Biotecnología Aplicada

Elfos scientiae
http://elfosscientiae.cigb.edu.cu/

REPORT

Novel molecular events related to CIGB-300 antineoplastic mechanism of action

✍Silvio Ernesto Perea Rodríguez, Yasser Perera Negrin, Arielis Rodríguez Ulloa, Yassel Ramos Gómez, Mauro Rosales Menzoney, Gabriel Padrón Palomares, Evelin Caballero Menéndez, Osmany Guirola Cruz, Alexis Musacchio Lasa, Jorge Fernández De Cossio, Luis Javier González López, Vladimir Besada López, George V Pérez Viltre, Daylen Aguilar Noriega, Dania Marcia Vázquez Blomquist, Ailyn de la Caridad Ramón Sánchez

1 Center for Genetic Engineering and Biotechnology, CIGB
Ave 31 No. 15802 e/ 158 y 190, Cubanacán, CP 11600, La Habana, Cuba
2 Faculty of Biology, University of Havana, Cuba
✍silvio.perea@cigb.edu.cu

ABSTRACT

CIGB-300 is an antitumor peptide that inhibits the CK2-mediated phos¬phorylation by direct targeting of substrates. This paper aims to describe breakthroughs about the CIGB-300 antineoplastic mechanism related to the inhibition of CK2-mediated phosphorylation and the synergism with anticancer drugs. CK2 phosphorylation assays were performed with catalytic CK2α subunit or the CK2 holoenzyme in presence or not of CIGB-300. CIGB-300/CK2 interaction was verified by pull-down experiments, in situ colocalization and phosphoproteomic analysis of CIGB-300-treated lung cancer cells were also performed. Synergism of the peptide with anticancer drugs was evaluated *in vitro* and for Cisplatin; it was also tested *in vivo*. Besides, comparative proteomics of CIGB-300 combined with Cisplatin was conducted. CIGB-300 targeted the CK2α subunit and inhi-bited the enzymatic activity of the holoenzyme in different experimental settings. Likewise, phosphoproteomic and Western Blot analysis allowed for knowing the early CK2 inhibition profile elicited by CIGB-300 at 10 and 30 min of treatment. Moreover, CIGB-300 did synergize with chemotherapeutics and EGFR inhibitors, and molecular events that reportedly support such synergistic interactions were also known. CIGB-300 inhibits the CK2-mediated phosphorylation by using an alternative mechanism of direct interaction with the enzyme itself. Besides, CIGB-300 synergizes with chemotherapeutics and EGFR inhibitors by modulating different proteins related to drug resistance.

Keywords: CIGB-300, CK2, anticancer drugs

RESUMEN

Nuevos eventos moleculares vinculados al mecanismo de acción antineoplásico del CIGB-300. El CIGB-300 es un péptido antitumoral inhibidor de la fosforilación de los sustratos de la proteína quinasa CK2. El presente trabajo persigue como objetivo fundamental exponer nuevos elementos vinculados al mecanismo antineoplásico del CIGB-300 relacionados con el modo de inhibición de dicha fosforilación y la sinergia del péptido con drogas anticáncer. Se realizaron ensayos de fosforilación mediada por la subunidad catalítica CK2α o la holoenzima en presencia o no del CIGB-300. La interacción CIGB-300/CK2 se verificó mediante ensayos de pull-down, colocalización in situ y además se realizó un análisis fosfoproteómico en células de cáncer de pulmón tratadas con el péptido. El sinergismo del CIGB-300 y las drogas anticáncer se evaluó *in vitro* y en el caso del cisplatino también *in vivo*. Además, se realizó un estudio de proteómica comparativa de la combinación del CIGB-300 con el cisplatino. El CIGB-300 interaccionó directamente con la subunidad CK2α e inhibió la actividad de la holoenzima en diferentes condiciones experimentales. Asimismo, el análisis de fosfoproteómica y Western blot permitió conocer el perfil de inhibición temprana de CK2 por el CIGB-300 a los 10 y 30 min. Adicionalmente, el CIGB-300 sinergizó con quimioterapéuticos e inhibidores del receptor del factor de crecimiento epidérmico (EGFR) y se conocieron los eventos moleculares que pueden sustentar la sinergia del péptido con las drogas anticáncer estudiadas. Como conclusiones, el CIGB-300 inhibe la fosforilación mediada por CK2 a través de un mecanismo alternativo de interacción directa con la enzima. Además, sinergiza con quimioterapéuticos e inhibidores del EGFR, modulando diferentes proteínas relacionadas con la resistencia a dichas drogas.

Palabras clave: CIGB-300, CK2, drogas anticáncer

How to cite (Vancouver style):
Perea-Rodríguez SE, Perera-Negrin Y, Rodríguez-Ulloa A, Ramos-Gómez Y, Rosales-Menzoney M, Padrón-Palomares G, *et al*. Novel molecular events related to CIGB-300 antineoplastic mechanism of action. Biotecnol Apl. 2022; 39(3):3501-5.

Introduction

The CK2 protein kinase is frequently deregulated in tumor cells and responsible for 20 % of the cellular phosphoproteome [1]. This kinase is associated with characteristic features of cancer, such as increased proliferation, cell survival, inhibition of apoptosis, angiogenesis, and metastasis [2]. To date, more than 300 cellular substrates for CK2 have been described, and there has been reported that it modulates the function of the tumor suppressors PTEN and PML, as well as the oncogenes AKT and c-myc [3-6]. Similarly, the aberrant expression of CK2 impacts multiple signaling cascades, its deregulation leading to malignant transformation, including Wnt, Hh, JAK/STAT, and PI3K/AKT [7].

1. Salvi M, Cesaro L, Pinna LA. Variable contribution of protein kinases to the generation of the human phosphoproteome: a global weblogo analysis. Biomol Concepts. 2010;1(2):185-95.

The CK2 enzymatic activity is displayed by the catalytic subunits CK2α and CK2α' separately, or bound to the dimer of CK2β regulatory subunits to form the holoenzyme [8]. Phosphorylation of so-called class III substrates can only be exerted by the holoenzyme, while class I substrates can be phosphorylated by the holoenzyme or single catalytic subunits [9]. Class II substrates are only phosphorylated by the catalytic subunits of CK2 [9].

CK2 is regarded as a promising and "drugable" target in cancer, due to successful in vitro and in vivo proofs of concept using small chemical molecules that inhibit its enzyme activity. This was achieved by blocking the ATP-binding site on the CK2α catalytic subunit, or by inhibiting the subunits' interaction to interfere the formation of the holoenzyme [10, 11]. Similarly, the use of antisense oligonucleotides against CK2α has served to validate the perspective of this enzyme as a target for cancer treatment [12].

CIGB-300 is a peptide that is currently being evaluated in the clinic and is part of CIGB's portfolio of new anticancer pharmaceutical developments. It inhibits CK2-mediated phosphorylation through its direct binding and blockade of the phosphor-acceptor domain conserved in the substrates for the enzyme [13]. Experimental evidences in solid tumor cell lines indicated that the B23/NPM1 protein is a CK2 substrate mostly bound by CIGB-300 in *in vivo* pull-down assays [14]. Similarly, in cell lysates, CIGB-300 binds different CK2 substrates, and, in chronic lymphocytic leukemia cells, it was found that CIGB-300 inhibited the phosphorylation of other substrates such as AKT and PTEN [15, 16].

Taking into account the mechanism described for CIGB-300 and the fact that a phosphor-acceptor domain has been reported in the CK2β subunit [17, 18], the study was aimed to elucidate if CIGB-300 could be capable of directly interacting with the enzyme and inhibiting its activity. Thus, the results validated this scientific hypothesis and also drafted the phosphoproteome regulated by CIGB-300 in tumor cells.

As part of this research, it was analyzed the probable antineoplastic synergy of CIGB-300 with chemotherapeutic agents and inhibitors of the epidermal growth factor receptor (EGFR). This was envisaged as to provide a molecular support to the synergy of CIGB-300 with those type of anticancer drugs.

Overall, this work comprised a contribution to the underlying antineoplastic mechanisms of action of CIGB-300, related to the inhibition of CK2's phosphorylation and the synergy of this peptide with anticancer drugs. Importantly, the results obtained not only have a scientific-technical impact, but also support the rational use of CIGB-300 in therapeutic schemes in combination with standard anti-cancer therapy.

Materials and methods

Radiometric and interaction assays

Phosphorylation reactions were performed with the holoenzyme or the CK2α subunit using 6000 Ci/mmol [γ^{32}P]-ATP in the presence of CIGB-300 and the peptide substrates M and 29, or the recombinant substrates GST-Olig2 and GST-Six1. Enzyme activity was expressed as direct counts values per minute (CPM). The interaction of the CK2 subunits with CIGB-300 was assayed by incubating GST-CK2α in the absence or presence of 50 μM CIGB-300, and adsorbing it to a glutathione-sepharose matrix. CK2β was then added and the enzymatic activity was determined. For interaction in solution, CK2α was incubated for 30 min in the presence or absence of 100 μM CIGB-300, and increasing amounts of biotinylated CIGB-300 (CIGB-300-B) adsorbed on streptavidin-sepharose matrix. Finally, the enzymatic reaction was started using peptide 29.

In situ pull-down and colocalization assays

Pull-down assays were run using the biotinylated CIGB-300 (CIGB-300-B) to capture the different proteins of interest such as CK2α, AKT, PTEN, or B23/NPM1 in cell lysates. Proteins bound to CIGB-300 were analyzed by Western blot. For fluorescent microscopy, NCI-H125 cells were treated with CIGB-300-B at different times. After fixation and permeabilization, cells were incubated with anti-CK2α, anti-CK2β, or anti-B23/NPM1 antibodies. Finally, the avidin-FITC conjugate or the corresponding secondary antibody conjugated to Alexa Fluor 594 was added, and results analyzed under a Leica Microsystem Confocal microscope.

Proteomics studies

The CK2 phosphoproteome regulated by CIGB-300 was explored in NCI-H125 cells, treated or not with CIGB-300 for 10 and 30 min. The cell extracts' phosphopeptides were isolated through enrichment by adherence to a TiO_2 matrix and subsequent LC-MS/MS identity analysis. For comparative proteomics, NCI-H125 cells were incubated with the combination CIGB-300 (50 μM/mL) + Cisplatin (0.5 μM/mL), or these agents separately, and nuclear extracts were prepared at the end of treatment. Then, proteins were isolated, subjected to tryptic digestion and differential isotopic labeling, and individual proteins were identified and quantified by LC-MS/MS. The results showed the changing factor of the levels of each protein.

Results and discussion

This research focused on answering two essential scientific questions on the mechanism of action of CIGB-300. First, to find out if this peptide was capable of inhibiting the enzyme itself, in addition to interacting with and inhibiting the phosphor-acceptor site of CK2 substrates. And secondly, to explore the synergism of CIGB-300 with anticancer drugs, with an approach to the molecular bases that can sustain such synergy. To clear up a possible direct effect of CIGB-300 on the CK2 enzyme, radiometric phosphorylation assays were performed, mediated both by CK2α (catalytic subunit) and by the holoenzyme (CK2α + CK2β), in the presence of serial doses of CIGB-300 (Figure 1) [19]. Interestingly, when using peptide M as a substrate, which is phosphorylated only by the CK2 holoenzyme, a marked dose-dependent inhibition of enzymatic activity was observed in the presence of CIGB-300 (Figure 1A). Similarly, two other specific substrates for the CK2 holoenzyme (GST-Olig2 and GST-Six1) were also inhibited in the presence of CIGB-300 (Figure 1B). In contrast,

2. Ruzzene M, Pinna LA. Addiction to protein kinase CK2: a common denominator of diverse cancer cells? Biochim Biophys Acta. 2010;1804(3):499-504.

3. Barata JT. The impact of PTEN regulation by CK2 on PI3K-dependent signaling and leukemia cell survival. Adv Enzyme Regulat. 2011;51(1):37-49.

4. Scaglioni PP, Yung TM, Cai LF, Erdjument-Bromage H, Kaufman AJ, Singh B, *et al*. A CK2-dependent mechanism for degradation of the PML tumor suppressor. Cell. 2006;126(2):269-83.

5. Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F, *et al*. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death Differ. 2005;12(6):668-77.

6. Channavajhala P, Seldin DC. Functional interaction of protein kinase CK2 and c-Myc in lymphomagenesis. Oncogene. 2002;21(34):5280-8.

7. Chua MM, Ortega CE, Sheikh A, Lee M, Abdul-Rassoul H, Hartshorn KL, *et al*. CK2 in cancer: Cellular and biochemical mechanisms and potential therapeutic target. Pharmaceuticals. 2017;10(1):18.

8. Pinna LA. Protein kinase CK2: a challenge to canons. J Cell Sci. 2002;115(Pt 20):3873-8.

9. Núñez de Villavicencio-Díaz T, Mazola Y, Perera Negrín Y, Cruz García Y, Guirola Cruz O, Perea Rodríguez SE. Predicting CK2 beta-dependent substrates using linear patterns. Biochem Biophys Rep. 2015;4:20-7.

10. Siddiqui-Jain A, Drygin D, Streiner N, Chua P, Pierre F, O'Brien SE, *et al*. CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits prosurvival and angiogenic signaling and exhibits antitumor efficacy. Cancer Res. 2010;70(24):10288-98.

11. Laudet B, Barette C, Dulery V, Renaudet O, Dumy P, Metz A, *et al*. Structure-based design of small peptide inhibitors of protein kinase CK2 subunit interaction. Biochem J. 2007;408(3):363-73.

12. Slaton JW, Unger GM, Sloper DT, Davis AT, Ahmed K. Induction of apoptosis by antisense CK2 in human prostate cancer xenograft model. Mol Cancer Res. 2004;2(12):712-21.

13. Perea SE, Reyes O, Puchades Y, Mendoza O, Vispo NS, Torrens I, *et al*. Antitumor effect of a novel proapoptotic peptide that impairs the phosphorylation by the protein kinase 2 (casein kinase 2). Cancer Res. 2004;64(19):7127-9.

14. Perera Y, Farina HG, Gil J, Rodriguez A, Benavent F, Castellanos L, *et al*. Anticancer peptide CIGB-300 binds to nucleophosmin/B23, impairs its CK2-mediated phosphorylation, and leads to apoptosis through its nucleolar disassembly activity. Mol Canc Ther. 2009;8(5):1189-96.

15. Perea SE, Baladron I, Garcia Y, Perera Y, Lopez A, Soriano JL, *et al*. CIGB-300, a synthetic peptide-based drug that targets the CK2 phosphoaceptor domain. Mol Cell Biochem. 2011;356(1-2):45-50.

16. Martins LR, Perera Y, Lúcio P, Silva MG, Perea SE, Barata JT. Targeting chronic lymphocytic leukemia using CIGB-300, a clinical-stage CK2-specific cell-permeable peptide inhibitor. Oncotarget. 2014;5(1):258-63.

peptide 29 and calmodulin, which are mainly phosphorylated by CK2α subunits, were not inhibited in the presence of CIGB-300, nor was autophosphorylation of CK2β regulatory subunit modified in the presence of serial doses of the peptide. Hence, these suggested that CIGB-300 does not block that particular phosphor-acceptor site (Figure 1C).

In this way, CIGB-300 inhibits the enzymatic activity of the CK2 holoenzyme without interacting with the CK2β subunit. Similarly, radiometric assays were conducted by incubating CK2α with increasing amounts of CK2β in the presence or absence of CIGB-300. As expected, the enzymatic activity of the holoenzyme increased proportionally to the addition of the CK2β subunit. Nevertheless, the activity was inhibited in the presence of CIGB-300 [19]. Despite, the peptide directly interacted with the CK2α subunit both in solution and when fixed to a solid support (Figure 2A and B, respectively) [19].

In order to corroborate this interaction in the cellular context, pull-down assays were performed using CIGB-300-B to capture CK2α in cell lysates. As shown in Figure 2C, it was demonstrated that CIGB-300 directly bound CK2α with over 90 % total capture in both cell lysates of NCI-H125 and HPB-ALL acute lymphocytic leukemia cells [19, 20]. The binding of CIGB-300 to the B23/NPM1 protein was used as positive control of the interaction in these assays (Figure 2C) [14]. Finally, to confirm the physical proximity of CIGB-300 with CK2α within the cell, in situ co-localization assays were performed by confocal fluorescence microscopy in NCI-H125 cells. An evident colocalization of CIGB-300 with the CK2α subunit was found in the cytoplasm of the cells, with an apparent enhancement in the perinuclear area and more slightly within the nucleus, as indicated by the orange fluorescence (Figure 3A) [19]. In contrast, the co-localization of CIGB-300 with B23/NPM1, used as positive control in this experiment, was more evident in the nucleus and nucleoli (Figure 3B).

In line with this hypothesis, we have observed that the treatment of HPB-ALL cells with CIGB-300 inhibited the phosphorylation of two key CK2 substrates, AKT and PTEN, without direct interaction of the peptide with these two substrates in pull-down assays [20]. This dual mechanism of inhibition of phosphorylation for CIGB-300, through the interaction with both, the substrate and the CK2 holoenzyme, strengthen its condition as a "First in Class" compound. This is also a distinctive element with respect to other CK2 inhibitors and of other kinases. Therefore, these findings provides an essential part of the scientific novelty of this work.

As part of the research related to the inhibition of CK2-mediated phosphorylation by CIGB-300, in our work, we explored for the first time the early-regulated cellular phosphoproteome. The global inhibitory profile of phosphorylation by CIGB-300 was unveiled. It was characterized by a large decrease in the phosphorylation of Ser-containing sites surrounded by acidic residues, which is typical of CK2 substrates [19]. The identity of the CK2 substrates to which the phosphosites inhibited by CIGB-300 belonged indicated that 8 and 10 substrates for this kinase were inhibited at 10 and 30 min, respectively [19]. In addition, the bioinformatic analysis indicated that the inhibited substrates

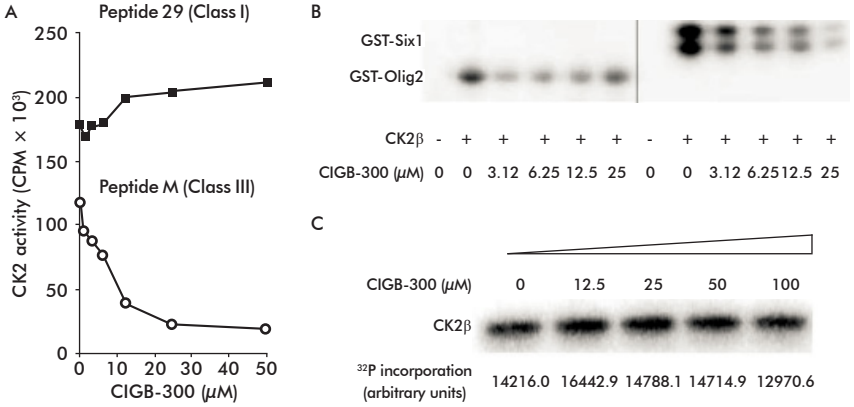

Figure 1. Effect of CIGB-300 on the activity of the CK2 holoenzyme. Radiometric assays for phosphorylation by the CK2 holoenzyme or the CK2α subunit were performed. There were used 28 ng of these proteins and increasing amounts of CIGB-300. Reactions were run in the presence of 10 mM $MgCl_2$ and 100 mM [γ32P]-ATP (6000 Ci/mmol), as well as 1 mM of different peptide substrates. A) M, 29. B) Recombinant substrates GST-Olig2 and GST-Six1. C) CK2β subunit.

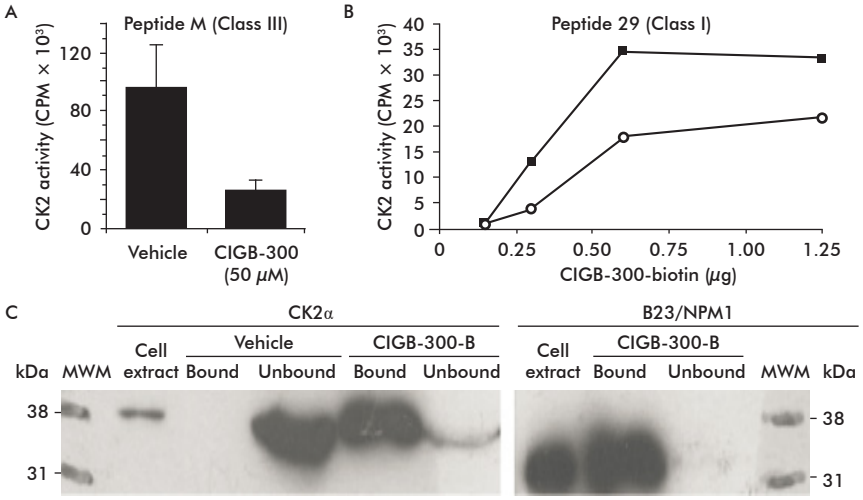

Figure 2. Evaluation of the interaction of CIGB-300 with CK2α. GST-CK2α fusion protein (80 ng) was incubated in the absence or presence of CIGB-300 (50 μM). A) Once adsorbed to a glutathione-sepharose matrix, 160 ng of CK2β were added and the enzymatic activity was determined using peptide M. B) Alternatively, CK2α (240 ng) was incubated in the presence or absence of 100 μM of CIGB-300 and increasing amounts of biotinylated CIGB-300 (CIGB-300-B) adsorbed on streptavidin-sepharose matrix. After several washings, the enzymatic reaction with peptide 29 was carried out. C) CK2α was captured in cell lysates by pull-down assays with CIGB-300-B adsorbed onto a streptavidin-sepharose matrix. After several washes, the proteins adhered to CIGB-300. MWM: Molecular weight marker.

were involved in processes such as ribosomal biogenesis, metabolism, RNA processing, apoptosis, gene expression and cell cycle, thereby contributing to the *in vitro* and *in vivo* antineoplastic effect of the CIGB-300.

Overall, we can conclude that CIGB-300 inhibits a set of CK2 substrates (Table 1), and that both mechanisms of CK2 inhibition by CIGB-300 could operate concomitantly in the cellular context. Furthermore, the integration of the results allows us to propose a model in which the inhibition of the CK2 holoenzyme occurs by the direct interaction of CIGB-300 with the CK2α subunit, giving rise to a "non-productive" or inactive form of the holoenzyme. This reinforces the scientific novelty of this work, by providing the first description of the global phosphoproteome regulated by

17. Boldyreff B, James P, Staudenmann W, Issinger OG. Ser2 is the autophosphorylation site in the beta subunit from bicistronically expressed human casein kinase-2 and from native rat liver casein kinase-2 beta. Eur J Biochem. 1993;218(2):515-21.

18. Pagano MA, Sarno S, Poletto G, Cozza G, Pinna LA, Meggio F. Autophosphorylation at the regulatory beta subunit reflects the supramolecular organization of protein kinase CK2. Mol Cell Biochem. 2005;274(1-2):23-9.

19. Perera Y, Ramos Y, Padrón G, Caballero E, Guirola O, Caligiuri LG, *et al*. CIGB-300 anticancer peptide regulates the protein kinase CK2-dependent phosphoproteome. Mol Cell Biochem. 2020;470(1-2):63-75.

CIGB-300 in tumor cells. Moreover, a wider overview of the inhibition exerted by CIGB-300 on the CK2-mediated phosphorylation within the cell was provided.

Considering the CIGB-300 antineoplastic mechanism of action, it was also investigated the ability of the peptide to synergize with some widely used chemotherapeutic agents in clinical oncology and EGFR inhibitors. Specifically, there were studied the effects of the combinations *in vitro* of CIGB-300 with cisplatin (an alkylating agent), paclitaxel (anti-mitotic), doxorubicin (anti-topoisomerase II), and 5-fluorouracil (antimetabolite) in human lung and cervix cancer cells [21].

To determine the type of interaction (synergistic, additive or antagonistic), a Latin square design was applied, where different doses of each drug were mixed and their effect on cell viability is quantified and processed by Calcusyn. This software generates important pharmacological parameters such as the combination index (CI), based on dose analysis-response of the combinations. CI values less than and equal to 0.5 indicated strong synergism, from 0.5 to 0.9 synergy/additivity, equal to 1.0 additivity, and greater than 1.0 antagonism. Interestingly, synergism of CIGB-300 with paclitaxel and cisplatin was observed with CI values of 0.30 and 0.83, respectively. In contrast, very little synergy and areas of slight antagonism was found for doxorubicin and 5-fluorouracil, for the dose range investigated. Additionally, the combination of CIGB-300 with Cisplatin was explored in vivo in a preclinical model of cancer [21]. The results showed a significant synergism of antitumor effect between both drugs, in terms of survival of tumor-bearing animals. The most relevant combinations were: CIGB-300 (50 µg) plus cisplatin (1 mg/kg) and CIGB-300 (200 µg) plus cisplatin (4 mg/kg). On the other hand, the results of the *in vitro* combination of CIGB-300 with EGFR inhibitors indicated peptide synergy with erlotinib, with CI values of 0.54 to 0.76 for NCI-H460 cells, and 0.35 to 0 .83 in A549 cells [22]. Similarly, an increase in the cytotoxic effect of the combination of CIGB-300 with nimotuzumab was observed, in the doses evaluated in A431 cells [23].

Overall, it was determined that CIGB-300 is not only capable of increasing its antineoplastic effect when combined with chemotherapeutic agents, but also in the presence of EGFR inhibitors, an aspect that strengthens the scientific novelty of this work. Finally, the molecular bases that support the synergism between CIGB-300 and anticancer drugs were studied. In this sense, the previously observed results related to the inhibition of CK2 phosphorylation in PTEN and AKT by CIGB-300 provide molecular support to the antineoplastic synergism of CIGB-300 with both chemotherapeutic agents and EGFR inhibitors. It is known that this biochemical event in both substrates plays a crucial role in tumor resistance [24]. Additionally, the NCI-H125 cell proteome was explored by means of comparative proteomics, in the presence of the combination CIGB-300 plus cisplatin and each agent separately. Up to 28 proteins linked to resistance to cisplatin were identified, their levels modified by its combination with CIGB-300 (Table 2) [25]. Other groups have described that the transcriptional factor NF-κB plays an essential role as mediating resistance against Cisplatin and other chemotherapeutic agents [26, 27]. Considering these, the impact of CIGB-300 on the levels of NF-κB under a resistant phenotype to Cisplatin in cell lung cancer cells was analyzed. For this purpose, a subline called

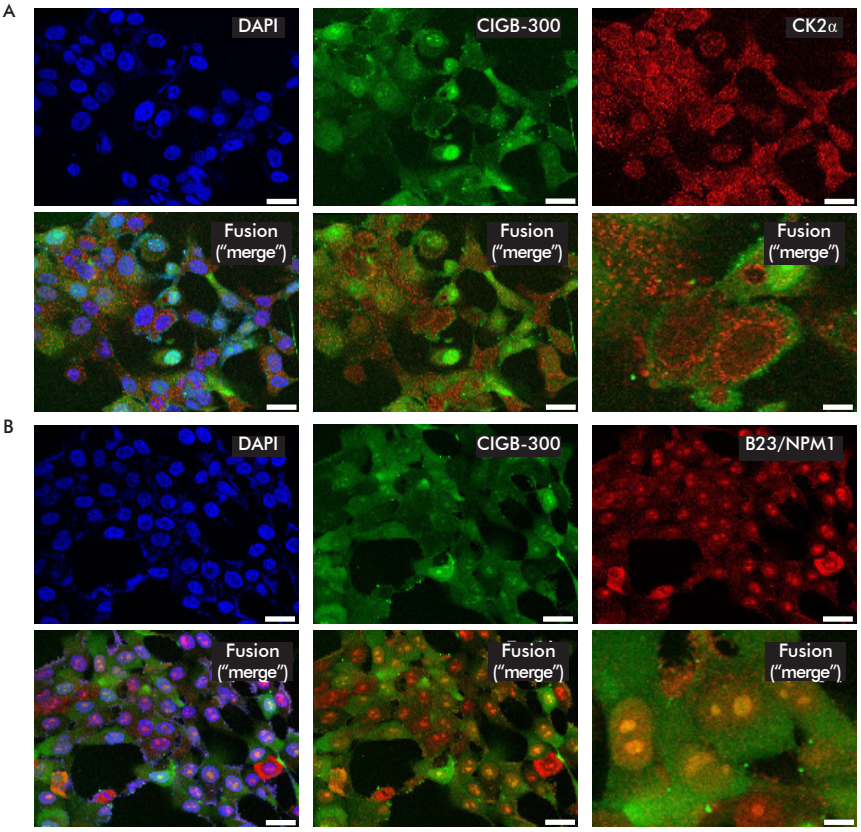

Figure 3. *In situ* colocalization of the CIGB-300 with CK2α. NCI-H125 cells were treated with CIGB-300-B (50 µM) for 10 min, then washed, fixed, and permeabilized. After blocking with BSA (4 %), they were incubated with the specific antibody. Finally, avidin-FITC conjugates, or secondary antibodies conjugated to Alexa Fluor 594, were added. Analysis was performed on a Leica Microsystem fluorescence microscope. Staining with 4′,6-diamino-2-phenylindole (DAPI; blue) was performed to contrast the cell nucleus. A) Incubation with anti-CK2α antibody. B) Incubation with anti-B23/NPM1 antibody, used as reference for colocalization analysis. Dimension barr: 20 µm.

Table 1. List of CK2 substrates inhibited by CIGB-300

Substrate	Name	Inhibition site	Methodology used [Reference]
B23/NPM1	Nucleophosmin	Ser125	Western blot/Metabolic labeling/ Phosphoproteomics [14, 19]
HDAC1	Histone deacetylase 1	Ser393	Phosphoproteomics [19]
PTEN	Phosphatidylinositol-3,4,5-triphosphate 3 phosphatase	Ser380	Western blot [16, 19]
AKT	Protein kinase B	Ser129	Western blot [16, 19]
EEF1D	Elongation factor 1 delta	Ser162	Phosphoproteomics [19]
USP7	Ubiquitin carboxyl-terminal hydrolase A7	Ser18	Phosphoproteomics [19]
STMN1	Statmin 1	Ser16/63	Phosphoproteomics [19]
ABCF1	ABC transporter Subfamily F1	Ser109	Phosphoproteomics [19]
PDCD5	Programmed cell death protein 5	Ser119	Phosphoproteomics [19]
PPP1R2	Protein phosphatase 2 inhibitor	Ser121/ Ser122	Phosphoproteomics [19]
SEPT2	Septin 2	Ser218	Phosphoproteomics [19]
HMGA1	High mobility group protein A1	Ser102/Ser103	Phosphoproteomics [19]
CDC37	HSP90 co-chaperone protein	Ser13	Phosphoproteomics [19]
MYH9	Myosin 9	Ser1943	Phosphoproteomics [19]
HSP90AB1	Heat shock protein 90 kDa	Ser255	Phosphoproteomics [19]
HSP90AB2P	Heat shock protein 90 kDa beta 2	Ser177	Phosphoproteomics [19]

A549-cispR was generated from parental A549 cells, which were chronically exposed to Cisplatin *in vitro* until developing a resistant phenotype. Consequently, the A549-cispR subline showed higher intrinsic levels of the NF-κB factor than the parental cell line, which were reduced by CIGB-300. Furthermore, the sensitivity levels of the A549-cispR subline were higher against CIGB-300 than those shown by parental cells [28].

Altogether, these results describe a group of molecular events modulated by the CIGB-300, which could contribute to the molecular bases supporting the synergy and additivity with other chemotherapeutic agents widely used in clinical oncology such as paclitaxel and cisplatin. Moreover, CIGB-300 could be simultaneously administered with EGFR inhibitors such as erlotinib and nimotuzumab, which have proven efficacy in the treatment of various types of solid tumors.

Our findings may have a scientific-technical and social impact, regarding the possibility of new therapeutic options for cancer treatment based on combinations of CIGB-300 with said drugs.

Conclusions

The scientific contributions of this research comprises the findings on the interaction of CIGB-300 directly with the CK2α subunit and the inhibition of the enzymatic activity of the holoenzyme. This gives rise to a dual inhibition mechanism not previously described for other inhibitors of CK2 and other protein kinases. The CIGB-300-regulated CK2 phosphoproteome was characterized, inhibiting phosphorylation of several CK2 substrates linked to relevant cellular processes in cancer such as ribosomal biogenesis, exacerbated metabolism and RNA processing, apoptosis, gene expression and cell cycle. Furthermore, CIGB-300 was found to synergize with paclitaxel, cisplatin, and EGFR inhibitors, of great value for medical practice, particularly for the design of future clinical trials with CIGB-300 in cancer. The inhibition by CIGB-300 of phosphorylation of PTEN and AKT, as well as the reduction of NF-κB levels and the modulation of a set of proteins associated with resistance to cisplatin, provides the molecular bases for the possible synergy of this peptide with chemotherapeutic agents and EGFR inhibitors.

Table 2. cisplatin-resistant phenotype proteins that changed their levels in response to CIGB-300 combined with cisplatin

Uniprot_ID	Protein	Change factor	Expression
P00558	Phosphoglycerate kinase 1	0	High
P38919	Eukaryotic initiation factor 4A-III	0	High
P22626	Heterogeneous nuclear ribonucleoprotein A2/B1	0.1	High
P09429	High mobility group protein B1	0.1	High
Q09028	Histone-binding protein RBBP4	0.1	High
P62888	60S ribosomal protein L30d	0.1	High
P33991	DNA replication licensing factor MCM4	0.1	High
Q02952	A-kinase anchor protein 12	0.1	High
Q15233	Non-POU domain-containing octamer-binding protein	0.1	High
P08670	Vimentin	0.2	High
Q04695	Keratin, type I cytoskeletal 17	0.2	High
P08107	Heat shock 70 kDa protein 1A	0.2	High
P21291	Cysteine and glycine-rich protein 1	0.2	High
P11142	Heat shock cognate 71 kDa protein	0.2	High
P13073	Cytochrome c oxidase subunit 4 isoform 1 mitochondrial	0.2	High
Q15149	Plectin	0.3	High
P60709	Actin, cytoplasmic 1	0.3	High
P0C0S5	Histone H2A.Z	0.3	High
P51858	Hepatoma-derived growth factor	0.3	High
P07910	Heterogeneous nuclear ribonucleoprotein	0.3	High
Q96PK6	RNA-binding protein 14	0.3	High
Q03252	Lamin-B2	0.3	High
P52272	Heterogeneous nuclear ribonucleoprotein M	0.3	High
P09382	Galectin-1	0.3	High
P04264	Keratin, type II cytoskeletal 1	3.3	Low
P47756	F-actin-capping protein subunit beta	3.6	Low
P31946	14-3-3 protein beta/alpha	8.2	High/Low
Q15070	Mitochondrial inner membrane protein OXA1L	42.3	Low

Change factor: CIGB-300 + cisplatin vs. Control. Expression: Expression in cisplatin-resistant cells.

Acknowledgements

We thank to Lorena G. Caligiuri, Norailys Lorenzo, Florencia Gottardo, Hernán G. Farina, Odile Filhol, Claude Cochet, Joao Barata, Estefano Cirigliano and Alejandro Ultreguer as direct collaborators of this work. This research was supported by the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

20. Perera Y, Melão A, Ramón AC, Vázquez D, Ribeiro D, Perea SE, *et al*. Clinical-grade peptide-based inhibition of CK2 blocks viability and proliferation of T-ALL cells and counteracts IL-7 stimulation and stromal support. Cancers. 2020;12(6):1377.

21. Perera Y, Toro ND, Gorovaya L, Fernandez DECJ, Farina HG, Perea SE. Synergistic interactions of the anti-casein kinase 2 CIGB- 300 peptide and chemotherapeutic agents in lung and cervical preclinical cancer models. Mol Clin Oncol. 2014; 2(6):935-44.

22. Perea SE, Perera Y, Baladrón I, González L, Benavent F, Fariña HG, *et al*. CIGB-300: a promising anti-casein kinase 2 (CK2) peptide for cancer targeted therapy. In: Ahmed K, *et al*. (eds.). Protein Kinase CK2 Cellular Function in Normal and Disease States. Cham: Springer; 2015. p. 281-98.

23. Perea SE, Baladrón I, Valenzuela C, Perera Y. CIGB-300: A peptide-based drug that impairs the protein kinase CK2-mediated phosphorylation. Sem Oncol. 2018;45(1-2):58-67.

24. Borgo C, Ruzzene M. Role of protein kinase CK2 in antitumor drug resistance. J Exp Clin Canc Res. 2019;38(1):287.

25. Rodriguez-Ulloa A, Ramos Y, Sanchez-Puente A, Perera Y, Musacchio-Lasa A, Fernandez-de-Cossio J, *et al*. The combination of the CIGB-300 anticancer peptide and Cisplatin modulates proteins related to cell survival, DNA repair and metastasis in a lung cancer cell line model. Curr Proteomics. 2019;16(4):338-49.

26. Jones DR, Broad RM, Madrid LV, Baldwin Jr AS, Mayo MW. Inhibition of NF-κB sensitizes non–small cell lung cancer cells to chemotherapy-induced apoptosis. Ann Thoracic Surg. 2000;70(3):930-6.

27. Mabuchi S, Ohmichi M, Nishio Y, Hayasaka T, Kimura A, Ohta T, *et al*. Inhibition of NFκB increases the efficacy of cisplatin in *in vitro* and *in vivo* ovarian cancer models. J Biol Chem. 2004;279(22):23477-85.

28. Cirigliano SM, Díaz Bessone MI, Berardi DE, Flumian C, Bal de Kier Joffé ED, Perea SE, *et al*. The synthetic peptide CIGB-300 modulates CK2-dependent signaling pathways affecting the survival and chemoresistance of non-small cell lung cancer cell lines. Canc Cell Int. 2017;17:42.

Published translated from: Perea-Rodríguez SE, Perera-Negrin Y, Rodríguez-Ulloa A, Ramos-Gómez Y, Rosales-Menzoney M, Padrón-Palomares G, *et al*. Nuevos eventos moleculares vinculados al mecanismo de acción antineoplásico del CIGB-300. An Acad Cienc Cuba. 2022;12(2). Available from: http://www.revistaccuba.cu/index.php/revacc/article/view/1110

Received in June, 2021.
Accepted in September, 2021.