REPORT

CIGB-552: A new penetrating peptide with antitumor action mediated by the increased levels of the COMMD1 protein in cancer cell lines

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ABSTRACT

A second-generation peptide CIGB-552, with cell-penetrating capacity, was developed by the modification of the primary structure of the L-2 peptide. The molecular mechanism underlying its cytotoxic activity remains partially unknown. In this study, it was shown that CIGB-552 binds and increases the levels of COMMD1, a protein involved in copper homeostasis, sodium transport, and the NF-κB signaling pathway. We found that CIGB-552 induces ubiquitination of RelA and inhibits the antiapoptotic activity regulated by NF-κB, whereas the knockdown of COMMD1 blocks this effect. We also found that CIGB-552 increases the levels of reactive oxygen species (ROS), decreases the cellular antioxidant capacity and induces the peroxidation of proteins and lipids in tumor cells. Altogether, our results bring new insights into the mechanism of action of CIGB-552. Moreover, its anti-tumoral effect was explored by subcutaneous administration in a therapeutic schedule in syngeneic murine tumors and patient-derived xenograft models. Outstandingly, a significant delay of tumor growth was observed after the administration of CIGB-552 in these experimental settings. Our data reinforce the perspectives of CIGB-552 for targeted therapy against cancer. This research granted the 2014 Award of the Cuban National Academy of Sciences.

Keywords: CIGB-552, COMMD1, cytotoxic peptide, oxidative stress, NF-kB, cancer animal models

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RESUMEN

CIGB-552: nuevo péptido penetrador con acción antitumoral mediada por el aumento de los niveles de la proteína COMMD1 en líneas celulares de cáncer. Se desarrolló un péptido con capacidad penetradora en las células, denominado CIGB-552, mediante la modificación de la estructura primaria del péptido L-2. Los mecanismos moleculares de su actividad citotóxica han sido caracterizados parcialmente. En este estudio se demostró que el CIGB-552 incrementa los niveles de la proteína COMMD1, involucrada en la homeostasis celular del cobre, el transporte de sodio y en la ruta de señalización de NF-κB. Se halló que el CIGB-552 induce la ubiquitinación de RelA e inhibe la actividad antiapoptótica regulada por NF-κB, mientras que la eliminación de COMMD1 bloquea dicho efecto. También se encontró que CIGB-552 incrementa los niveles de especies reactivas de oxígeno (ERO), disminuye la capacidad antioxidante celular e induce peroxidación de proteínas y lípidos en las células tumorales. De conjunto, nuestros resultados permitieron profundizar en el mecanismo de acción del CIGB-552. Además, se exploró su efecto antitumoral, mediante la administración subcutánea en un esquema terapéutico en el modelo de tumores singénicos murinos y en el de xenotrasplante derivado de pacientes. De forma muy relevante, se observó una demora significativa en el crecimiento tumoral tras la administración del CIGB-552 en condiciones experimentales. Estos resultados refuerzan las perspectivas del uso del péptido CIGB-552 en la terapia dirigida contra el cáncer. Esta investigación mereció el Premio de la Academia de Ciencias de Cuba en 2014.

Palabras clave: CIGB-552, COMMD1, péptido citotóxico, estrés oxidativo, NF-kB, modelos animales de cáncer

*I*ntroduction

The development of new, increasingly-selective and effective drugs against cancer, that minimize toxicity and able to be used in combination with standard therapy is a challenge today. Therapies directed against molecular targets important in survival, proliferation and spread of tumor cells are revolutionizing

1. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell. 2009;136(5): 823-37.

the paradigm of cancer treatment and will probably be used in most patients in the next ten years [1].

The Antimicrobial Peptides (AMP), derived from plants and animals, are one of such potential source of new antitumor agents. The cyclic peptide $LALF_{31-52}$, derived from horseshoe crab (*Limulus polyphemus*) LALF protein, was initially identified by its ability to bind LPS and the inhibition of the coagulation cascade mediated by the activation of endotoxins [2]. Studies published by Vallespi e*t al*. identified that the linear variant, the LALF32-51 peptide, exhibited antiviral activity mediated by the induction of α and γ interferons. Furthermore, the effectiveness of the peptide was demonstrated in murine models of bacteria-mediated sepsis [3, 4].

Based on the relationship between the primary structure and the biological function of $LALF_{32-51}$, a chemical library of peptides was studied by alanine-scanning. The essential amino acids conferring LPS-binding capacity in $LALF_{31-52}$ and determining or influencing its cytotoxic activity were identified, leading to the design of the L-2 peptide, devoid of LPS-binding activity and showing a stronger cytotoxic effect than the original AMP $LALF_{32-51}$ peptide. Then, a second generation peptide, named CIGB-552, was generated by replacing amino acids at positions 6 and 12 for their respective D-amino acids in L-2, and blocking its N-terminus by acylation, making it resistant to proteolytic degradation [5, 6].

Subsequently, the molecular basis of the CIGB-552 peptide antitumor effect was established, the COMMD1 protein being identified as a new molecular target in cancer therapy. These results support the capacity of CIGB-552 to act against cancer cells through apoptosis-mediated mechanisms and the inhibition of tumor angiogenesis. From a practical point of view, the results of pharmacological and toxicological studies provide evidences on the safety of CIGB-552 for its use in humans. This research granted the 2014 Award of the Cuban National Academy of Sciences.

Results and discussion

Design of a family of second-generation peptides and evaluation of its cytotoxic activity

The proteolytic stability of natural peptides is a major limitation for its use as drug candidates. Therefore, amino acids substitutions were performed introducing D-amino acids (D-aa.) in specific positions in the original sequence of the L2 peptide, as shown in Table 1. In one case, the N-terminal end was further blocking by acetylation.

Subsequently, the proliferative capacity of tumor cells from different origins was challenged with different amount of the peptides. Cells were seeded into 96-well plates and incubated with increasing amounts of the peptides. Cytotoxicity was estimated with Sulforhodamine B and IC_{50} values were calculated using the Calcusyn software (Biosoft®, United Kingdom). As shown in figure 1, the CIGB-552 peptide displayed the best cytotoxic effect on tumor cell lines as evidenced by the IC_{50} values. This finding suggests that the incorporation of unnatural amino acids in the sequence may have improved the metabolic stability of the peptide.

Table 1. Second generation antitumor therapeutic peptides generated from the L-2 peptide with potentially improved cytotoxicity and chemical stability

Peptide	Amino acid sequence	Properties
L2	HARIKPTFRRLKWKYKGKFW	Original peptide
L551	HARIKPTFRRLKWKYKGKFW	Peptide with D-aa in positions P-6 and L-11
CIGB-552	Ac-HARIKPTFRRLKWKYKGKFW	Peptide with D-aa in the P-6 and L-11 positions, and acetylated (Ac) at its N-terminus
L553	HARIKPTFRRLKWKYKGKFW	Peptide with D-aa at positions K-14 and G-17
L554	HARIKPTFRRLKWKYKGKFW	Peptide with D-aa at positions R-3 and P-6

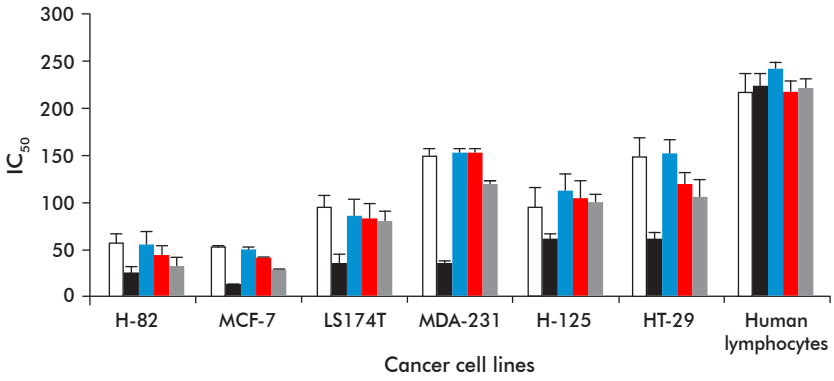

Figure 1. Antiproliferative effect of L-2 derived peptides on tumor cells of different histological origins. IC_{50}: half maximal inhibitory concentration.

Identification of proteins interacting with the CIGB-552 peptide

To identify proteins that interact with CIGB-552 peptide, a yeast-two-hybrid assay was performed. For that purpose, 38 positive clones were identified and sequenced. Similarity analysis of the sequences of the clones evidenced an expected value with an exponential lower than -25 (Table 2).

Among the proteins identified, alpha-2-HS-glycoprotein (AHGS) is produced abundantly in the liver and is present in serum and in the extracellular matrix. High levels of expression in liver explain the identification of three independent clones in the two hybrid assay, since the cDNA library used was derived from a normal human liver tissue (Clontech, USA).

The AHSG protein is capable of binding to cytokines of the Transforming Growth Factor (TGF) family, through a domain which has homology to the extracellular TGF-receptor (TGFR) domain. In fact, it has been shown that high levels of expression of AHSG contribute to the inhibition of tumor progression in different models [7], this protein mainly located in the extracellular space. The need for internalization of the CIGB-552 family of peptides to exert its effect makes it unlikely that the antitumor mechanism could be mediated by the AHSG-TGFR interaction [6]. The implications of that specific interaction should be properly addressed in further experimental settings.

2. Vallespi MG, Glaria LA, Reyes O, Garay HE, Ferrero J, Arana MJ. A Limulus antilipopolysaccharide factor-derived peptide exhibits a new immunological activity with potential applicability in infectious diseases. Clin Diagn Lab Immunol. 2000;7(4):669-75.

3. Vallespi MG, Alvarez-Obregon JC, Rodriguez-Alonso I, Montero T, Garay H, Reyes O, *et al*. A Limulus anti-LPS factor-derived peptide modulates cytokine gene expression and promotes resolution of bacterial acute infection in mice. Int Immunopharmacol. 2003;3(2):247-56.

4. Vallespi MG, Colas M, Garay H, Reyes O, Arana MJ. Differential regulation of Th1/Th2 in relevant tissues for sepsis pathogenesis with a Limulus anti-LPS factor-derived peptide increases survival in Gram-positive sepsis. Int Immunopharmacol. 2004;4(10-11):1343-51.

5. Fernandez Masso JR, Oliva Arguelles B, Tejeda Y, Astrada S, Garay H, Reyes O, *et al*. The Antitumor Peptide CIGB-552 Increases COMMD1 and Inhibits Growth of Human Lung Cancer Cells. J Amino Acids. 2013;2013:251398.

6. Vallespi MG, Fernandez JR, Torrens I, Garcia I, Garay H, Mendoza O, *et al*. Identification of a novel antitumor peptide based on the screening of an Ala-library derived from the LALF(32-51) region. J Pept Sci. 2010;16(1):40-7.

7. Mori K, Emoto M, Inaba M. Fetuin-A: a multifunctional protein. Recent Pat Endocr Metab Immune Drug Discov. 2011;5(2):124-46.

Table 2. Second generation antitumor therapeutic peptides generated from the L-2 peptide with potentially improved cytotoxicity and chemical stability

Clone number	NCBI access number	Properties	Properties
1	NP_001613	alpha-2-HS-glycoprotein (AHSG)	NP_001613
3	NP_001613	alpha-2-HS-glycoprotein (AHSG)	NP_001613
16	NP_001613	alpha-2-HS-glycoprotein (AHSG)	NP_001613
21	NP_689729.1	COMM domain-containing protein 1	NP_689729.1

The second identified interaction corresponded to the COMMD1 protein. COMMD1 is the prototype protein of the COMMD protein family. The ten members of that family are highly conserved and ubiquitously expressed in multicellular organisms [8], their biological functions mostly unknown. COMMD1 function has been linked to copper homeostasis, sodium transport, the signaling mediated by transcription factor NF-kB and the hypoxia-induced factor 1 (HIF-1), among others processes. In the case of the COMMD1 protein, it inhibits the transcription factor NF-kB, an important element activating genes involved in resistance to apoptosis in the cancer cells, stimulates oncogene activation, angiogenesis and cell proliferation. COMMD1 also regulates HIF-1, this protein playing a key role in cell survival in hypoxic tissue, a common feature of tumor [9]. In line with our results, it was recently discovered that COMMD1 is expressed but at very low levels in cancer cells, this also associated with more invasive tumor types [10].

Identification of pathways associated with the cytotoxic effect

Analysis of data obtained from a microarray study, by comparing treated vs. untreated NCI-H460 cells at 0, 2, 4 and 8 h at the CIGB-552 IC_{50} (25 μM), identified up to 349 differentially-expressed genes using the relative expression criteria of the modular value of fold change ($|FC| \geq 1.5$ and adjusted p values by the Benjamini and Hochberg method [11] lower than 0.05. Bioinformatics analysis of the experimental data revealed pathways modulated by CIGB-552 treatment (Figure 2).

The pathway enrichment in differentially-expressed genes in the cell line NCI-H460 treated with the CIGB-552 peptide, identified pathways related with the immune system, which comprise genes associated with the inflammatory response. These pathways correspond to the previously described immunomodulatory action of the $LALF_{32-51}$ peptide and are common to all AMPs [2, 3]. Surprisingly, the oxidative stress response was found associated with the mechanism of cytotoxicity of the CIGB-552 peptide. This was unprecedented, in spite of the intense debate on the relationship between cancer and oxidative stress [12]. The cellular oxidative damage has been associated with carcinogenesis and apoptosis. Additionally, increased levels of oxidative stress have been considered a successful strategy to induce apoptosis in malignant cells [13, 14].

Moreover, other identified pathways as those triggered by MAPK kinase and NF-κB signaling are also modulated by the oxidative stress. Overall, the results allowed us to identify oxidative stress as a key mechanism mediating the activation of multiple signaling pathways (MAPK, TP53, NF-κB), that explain the cytotoxicity of the CIGB-552 peptide.

CIGB-552 accumulates COMMD1 in human cancer cells and promotes ubiquitination and degradation of the RelA subunit of NF-kB

Since COMMD1 was identified as a protein that is associated with antitumor peptides, the role of COMMD1 was studied in the mechanism of action of CIGB-552. First, COMMD1 expression in lysates of human cancer cells of different histological origin was determined using Western blot analysis. These experiments revealed an increase in COMMD1 levels after 5 h of treatment with the peptide. COMMD1 cellular accumulation was also identified by immunofluorescence in human cancer cells [4]. Both cell lines tested, MCF7 and HT29, showed accumulation of COMMD1 after 5 h of treatment with the CIGB-552.

COMMD1 overexpression accelerated the ubiquitination and degradation of RelA subunit of NF-κB. Given the above data, the effect of CIGB-552 levels of ubiquitinated RelA was addressed. Immunoprecipitation of endogenous RelA was performed using mouse anti-RelA, followed by Western Blot with an anti-ubiquitin antibody. Increased amounts of ubiquitinated RelA in response to the peptide was found as early as 2 h after treatment. Then, the possible induction of ubiquitination by CIGB-552 and the subsequent degradation of RelA through a proteasome-dependent process were evaluated through the effect of MG132 and CIGB-552 in the basal levels of RelA. The treatment with CIGB-552 decreases the basal levels of RelA. This result suggests that CIGB-552 induces ubiquitination of RelA and promotes its proteasomal degradation [4].

The functional role of COMMD1 accumulation in the antitumor activity of CIGB-552 was confirmed by transduction of H460 cells with a retrovirus expressing a shRNA targeting COMMD1. In this cell line, the cytotoxic activity of CIGB-552 decreased compared to control cells, possibly with decreased ubiquitination and downstream degradation of RelA [4].

The CIGB-552 alters the redox state in H-460 cells

Since microarray studies indicated that the CIGB-552 modulates the expression of oxidative stress-related genes, the relevance of COMMD1 accumulation on the oxidative stress balance was assessed by measuring the redox balance in NCI-H460 cells.

We observed that treatment with CIGB-552 caused an increase in the levels of reactive oxygen species (ROS) $O2^{\bullet-}$ and $^{\bullet}ON$, which explains the activation of genes involved in the process of oxidative stress response observed in the experiments of differential

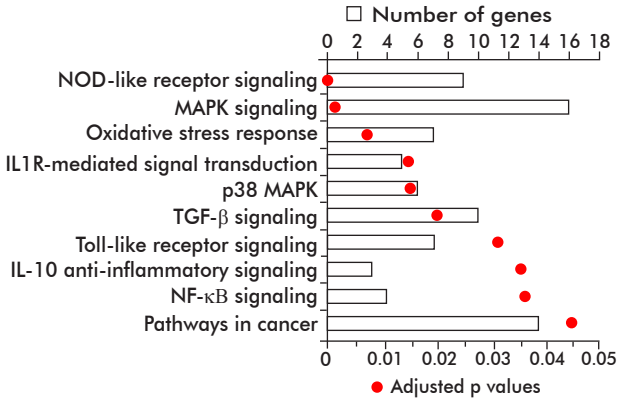

Figure 2. Classification of differentially-expressed genes in the cell line NCI-H460, according to biological pathways. The significance of the process was estimated according to the method implemented in David software. Number of genes: identified as differentially expressed in the pathway; Adjusted p values: Adjusted p values: a modified Fisher Exact P-Value, for gene-enrichment analysis (https://david.ncifcrf.gov/home.jsp).

8. Maine GN, Burstein. COMMD proteins: COMMing to the scene. Cell Mol Life Sci. 2007;64(15):1997-2005.

9. Bartuzi P, Hofker MH, van de Sluis B. Tuning NF-kappaB activity: a touch of COMMD proteins. Biochim Biophys Acta. 2013;1832(12):2315-21.

10. van de Sluis B, Mao X, Zhai Y, Groot AJ, Vermeulen JF, van der Wall E, *et al*. COMMD1 disrupts HIF-1alpha/beta dimerization and inhibits human tumor cell invasion. J Clin Invest. 2010;120(6):2119-30.

11. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J Roy Soc . 1995;57(1):289-300.

12. Dayem AA, Choi HY, Kim JH, Cho SG. Role of oxidative stress in stem, cancer, and cancer stem cells. Cancers (Basel). 2010;2(2):859-84.

13. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov. 2013;12(12):931-47.

14. Verrax J, Taper H, Buc Calderon P. Targeting cancer cells by an oxidant-based therapy. Curr Mol Pharmacol. 2008;1(1):80-92.

gene expression. This is in agreement with previous findings which describe COMMD1 as an interaction partner of superoxide dismutase 1 (SOD1) [15]. Maturation and activation of SOD1 are highly regulated processes requiring several posttranslational modifications, with COMMD1 deteriorating the SOD1 activity by reducing the expression levels of enzymatically-active homodimers in the process of SOD1 posttranslational maturation. A relevant finding was the decrease in the total antioxidant capacity by treating cells with CIGB-552. The increased ROS concentrations and the overall decrease of total antioxidant capacity in cells after the treatment at the IC_{50} of CIGB-552 are shown in Figure 3. In summary, these results could explain the observed increase in the oxidation of lipids and proteins and the reduction of the mitochondrial membrane potential inducing the apoptosis as part of the cytotoxic mechanism.

Synergistic effect of the combination of CIGB-552 and standard chemotherapy

For this assay, tumor cells lines HT-29 and H-125 were added with culture medium containing the CIGB-552 peptide in a dose range from 9 to 300 μM. The cytostatic 5-FU and cisplatin were added to cell cultures at ten-fold and 1:10 dilutions of their respective IC_{50} as reported for each cell line. The effect of concomitant treatment-cytostatic peptide was analyzed by the CalcuSyn computer program for the study of drug combinations.

As shown in table 3, the peptide-cytostatic combinations reduced the amount of cytostatic required, given by the values shown in the Reduction Index (RI). These results indicate that the peptide can be administered together with standard chemotherapy to provide an effective treatment (the fraction of affected cells: 89-94 %) with minor amounts of each standard drug. Combining 5-FU with CIGB-552 allows a 20-fold reduction (RI) in the amount of 5-FU in the cell line HT-29 required. For cisplatin, its combination with CIGB-552 allowed a 5-fold reduction in the cytostatic concentration required to attain the effect in H-125 cells. These results indicate that CIGB-552 can be administered in combination with standard treatment for human colorectal and lung cancer (HT-29 and H-125 cell lines, respectively), facilitating a reduction in the dose of cytostatic. This may reduce the adverse effects associated with chemotherapy.

Efficacy of CIGB-552 in the therapy of solid tumors in mice

To evaluate the in vivo antitumor activity of CIGB-552, it was administered subcutaneously in the murine model of tumor cells CT-26 in BALB/c mice. Both the tumor accumulation of the 131I-labeled CIGB-552 and the inhibition of tumor growth after treatment with CIGB-552 were evaluated. A human colon tumor xenograft model in nude mice was also used to further evaluate the anticancer effect of CIGB-552.

The results demonstrated that two injections a week of CIGB-552 for 2 weeks elicited an antitumor activity mediated by the inhibition of tumor growth (50 % inhibition). In addition, the peptide reduces the density of blood vessels in the tumors [16]. Together, these data prove that the second-generation peptide CIGB-552 is able to induce a significant antitumor response after systemic delivery.

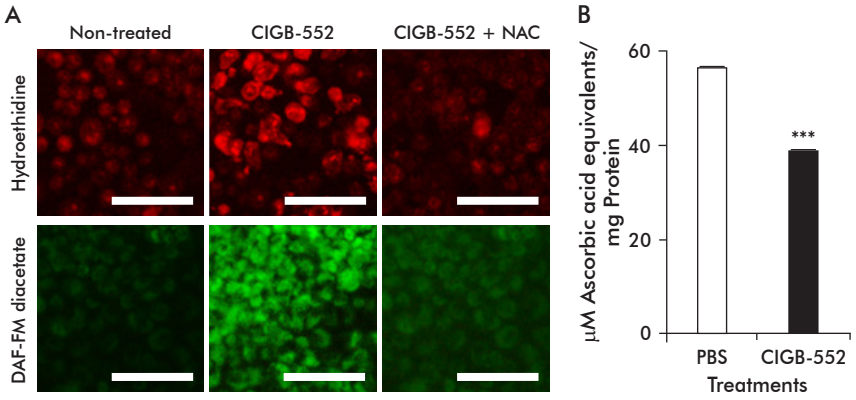

Figure 3. Redox state modification in cancer cell lines treated with the CIGB-552 peptide. A) Effect of CIGB-552 treatments on the generation of reactive oxygen species. NCI-H460 cells were incubated with 25 μM of CIGB-552, the combination of 25 μM of CIGB-552 and 3 mM of the antioxidant N-acetyl-cysteine (NAC) and as the control cells treated with PBS (NT). After 3 h of treatment, cells were labeled with 10 μM of hydroethidine for 30 min or 3 μM of DAF-FM diacetate for 45 min. Three independent experiments were performed in triplicate. Representative images of an experiment are shown. Images were acquired in the inverted Olympus 10X objective CKX41SF with fluorescent microscope. Bar stands for 100 microns. B) Evaluation of the total antioxidant capacity of the NCI-H460 cell line treated with CIGB-552. Total antioxidant capacity measured as ferric iron reducing capacity (FRAP). Complex formation Fe^{2+}-2,4,6-tripyridyl-s-triazine was detected at 593 nm. The average values of three determinations per experimental point obtained from two independent experiments are shown in the graph. Means ± SD (n = 6). NT: cells treated with PBS. The comparison between groups was performed using unpaired t-test (*** $p < 0.001$). Bars stands for 50 μm.

Table 3. Synergism of the combination therapy between CIGB-552 peptide and the cytostatic 5-FU and cisplatin

Cell line	Cell affected fraction (% ± CI)	Treatment concentration (μM)		Reduction Index	
		5-FU	CIGB-552	5-FU	CIGB-552
HT-29	89 ± 0.3	5000	700	20	5
H-125	94 ± 0.5	273	308	5	3

CI: confidence interval

*S*cientific relevance of the study

The study of the differential gene expression identified the oxidative stress response among the routes and biological processes being modulated by the CIGB-552 in the lung cancer cell line NCI-H460. It was shown that the CIGB-552 causes the increase of ROS, causing damage to mitochondria and increasing the oxidative damage of lipids and proteins. Moreover, proteomic studies revealed that the CIGB-552 interacts with COMMD1, the treatment with CIGB-552 increasing the expression of COMMD1 in cell lines which leads to apoptosis-mediated cell death. Altogether, these findings provide the molecular basis to fully unravel the cytotoxic mechanism of CIGB-552 in cancer cells.

From a practical point of view, the results of the pharmacological and toxicological studies support the safety of CIGB-552 for use in humans. The preclinical studies have demonstrated: 1) the antitumor effect of CIGB-552 in animal models of lung and colon cancer; 2) the antiangiogenic activity; 3) the treatment tolerability through the monitoring of body weight in treated mice; and 4) the accumulation of the peptide in tumors, supporting the potential safety based on the

15. Vonk WIM, Wijmenga C, Berger R, van de Sluis B, Klomp LWJ. Cu,Zn superoxide dismutase maturation and activity are regulated by COMMD1. J Biol Chem. 2010;285(37):28991-9000.

16. Vallespi MG, Pimentel G, Cabrales-Rico A, Garza J, Oliva B, Mendoza O, *et al*. Antitumor efficacy, pharmacokinetic and biodistribution studies of the anticancer peptide CIGB-552 in mouse models. J Pept Sci. 2014;20(11):850-9.

bioavailability of the CIGB-552 peptide in Phase I clinical trials in patients with solid tumors. These results support the therapeutic possibilities of this new therapeutic candidate for the treatment of cancer. Significantly, metabolic pathways activated by CIGB-552 peptide makes it unique, constituting the first synthetic peptide aimed at stabilizing COMMD1 as a way to downregulate NF-kB and ultimately lead to cancer cell cytotoxicity.

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