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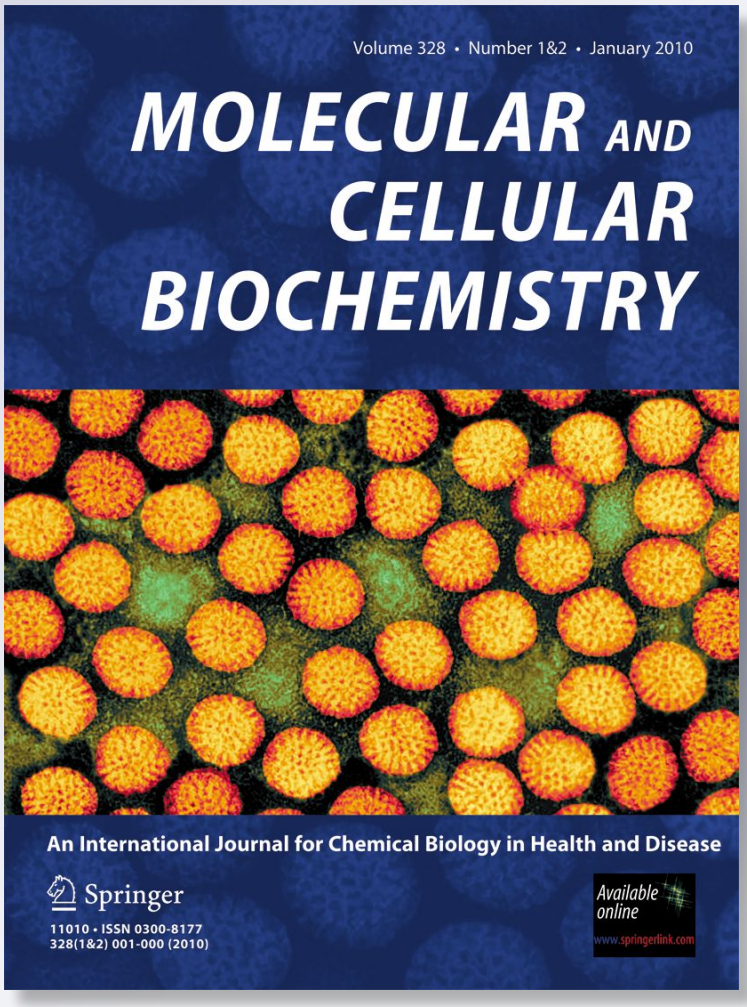

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CIGB-300, a synthetic peptide-based drug that targets the CK2 phosphoaceptor domain. Translational and clinical research

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Abstract CK2 represents an oncology target scientifically validated. However, clinical research with inhibitors of the CK2-mediated phosphorylation event is still insufficient to recognize it as a clinically validated target. CIGB-300, an investigational peptide-based drug that targets the phosphoaceptor site, binds to a CK2 substrate array in vitro but mainly to B23/nucleophosmin in vivo. The CIGB-300 proapoptotic effect is preceded by its nucleolar localization, inhibition of the CK2-mediated phosphorylation on B23/nucleophosmin and nucleolar disassembly. Importantly, CIGB-300 shifted a protein array linked to apoptosis, ribosome biogenesis, cell proliferation, glycolisis, and cell motility in proteomic studies which helped to understand its mechanism of action. In the clinical ground, CIGB-300 has proved to be safe and well tolerated in a First-in-Human trial in women with cervical malignancies who also experienced signs of clinical benefit. In a second Phase 1 clinical trial in women with cervical cancer stage IB2/II, the MTD and DLT have been also identified in the clinical setting. Interestingly, in cervical tumors the B23/nucleophosmin protein levels were significantly reduced after CIGB-300 treatment at the nucleus compartment. In addition, expanded use of CIGB-300 in case studies has evidenced antitumor activity when administered as compassional option. Collectively, our data outline important clues on translational and clinical research from this novel peptide-based drug reinforcing its perspectives to treat cancer and paving the way to validate CK2 as a promising target in oncology.

Keywords CIGB-300 · Nucleophosmin · Protein kinase CK2 · Cell penetrating peptide · Cancer targeted therapy

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Abbreviations

MTD	Maximal tolerated dose
DLT	Dose-limiting toxicity
CIGB-300-B	CIGB-300 conjugated to biotin
BFS	Bovine fetal serum
TBB	4,5,6,7-Tetrabromobenzotriazole
PBS	Phosphate buffer solution
RIPA	Radioimmunoprecipitation assay
HPV	Human papillomavirus
^{99}Tc	Technetium-99
MRI	Magnetic resonance images

Introduction

Exploitation of human kinome as cancer therapeutic targets is continuously growing and its clinical validation becomes a reality as therapies with some kinase inhibitors have showed clinical benefit in cancer patients [1, 2]. CK2 is a serine/threonine kinase that phosphorylates around 300 substrates [3] and is responsible for the generation of a substantial proportion of the eukaryotic phosphoproteome [4]. Data from different groups have evidenced an essential role for CK2 on cell survival [5], chromatin remodeling [6], protection of cells against apoptosis [7], and angiogenesis [8]. Likewise, additional experimental validation of the CK2 role in cancer cell biology has come from different authors who have confirmed its oncogenic potential and tumorigenicity [9, 10] and the overexpression in human cancer [11, 12]. Therefore, the CK2-mediated phosphorylation has been suggested as a druggable event which could impact different hallmarks in cancer [13, 14].

In the past 10 years, mounting evidences have also contributed to the scientific validation of CK2 as a putative target in cancer using pharmacological and genetic approaches to impair the enzymatic activity. For instance, condensed polyphenolic compounds, tetrabromobenzimidazole/triazole derivatives and indoloquinazolines, have been showed to selectively inhibit the CK2 enzyme and exhibit a remarkable pro-apoptotic efficacy on a variety of tumor cell lines [15]. Similarly, CK2 down-regulation and antitumor effect have been observed using antisense CK2 alpha oligodeoxynucleotide in PC3-LN4 xenograft tumors in nude mice [16]. More recently, the CX-4945 compound, another CK2 inhibitor that blocks the ATP-binding site on the catalytic subunit has recently entered into the clinical ground for testing in cancer patients [17]. Thus, the development and exploitation of CK2 inhibitors could provide new hopes for cancer therapy.

The anti-CK2 approaches so far described have been focused to target the enzyme itself, however, our approach describes a peptide (CIGB-300) targeting the acidic phosphoaceptor site on the CK2 substrates [18]. Recent biochemical and biological characterization of CIGB-300 allowed knowing the CK2 substrates that bind to this peptide in vitro as well as the major targets in vivo. We also accumulated evidences that suggest a putative impact of CIGB-300 over ribosome biogenesis and other relevant processes in cancer. Finally, CIGB-300 has reached the clinical ground under a novel scientific rational which exploits the targeting of substrates outcompeting the protein kinase CK2 [19]. We provide here the most recent advances in clinical research with CIGB-300 in patients with cancer.

Materials and methods

CIGB-300

CIGB-300 was synthesized on solid phase and purified by reverse-phase high performance liquid chromatography (RP-HPLC) to >95% purity on an acetonitrile/H_2O-trifluoroacetic acid gradient [20] and confirmed by ion-spray mass spectrometry (Micromass, Manchester, UK).

Pull-down assay

To look the CIGB-300 interacting proteins in vitro, cell extract from NCI-H125 cell line was prepared, and 300 μg of total protein was added to 50 μl of pre-equilibrated Streptavidin–Sepharose matrix (Sigma). After 1 h of incubation at 4°C, the matrix was collected by short spin, extensively washed with PBS 1 mM DTT and treated for western blot analysis or stored to −70°C in double distilled water for mass spectrometry analysis. For in vivo pull-down experiments, cells were seeded in appropriate vessels at 4×10^5 cell/ml (NCI-H82) and cultured during 18–20 h. Next day, the CIGB-300-B was added to the cell cultures at a final concentration of 100 μM and incubated for 30 min. Subsequently, cells were collected by centrifugation, washed two times with cold PBS and lysed in hypotonic PBS solution (0.1×) containing 1 mM dithiothreitol (DTT, Sigma) and complete protease inhibitor (Roche, Germany) by five freeze (N_2)–thaw (37°C) cycles. Then, cellular lysate was cleared by centrifugation at 12,000 rpm 4°C for 15 min, and 300 μg of total protein was mixed with 50 μl of pre-equilibrated Streptavidin–Sepharose matrix (Sigma). After 1 h of incubation at 4°C, the matrix was collected by short spin, extensively washed with PBS 1 mM DTT and treated for western blot analysis or stored to −70°C in double distilled water for mass spectrometry analysis.

In vivo phosphorylation

For [^{32}P] orthophosphate labeling, NCI-H82 cells were grown in RPMI at 10% BFS for 20 h (non-arrested condition) or 0.2% BFS for 48 h (arrested conditions). Serum-deprived cultures were further grown in RPMI 10% FBS for 2 h to commit cells to re-enter the cell cycle. Subsequently, cells were incubated in phosphate-free medium supplemented with 10% BFS for 1 h. Fresh medium containing 1 mCi/ml [^{32}P] orthophosphate (Amersham) was then added. Different concentrations of selected peptides or TBB inhibitor were included in the culture medium and further incubated for 30 min. Cells were washed twice with cold PBS and lysed in RIPA buffer containing 2 mM of each NaF, Na3VO4, and B-glycerophosphate. Lysates were clarified at 12,000×*g* 4°C for 15 min and supernatants collected for immunoprecipitation. For each reaction, 200 μg of total proteins were mixed with 3 μg of anti-B23 Mab. Subsequent steps were performed as recommended in the Protein G immunoprecipitation Kit (Sigma). Finally, immunoprecipitates were analyzed by SDS-PAGE, and gels were blue-stained, dried, and further exposed. Image analysis from the X-rays films (FUGI, Japan) and gels was performed with ImageJ 1.37v (NIH, USA). The phosphorylation inhibition was calculated relative to ^{32}P signal from non-treated cells (100%) at each condition. Immunoprecipitation efficiency and gel loading were normalized by dividing the ^{32}P signal by their corresponding blue-stained protein signal for each lane.

Clinical evaluation of CIGB-300

First-in-human trial

Thirty-one women with colposcopic and histological diagnosis of microinvasive or pre-invasive cervical cancer were enrolled in a dose escalating study. CIGB-300 was administered sequentially at 14, 70, 245, and 490 mg by intralesional injections during five consecutive days to groups of 7–10 patients. Toxicity was monitored daily until 15 days after the end of treatment, when patients underwent conization. Digital colposcopy, histology, and HPV DNA status were also evaluated by polymerase chain reaction.

Phase 1 study

Fourteen women with cervical cancer stage IB2/II were enrolled in a dose escalating study. CIGB-300 was administered sequentially to groups of 6 patients at 35, 70, 245, and 490 mg during five consecutive days by intratumor injections until maximal tolerated dose was found. Systemic and local toxicity was monitored daily until the end of treatment and histamine levels in plasma were kinetically measured after the first drug administration by ELISA. Tumor uptake, whole body biodistribution, and drug levels in blood were performed after the first drug administration with ^{99}Tc-labeled CIGB-300. The modulation of B23/nucleophosmin and CK2β in tumors was studied by immunohistochemistry in paraffin-embedded tumor biopsies.

Case study

A 24-year-old man diagnosed as Germinoma with a big tumor mass at the L4-L5 intervertebral space was treated with intralesional injections of CIGB-300 in a consecutive 5 day cycle at day 0 and 30; using 35 mg in each dose. Clinical response was recorded by resonance MRI.

All the clinical experimentation in human beings was performed under *Good Clinical Practices* with approval of ethical committees and informed consent from each patient was obtained.

Results and discussion

In this study, we have outlined novel biochemical and biological features of CIGB-300, a peptide-based approach which targets the CK2 phosphoaceptor site on the substrates. In particular it was investigated the in vitro and in vivo CIGB-300 interacting proteins guided by the fact that the CK2 phosphoaceptor site is very conserved among the

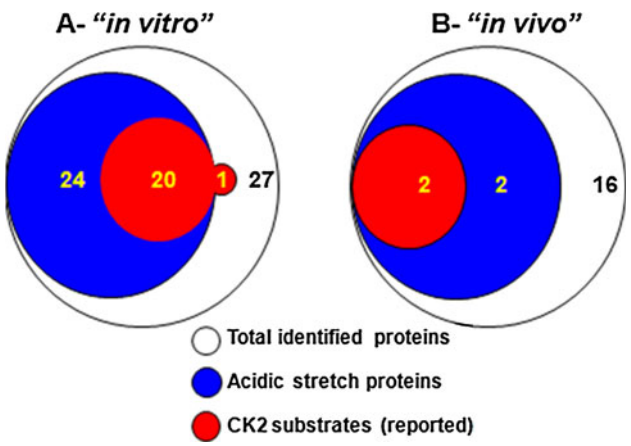

Fig. 1 CIGB-300 interactome and pull-down experiments. Cell extract from NCI-H-125 cell line was incubated 1 h with Streptavidin–Sepharose matrix in vitro. After extensive washes, attached proteins were analyzed by western blot and mass spectrometry (**a**). For in vivo pull-down experiments, 4 × 10^5 cells/ml (NCI-H82) were incubated with CIGB-300 peptide conjugated to CIGB-300-B to a final concentration of 100 μM for 30 min. Then, cellular lysates were incubated with Streptavidin–Sepharose matrix for 1 h at 4°C, the matrix was collected by short spin, extensively washed and treated for WB analysis or stored to −70°C in double distilled water for mass spectrometry analysis (**b**)

near 300 substrates recognized today for CK2. Interestingly, data from in vitro studies indicated that 21 different CK2 substrates did bind to CIGB-300 in our experimental conditions whose functions are implied in cell signaling, translation, ribosome biogenesis, chaperone activity, cytoskeleton, and DNA/RNA processing among others (Fig. 1). Otherwise, in vivo experiments indicated that two CK2 substrates were mainly targeted by CIGB-300, the nucleolar proteins: B23/nucleophosmin and C23/nucleolin. Interesting, 13 structural ribosomal proteins from the small subunit and one from the large subunit were also identified in vivo. Considering that B23 and C23 are usually found associated with ribosomal RNA or small-nucleolar RNA complexes in the ribosomal biogenesis pathway [21]; we treated the pull-down fractions with excess amounts of RNase. Data from such experimentation demonstrated that CIGB-300-C23 but not CIGB-300-B23 interaction is mediated by RNA hence suggesting that B23 but not C23 is the actual in vivo target for CIGB-300. In line with the results from pull-down experiments, we were able to evidence that CIGB-300 colocalizes with B23/nucleophosmin at the nucleolar compartment. Such physical interaction leads to reduction of the CK2-mediated phosphorylation on this B23/nucleophosmin in a dose-dependent manner (Fig. 2). Parallely, evidences of the nucleolar breakdown were obtained from the CIGB-300-B23 colocalizations experiments performed at the same peptide concentrations. Thus, using B23/npm stain as a marker for the granular component of the nucleolus, we observed a red punctuate pattern within the cell nucleoplasm after 1 h of incubation with CIGB-300, which indicate the lost of nucleolar architecture. All of these biochemical events were paralleled by the rapid induction of cell death by apoptosis as determined by annexin V labeling.

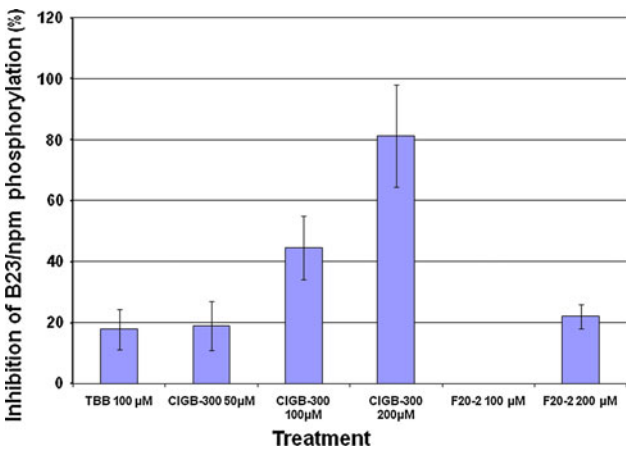

Fig. 2 CIGB-300 inhibits the CK2-mediated phosphorylation on B23/nucleophosmin. Cells were incubated in phosphate-free medium supplemented with 10% FBS for 1 h. Fresh medium containing 1 mCi/ml [^{32}P] orthophosphate (Amersham) was then added. Subsequently, CIGB-300, F20-2 (negative control peptide) or TBB inhibitor were included in the culture medium and further incubated for 30 min. Lysates were prepared with RIPA buffer, clarified and supernatants collected for immunoprecipitation. For each reaction, 200 µg of total proteins were mixed with 3 µg of anti-B23 Mab. Subsequent steps were performed as recommended in the Protein G immunoprecipitation Kit (Sigma). Finally, immunoprecipitates were analyzed by SDS-PAGE, and gels were blue-stained, dried, and further exposed. Image analysis from the X-rays films and gels was performed with ImageJ 1.37v (NIH, USA). The phosphorylation inhibition was calculated relative to ^{32}P signal from non-treated cells (100%) at each condition. Immunoprecipitation efficiency and gel loading were normalized by dividing the ^{32}P signal by their corresponding blue-stained protein signal for each lane

In the clinical ground, we initially conducted a First-in-Human clinical trial in 31 women with cervical malignancies in a dose escalation study. CIGB-300 was administered sequentially at 14, 70, 245, and 490 mg for five consecutive daily intralesional injections to groups of 7–10 patients. Results from this clinical trial indicated a dose-dependent toxicity pattern. The most frequent local events were pain, bleeding, hematoma, and erythema at the injection site. The systemic adverse events were rash, facial edema, itching, hot flashes, and localized cramps. Efficacy signs were registered as follows: 75% of the patients experienced a significant lesion reduction, 19.3% of the patients exhibited full histological regression, and 50% of the previously HPV DNA-positive patients become negative by the end of treatment.

Remarkably, in a Phase 1 study in 14 women with cervical cancer stage IB2/II, we identified the MTD for CIGB-300 using local application neoadjuvant to chemoradiotherapy. Likewise, the DLT was related to allergic reactions that in turns did correlate with histamine levels in plasma around 15 min after CIGB-300 administration. Importantly, high CIGB-300 tumor uptake was evidenced using ^{99}Tc-labeled peptide which was also detected in other organs like kidneys, liver, and spleen. Interestingly, CIGB-300 treatment significantly reduced the B23/nucleophosmin levels in tumor biopsies.

Finally, CIGB-300 is being also tested in the clinical ground as expanded use in compassional treatment. A chemoradio refractory patient diagnosed as Germinoma with a big tumor mass at the L4-L5 intervertebral space in the lumbar region was treated with intralesional injections of CIGB-300 in two independent consecutive 5 day cycles using 35 mg in each dose. Importantly, 7 days after concluding the first cycle of CIGB-300, a significant tumor shrinking was observed and patient experienced amelioration of the clinical symptoms. Such antitumor response was further maintained after the second cycle with CIGB-300 (day 30) and thereafter until complete 12 months (Fig. 3). Thus, the use of CIGB-300 improved the life quality for this cancer patient at least during the 1 year of follow-up.

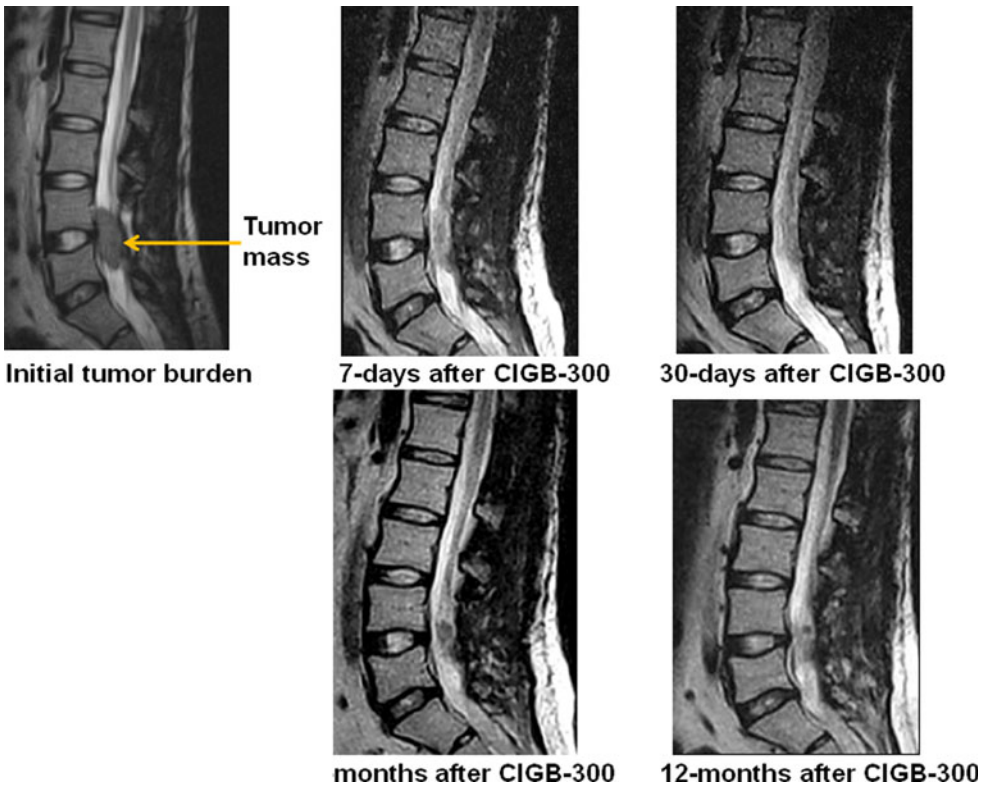

Fig. 3 Compassional use of CIGB-300 in a cancer patient. A chemo-radio refractory patient with a Germinoma and a big tumor mass at the L4-L5 intervertebral space in the lumbar region received intralesional injections of CIGB-300 in a consecutive 5 day cycle at day 0 and 30; using 35 mg in each dose. Antitumor effect was periodically recorded by MRI

Further clinical development of CIGB-300 is currently in course with different clinical trials running in cancer patients using alternative routes of delivery.

Altogether, data provided here uncover novel biochemical insights among the mechanism of action of CIGB-300 and give important clues not only for the clinical development of CIGB-300 but also for paving the way toward the clinical validation of CK2-mediated phosphorylation as a promising oncology target.

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