-			ani-	ani-	ani-	ani-	ani-		
Groups Groups ma		mals)	mals)	mals)	mals)	mals)	mals)	Incidence	e
(n=10)	(n=10)	a	a	a	a	a	a	(%) ^b	
		Piloerectio		Fallen	Bending	Bending			
				hind					
				\mathbf{legs}					
PBS	2/10	2/10	3/10	1/10	3/10	40	40	40	40
1X	,	,	,	,	,				

Experimer Groups (n=10)	n Ea lperimer Groups (n=10)	Physical deterio- ration after 36 days post- implantati (Num- ber of affected ntami- mals) a	Physical deterio- ration after 36 days post- omplantati (Num- ber of affected ani- mals) a	on Incidence (%) ^b					
CIGB- 552 (1 mg/kg)	1/10	1/10	-	-	-	10 *	10 *	10 *	10 *
$\begin{array}{c} { m CDDP} \\ (0.4 \\ { m mg/kg}) \end{array}$	1/10	1/10	4/10	2/10	1/10	50	50	50	50
CIGB- 552 (1 mg/kg) + CDDP	-	-	-	-	-	0 *	0 *	0 *	0 *

(0.4 mg/kg)

		Physical	Physical	Physical	Physical	Physical	Physical		
		deterio-	deterio-	deterio-	deterio-	deterio-	deterio-		
		ration	ration	ration	ration	ration	ration		
		after 36	after 36	after 36	after 36	after 36	after 36		
		days	days	days	days	days	days		
		post-	post-	post-	post-	post-	post-		
		implantat	iomplantat:	iomplantat	iomplantat	iomplantat	iomplantat	ion	
		(Num-	(Num-	(Num-	(Num-	(Num-	(Num-		
		ber of	ber of	ber of	ber of	ber of	ber of		
		affected	affected	affected	affected	affected	affected		
Experime	n Eal perime	n taali -	ani-	ani-	ani-	ani-	ani-		
Groups	Groups	$\mathbf{mals})$	$\mathbf{mals})$	$\mathbf{mals})$	$\mathbf{mals})$	$\mathbf{mals})$	$\mathbf{mals})$	Incidence	
(n=10)	(n=10)	а	а	а	a	а	а	(%) ^b	
^a Num-	^a Num-	^a Num-	^a Num-	^a Num-	^a Num-	^a Num-	^a Num-	^a Num-	^a Num-
ber of	ber of	ber of	ber of	ber of	ber of	ber of	ber of	ber of	ber of
ani-	ani-	ani-	ani-	ani-	ani-	ani-	ani-	ani-	ani-
mals	mals	mals	mals	mals	mals	mals	mals	mals	mals
with	with	with	with	with	with	with	with	with	with
each	each	each	each	each	each	each	each	each	each
symp-	symp-	symp-	symp-	symp-	symp-	symp-	symp-	symp-	symp-
$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\mathrm{tom}/$	$\operatorname{tom}/$
total);	total);	total);	total);	total);	total);	total);	total);	total);	total);
^b Num-	^b Num-	^b Num-	^b Num-	^b Num-	^b Num-	^b Num-	^ь Num-	^b Num-	^b Num-
ber of	ber of	ber of	ber of	ber of	ber of	ber of	ber of	ber of	ber of
ani-	ani-	ani-	ani-	ani-	ani-	ani-	ani-	ani-	ani-
mals	mals	mals	mals	mals	mals	mals	mals	mals	mals
with	with	with	with	with	with	with	with	with	with
one or	one or	one or	one or	one or	one or	one or	one or	one or	one or
more	more	more	more	more	more	more	more	more	more
symp-	symp-	symp-	symp-	symp-	symp-	symp-	symp-	symp-	symp-
toms/	toms/	toms/	toms/	toms/	toms/	toms/	toms/	toms/	toms/
total	total	total	total	total	total	total	total	total	total
$(\inf \%);$	(in %);	(in %);	(in %);	(in %);	(in %);	(in %);	(in %);	(in %);	(in %);
* p <	* p <	* p <	* p <	* p <	* p <	* p <	* p <	* p <	* p <
0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
com-	com-	com-	com-	com-	com-	com-	com-	com-	com-
pared	pared	pared	pared	pared	pared	pared	pared	pared	pared
to the	to the	to the	to the	to the	to the	to the	to the	to the	to the
CDDP	CDDP	CDDP	CDDP	CDDP	CDDP			CDDP	
treated	treated	treated	treated	treated	treated	treated	treated	treated	treated
group	group (One	group (One	group	group	group (One	group (One	group (One	group (One	group (One
(One	(One	(One	(One	(One	(One	(One	(One	(One	(One
way Anove	way Anowo	way Anows	way Anove	way Anove	way Anows	way Anorro	way Anows	way Anowo	way Anows
and	and	and	and	and	and	and	and	and	and
Dunnot	Dunnot	Dunnot	Dunnot	Dunnot	Dunnot	Dunnot	Dunnot	Dunnot	Dunnot
post	post	post	post	post	post	post	post	post	post
post tost)	tost)	post test)	post test)	post test)	post)	post	post	post test)	post test)
uest)	uesu)	uesu)	uest)	uesu)	uesu)	uesu)	uesu)	uesu)	uesu)

Table 3. Effectiveness of the combination therapy in the syngeneic mouse model of TC-1 lung cancer

Treatment CIGB-552 (1 mg/kg) s.c CDDP (0.4 mg/kg) i.p CIGB-552 (1 mg/kg) s.c + CDDP (0.4 mg/Kg) i.p TGI, tumor growth inhibition; TGD, tumor growth delay; T/C, treated and control animals ratio (<15 highly effective, <50

FIGURE CAPTIONS

Figure 1.Antiproliferative effects of CIGB-552, CDDP and Paclitaxel in NCI-H460 cells. A)Proliferation inhibition induced by CIGB-552 and CDDP (A) or Paclitaxel(B) alone and combined at selected concentrations in μ M.C) Color surface interaction map, based on CI values from *in vitro* drug combination assay for CIGB-552 with CDDP and Paclitaxel under concomitant and pre-treatment settings. * p<0.05, ** p<0.01 for the combination in comparison to individual treatments (One way ANOVA and Dunnet post test).

Figure 2. Cell cycle profile of NCI-H460 cells treated with CIGB-552 (37.5 μ M), " $\Delta\Delta\Pi$ (5 μ M) and the complementation of both deuxe for 12 (A) and 24 house (B). The DNA content was assessed by Propidium Iodide (PI) staining and flow cytometry. The percentage of cells in each cell cycle phase is represented in the corresponding histograms.

Φιγυρε 3. Αποπτοσις ινδυςτιον ιν N^{*}I-H460 ςελλς μεδιατεδ βψ^{*}IΓB-552 (37.5 μM) ανδ ^{*}ΔΔΠ (5 μM) αλονε ανδ ςομβινεδ. A) Western Blott of apoptosis-related proteins in response to 12 and 24 hours of exposure to the products. Staurosporin (STS) 1 μM, was used as positive control. B) Cell distribution on the Annexin V/Propidium Iodide (AV/PI) double staining assessed by flow cytometry after 48 of exposure to the drugs. STS (1 μM) was also included as positive control for the experiment. C) Percentage of apoptotic cells (AV + AV/PI) from the double staining assay in response to each treatment. * p<0.05, for the combination in comparison to individual treatments (One way ANOVA and Dunnet post test).

Figure 4. Superoxide anion ($O2^{*-}$) $\alpha \zeta \circ \mu \nu \lambda \alpha \tau \circ \alpha \sigma \alpha \psi \zeta \iota \nu \rho \varepsilon \sigma \tau \circ \tau \sigma \tau \Gamma B-552$ (37.5 μM) $\alpha \lambda \delta \Delta \Lambda \Pi$ (5 μM) $\alpha \lambda \circ \nu \varepsilon \alpha \nu \delta \iota \nu \zeta \circ \mu \beta \iota \nu \alpha \tau \iota \circ \nu$. A)Fluorescence microscopy images from Hydroethidine (HE) staining in NCI-H460 (A) and MRC-5 cells (B) to detect O2*- accumulation in response to 1 hour exposure to the products. C)HE fluorescence intensity as fold of control in NCI-H460 cells in response to the treatments, quantified from microscopy images with ImageJ 1.41 software. ** p<0.01 for the combination in comparison to individual treatments (One way ANOVA and Dunnet post test). D) HE positive cells in percent, after 12 hours of exposure to the products in NCI-H460 cell line, assessed by flow cytometry. * p<0.05 for the combination in comparison to individual treatments (Unpaired T test). H₂O₂(2.5 mM) was included as a positive control for all experiments.

Figure 5. In vivo antitumor activity of CIGB-552 (1 mg/kg s.c.) and CDDP (0.4 mg/kg i.p.) in a TC-1 lung cancer model in C57/BL6 mice. A) Mean tumor volume of treated and control mice from 10 to 24 days post-implantation of TC-1 cells. B) Tumor volume of animals from the four experimental groups at day 21 post-implant. Control animals only received saline solution (PBS 1X s.c.) * p<0.05 for the combination in comparison to individual treatments (Unpaired T test).C) Mean body weight of treated and control mice measured once a week during the three weeks of the experiment. D) Kaplan-Meier survival curves of control and treated animals from implantation to 60 days post-implant. Log-rank analysis showed statistical differences between treated and control animals (p<0.01).

Figure 6. Hypothetical mechanism proposed to explain the synergic effects between CIGB-552 and CDDP in lung cancer cells. Both drugs target oxidative stress and apoptosis in tumor cells but acting through different pathways. CDDP intercalates in the DNA inducing DNA damage and cell death by apoptosis and at the same time, it causes a progressive reactive oxygen species (ROS) accumulation and oxidative stress as a result of its enzymatic biotransformation. On the other hand, CIGB-552 stabilizes and

accumulates COMMD1 leading to the downregulation of the NF-kB signaling and consequently it inhibits the transcription of antiapoptotic and pro-survival genes. This effect enhances the CDDP-induced apoptosis and decreases chemoresistance. In addition, the COMMD1-mediated inhibition of SOD1 enzyme induced by CIGB-552 also triggers oxidative stress. The sustained and synergic oxidative stress activation by both products eventually leads to apoptotic cell death as well. CDDP: Cisplatin, CTR1: High affinity copper uptake protein 1; CIGB-552: anticancer cell penetrating peptide; COMMD1: Copper Metabolism Murr 1 Domain Containing Protein 1; SOD1: Superoxide Dismutase 1; p50/p65: NF-kB subunits. Black arrows indicate individual effects and red arrows indicate synergic effects. Red crosses indicate inhibition.











