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CIGB-300, a novel proapoptotic peptide that impairs the CK2 phosphorylation and exhibits anticancer properties both in vitro and in vivo

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Abstract Protein Kinase CK2 is a serine-threonine kinase frequently deregulated in many human tumors. Here, we hypothesized that a peptide binder to the CK2 phosphoacceptor site could exhibit anticancer properties in vitro, in tumor animal models, and in cancer patients. By screening a random cyclic peptide phage display library, we identified the CIGB-300 (formerly P15-Tat), a cyclic peptide which abrogates the CK2 phosphorylation by blocking recombinant substrates in vitro. Interestingly, synthetic CIGB-300 led to a dose-dependent antiproliferative effect in a variety of tumor cell lines and induced apoptosis as evidenced by rapid caspase activation. Importantly, CIGB-300 elicited significant antitumor effect both by local and systemic administration in murine syngenic tumors and human tumors xenografted in nude mice. Finally, we performed a First-in-Man trial with CIGB 300 in patients with cervical malignancies. The peptide was found to be safe and well tolerated in the dose range studied. Likewise, signs of clinical benefit were clearly identified after the CIGB-300 treatment as evidenced by significant decrease of the tumor lesion area and histological examination. Our results provide an early proof-of-principle of clinical benefit by using an anti-CK2 approach in cancer. Furthermore, this is the first clinical trial where an investigational drug has been used to target the CK2 phosphorylation domain.

Keywords Casein kinase 2 · Apoptosis · Cancer targeted therapy

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Introduction

Exploitation of kinases as cancer therapeutic targets is continuously growing and its clinical validation becomes a reality as therapies with some specific inhibitors have showed clinical benefit in cancer patients [1, 2]. Protein Kinase CK2 is a very conserved serine/threonine kinase involved in the phosphorylation of a plethora of substrates, which display a very conserved acidic phosphorylation

domain [3]. CK2 is essential for different cell functions like gene expression [4], cell growth [5], cell survival [6], chromatin remodeling [7], and protection of cells against apoptosis [8]. Further to the findings mentioned above the potential of CK2 as a particularly suitable target for cancer treatment has been experimentally validated by different groups. For example, studies in transgenic models of cancer have demonstrated that CK2 overexpression displays a great oncogenic potential and tumorigenicity [9, 10]. Likewise, CK2 signal is uniformly dysregulated 3- to 7-fold in different cancer types [11], and also it has been associated with aggressive tumor behavior in human squamous cell carcinoma of head and neck cancer [12].

Other important hallmark that meets CK2 as an ideal target for cancer therapy is the cell death observed after its targeting by different agents. For instance, condensed polyphenolic compounds, tetrabromobenzimidazole/triazole derivatives and indoloquinazolines have been showed to selectively inhibit the CK2 enzyme and exhibit a remarkable proapoptotic efficacy on a variety of tumor cell lines [13]. Similarly, CK2 down-regulation and antitumor effect have been observed using antisense CK2 alpha oligodeoxynucleotide in PC3-LN4 xenograft tumors in nude mice [14]. 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 either by biochemical or genetic procedures; however, our approach describes a peptide (CIGB-300) targeting the acidic phosphoacceptor site on the CK2 substrates, exhibits antitumor activity in cancer animal models, and showed efficacy signs when assayed in a First-in-Man Clinical trial patients with cervical malignancies. Collectively, our data reinforces the potential of CIGB-300 as the first peptide-based drug for cancer targeted therapy which focuses on the CK2 substrates.

Materials and methods

Peptides

Synthetic peptide CIGB-300 and F20.2 control previously described [15] were synthesized on solid phase and purified by reverse-phase high performance liquid chromatography (RP-HPLC) to >95% purity on an acetonitrile/H2O-trifluoroacetic acid gradient [16] and confirmed by ion-spray mass spectrometry (Micromass, Manchester, UK).

In vitro phosphorylation of HPV-16 E7-GST fusion protein

The recombinant HPV-16 E7-GST fusion protein which served as a CK2 substrate for the in vitro phosphorylation in this work was purified by Glutathione Sepharose^{TM} 4B. For in vitro phosphorylation, about 30 µl of Glutathione Sepharose beads containing 10 µg of HPV-16 E7-GST in CK2 kinase buffer (20 mM Hepes pH 7.8, 20 mM $MgCl_2$) were used in each reaction. Prior to the phosphorylation reaction, either 10^9 Transducing Units of peptide-presenting phages or 100 µM from synthetic peptides were pre-incubated with the E7-GST beads for 1 h at 37°C with occasional shaking. Phosphorylation was performed by adding 10 µCi of [γ^{32}P] ATP, 100 µM of cold ATP (Amersham Biosciences), 1 U of recombinant CK2 (Promega), and further incubation for 30 min at 37°C. The CK2 activity was expressed as phosphorylated HPV-16 E7 relative levels respect to the blue-stained HPV-16 E7 levels.

Cell viability assay

Cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 or DMEM supplemented with 10% fetal calf serum (FCS). The cytotoxicity of peptides was monitored by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS$^{(a)}$] assay (Promega). Peptide concentrations ranging from 0 to 325 µM were added to 10,000 cells per well and incubated for 72 h at 37°C in 5% CO_2. Subsequently, 20 µl of the MTS solution was added to the wells and cells were further incubated for 1 h at 37°C . Finally, absorbance was taken at λ 492 nm. Background corresponding to the absorbance of wells without cells was subtracted. Each sample point was performed in triplicate and the experiments were carried out twice. IC50 values were obtained from the respective growth curves.

In vivo administration of CIGB-300

The animal models used for this experimentation were either C57/BL6 or athymic nu/nu BALB/c mice. Either tumor-bearing mice or animals before tumor debut received daily injections for five consecutive days with CIGB-300. In some experiments, F20.2-Tat or vehicle PBS were used as control. Animals were maintained in pathogen-free conditions, and procedures were performed in accordance with recommendations for the proper use and care of laboratory animals. Tumors were measured with a caliper and the respective volumes were calculated using the formula: Volume = width^2 (mm^2) × length (mm)/2.

Results and discussion

In the present study, we have characterized a peptide-based approach which was used to interfere CK2 phosphoacceptor site on its substrates. The antineoplastic effect of such a peptide inhibitor, the CIGB-300, was investigated

on tumor cell lines, in cancer animal models and in patients with cervical malignancies. The CIGB-300 peptide arose from screening a random cyclic peptide phage library using a synthetic CK2 phosphoacceptor site spanning from *Leu*28 to the *Iso*38 of the HPV-16 E7 oncoprotein. Thus, the synthetic CIGB-300 is a chimera containing the cyclic peptide identified from the library screening and fused to the Tat cell penetrating peptide.

The synthetic CIGB-300 largely abrogated the phosphorylation of recombinant E7-GST when pre-incubated with the substrate (Fig. 1). Otherwise, the Tat peptide did not modify significantly such biochemical event. Similarly to the data using chemical compounds targeting the CK2 ATP binding site, CIGB-300 induced a rapid induction of apoptosis on tumor cells as determined by caspase activation [15]. The biological effect of CIGB-300 was also examined in various tumor cell lines from different origins; importantly, a differential antiproliferative effect on various tumor cell lines was observed as determined by the IC50 values (Fig. 2) and primary fibroblasts seemed to be more resistant to this effect. In line with the ability of CIGB-300 to induce apoptosis, proteomic studies on H-125 cells indicated the up- and down-regulation of a set of proteins in the presence of this peptide which are involved in the apoptotic intrinsic pathways (Table 1). Additionally, studies on the functional characterization of the CIGB-300 indicated that different CK2 substrates are bound by this peptide using pull-down experiments both in vitro and in vivo.

Additionally, we explored whether CIGB-300 peptide could represent a therapeutic strategy to treat exponential growing tumors and therefore, the efficacy of daily administration both by intratumor and systemic injections of CIGB-300 was investigated in different cancer animal models. Data shown in Fig. 3 clearly demonstrated that

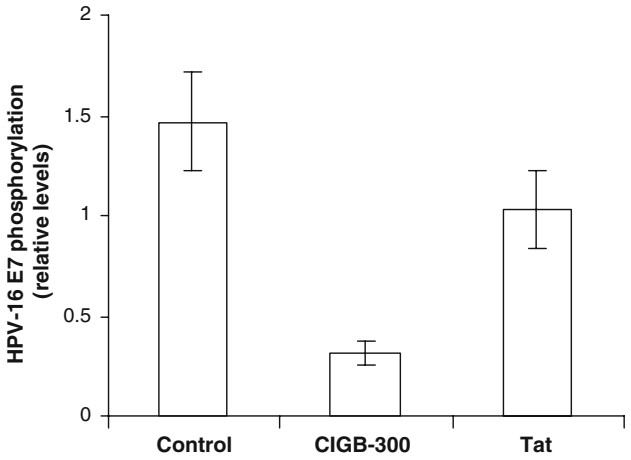

Fig. 1 HPV-16 E7 phosphorylation assay: Recombinant HPV-16 E7 as a GST-fusion protein was in vitro phosphorylated by CK2 in presence of equimolar concentration of CIGB-300 or Tat peptide. Phosphorylation levels were normalized with the blue-stained protein

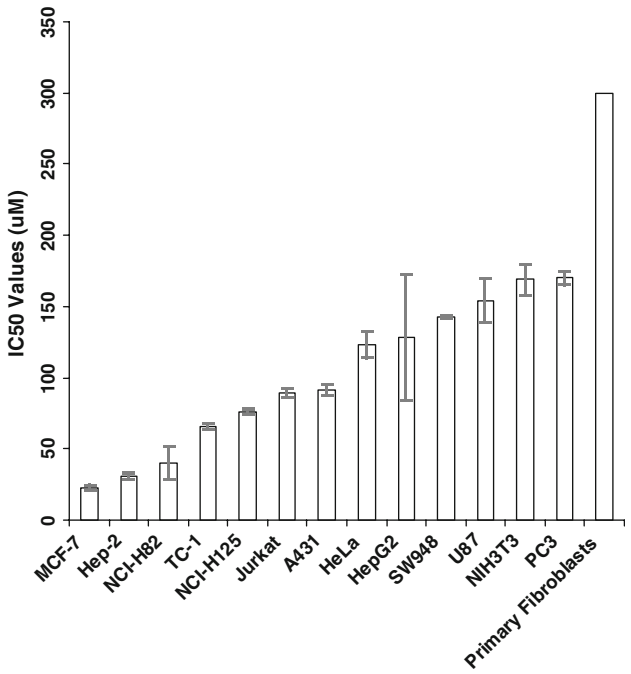

Fig. 2 Effect of CIGB-300 on cell proliferation. Peptide concentrations ranging from 0 to 200 μM were added to 1,000 cells per well and incubated for 72 h at 37°C in 5% CO_2. Results are expressed as IC50 values

intratumor administration of CIGB-300 led to a significant reduction of tumor growth compared to the group treated with Tat peptide as negative control. By day 15, $P < 0.0001$ was achieved between the CIGB-300 versus Tat group as determined by the U-Mann Whitney test (Confidence intervals 95%). Interestingly, the differences between CIGB-300 and the control group were maintained after cessation of treatment and until the end of the assay. In other set of experiments, the peptide was administered either intraperitoneal or intravenously during five consecutive days and using doses from 2 to 40 mg/kg. Interestingly, systemic administration of CIGB-300 also inhibited the tumor growth and induced apoptosis on the tumor mass [17].

Finally, to investigate the safety and tolerability of CIGB-300 in patients with cervical malignancies, we conduced the First-in-Man clinical trial with the CIGB-300. For the purpose, 31 women with colposcopically and histologically diagnosed microinvasive or pre-invasive cervical cancer were enrolled in a four-dose escalating study. CIGB-300 was administered sequentially at 14, 70, 245, and 490 mg by five consecutive daily intralesional injections to groups of 7–10 patients. Local and systemic toxicities were monitored daily until 15 days after the end of treatment, when patients underwent conization. Clinical benefit of CIGB-300 was evaluated by digital colposcopy and histology of colposcopically- directed biopsies. HPV status was also analyzed by PCR on biopsies before and

Table 1 CIGB-300 regulated proteome on Non-Small Cell Lung Cancer (NSCLC) H-125 cell line

Down-regulated proteins by the P15 peptide ingredient	Inhibition rate on the protein expression
Nucleofosmin	48
T-Plastin	3.34
Heat Shock Proteins (HSP-27, -70 y -90)	2.5
Y-box1 transcription factor	3.33
Eritropoietin precursor	120
S-gluthathione transferase	4.87
Proteasome activator complex	3.35
Ubiquitin activated E1 enzyme	2.49
Glucose-6-phosphate isomerase	8.53
Gliceraldehyde 6-phosphate deshydrogenase	6.62
Piruvate kinase	8.34
Translational controled tumor protein	4.32
Up-regulated proteins by the P15 peptide ingredient	**Activation rate on the protein expression**
Prohibitin	2.28
Tubulin alpha-1	3.23
Tubulin beta-2	2.56
Tubulin beta-3	3.15

4×10^5 cells per ml of H-125 cell line were incubated with 200 μM of CIGB-300 during 45 min at 37°C, 5% CO_2. To identify the CIGB-300 regulated proteome, nuclear extracts were prepared and further analyzed by Nano HPLC-Mass Spectrometry technology. Change in the protein expression levels in the presence of CIGB-300 was quantified by ISOTOPICA software, and data are represented as a rate of either inhibition or activation respect to untreated cells

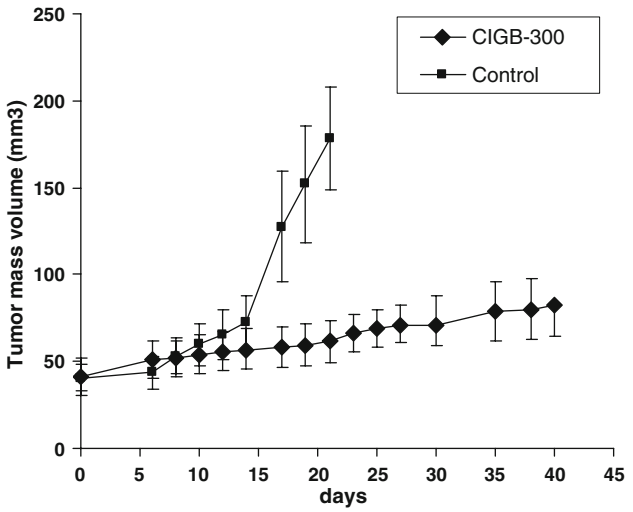

Fig. 3 Antitumor effect of CIGB-300 on human tumors xenografted in nude mice. Two million human cervical cancer cells (SiHa) were subcutaneously inoculated at the dorsal region and after several days, tumor-bearing mice received daily intratumor injections for five consecutive days with 200 μg of CIGB-300 or Tat peptide as negative control. Tumors were measured with a caliper and the respective volumes were calculated

after treatment. Results from this clinical trial indicated a dose-dependent toxicity pattern; neither a specific maximum-tolerated dose nor a dose-limiting toxicity was achieved. 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. Colposcopy indicated that 75% of the patients experienced a significant lesion reduction and 19.3% of the patients exhibited full histological regression. HPV became undetectable in 50% of the previously positive patients. Therefore, CIGB 300 was found to be safe and well tolerated in the dose range studied. The results provide an early proof-of-principle of clinical benefit by using a CK2 inhibitor in cervical malignancies, and this is the first clinical trial where a drug has been used to target the CK2 phosphoacceptor domain.

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References

1. Baselga J (2006) Targeting tyrosine kinases in cancer. Science 312:1175–1178. doi:10.1126/science.1125951
2. Kantarjian HM, Cortes J (2006) New strategies in chronic myeloid leukemia. Int J Hematol 83:289–293. doi:10.1532/IJH97.06024
3. Meggio F, Pinna LA (2003) One-thousand-and-one substrates of protein kinase CK2? FASEB J 17:349–368. doi:10.1096/fj.02-0473rev
4. Johnston IM, Allison SJ, Morton JP, Schramm L, Scott P, White RJ (2002) CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. Mol Cell Biol 22:3757–3768. doi:10.1128/MCB.22.11.3757-3768.2002
5. Guerra B, Issinger O-G (1999) Protein kinase CK2 and its role in cellular proliferation, development, and pathology. Electrophoresis 20:391–408. doi:10.1002/(SICI)1522-2683(19990201)20:2≤391::AID-ELPS391≥3.0.CO;2-N
6. Ahmed K, Gerber DA, Cochet C (2002) Joining the cell survival squad: an emerging role for protein kinase CK2. Trends Cell Biol 12:226–230. doi:10.1016/S0962-8924(02)02279-1
7. Barz T, Ackermann K, Dubois G, Eils R, Pyerin W (2003) Genome-wide expression screens indicate a global role for protein kinase CK2 in chromatin remodeling. J Cell Sci 116:1563–1577. doi:10.1242/jcs.00352
8. Piazza FA, Ruzzene M, Gurrieri C, Montini B, Bonanni L, Chioetto G et al (2006) Multiple myeloma cell survival relies on high activity of protein kinase CK2. Blood 108:1698–1707. doi:10.1182/blood-2005-11-013672
9. Landesman-Bollag E, Channavajhala PL, Cardiff RD, Seldin DC (1998) P53 deficiency and misexpression of protein kinase CK2α collaborate in the development of thymic lymphomas in mice. Oncogene 16:2965–2974. doi:10.1038/sj.onc.1201854
10. Landesman-Bollag E, Song DH, Romieu-Mourez R, Sussman DJ, Cardiff RD, Sonenshein GE et al (2001) Protein kinase CK2: signaling and tumorigenesis in the mammary gland. Mol Cell Biochem 227:153–165. doi:10.1023/A:1013108822847
11. Tawfic S, Yu S, Wang H, Faust R, Davis A, Ahmed K (2001) Protein kinase CK2 signal in neoplasia. Histol Histopathol 16:573–582

12. Faust RA, Gapany M, Tristani P, Davis A, Adams GL, Ahmed K (1996) Elevated protein kinase CK2 activity in chromatin of head and neck tumors: association with malignant transformation. Cancer Lett 101:31–35. doi:10.1016/0304-3835(96)04110-9
13. Serno S, Salvi M, Battistutta R, Zanotti G, Pinna LA (2005) Features and potentials of ATP-site directed CK2 inhibitors. Biochim Biophys Acta 1754:263–270
14. Slaton JW, Unger GM, Sloper DT, Davis AT, Ahmed K (2004) Induction of apoptosis by antisense CK2 in human prostate cancer xenograft model. Mol Cancer Res 2:712–720
15. Perea SE, Reyes O, Puchades Y, Mendoza O, Vispo NS, Torrens I et al (2004) Antitumor effect of a novel proapoptotic peptide that impairs the phosphorylation by the Casein Kinase 2 (CK2). Cancer Res 64:7127–7129. doi:10.1158/0008-5472.CAN-04-2086
16. Fields R (1990) Nobel, Solid phase peptide synthesis utilizing 9-fluorenylmethoxycabonyl amino acid. Int J Pept Protein Res 35:161–214
17. Perera Y, Farina HG, Hernández I, Mendoza O, Serrano JM, Reyes O et al (2008) Systemic administration of a peptide that impairs the Protein Kinase (CK2) phosphorylation reduces solid tumor growth in mice. Int J Cancer 122:57–62. doi:10.1002/ijc.23013