

## Rational design of an antitumor peptide based on the 32-51 region of the *Limulus anti-LPS* factor protein obtained from a chemical library

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### ABSTRACT

In previous work, we identified the peptide comprising amino acids 32-51 on the antimicrobial protein *limulus anti-LPS* factor (LALF) from *Limulus polyphemus*, based on its capacity to bind to the bacterial lipopolysaccharide (LPS). In this study, we designed a chemical peptide library by alanine scanning from the sequence of the LALF<sub>32-51</sub> peptide. Four new peptides, named L-2, L-8, L-12 and L-20 by the Ala residue substituted in the given position of the LALF<sub>32-51</sub> peptide, were identified as retaining their penetrating activity but also showing antitumor effects when subcutaneously administered in female C57Bl/6 mice after the implantation of malignant TC-1 lung epithelial cells. They significantly increased animal survival ( $p < 0.05$ ) as compared to LALF<sub>32-51</sub>. The substitution of Tyr residue to Ala at position 2 in the peptide sequence enhanced the cytotoxic effect, while residues Phe8, Lys12 and Trp20 were essential for the antitumor activity. Moreover, the administration of the L-2 peptide significantly reduced tumor growth in comparison to PBS- or L-20 peptide-treated lung TC-1 cells in C57Bl/6 mice. L-2 was found to deregulate the tumor cell cycle and induces apoptosis, through mechanisms affecting glycolytic and protein biosynthesis pathways. It also significantly increased survival in human colon cancer LS-174T xenotransplanted nude mice for up to 32 days, two of the animals being tumor free. The L-2 peptide could serve as peptide-based prototype drug with potential to reduce tumor load or could be coadministered with conventional chemotherapy. This research granted the 2013 Award of the Cuban National Academy of Sciences.

Keywords: antitumor peptide, chemical peptide library, *Limulus*, LALF<sub>32-51</sub>, alanine scanning

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### RESUMEN

**Diseño racional de un péptido antitumoral mediante el uso de una librería química generada de la región 32-51 de la proteína Factor Anti-LPS de *Limulus*.** En este estudio se diseñó una biblioteca química de péptidos mediante el barrido de alanina del péptido LALF<sub>32-51</sub>, derivado de la proteína antimicrobiana factor anti-LPS de *Limulus polyphemus* (LALF) con capacidad de unión al lipopolisacárido bacteriano (LPS). Se identificaron cuatro nuevos péptidos, L-2, L-8, L-12 y L-20 con el residuo Ala en la posición indicada en cada caso, que retuvieron la penetrabilidad celular original y mostraron actividad antitumoral al ser administrados en ratones C57Bl/6 a los que previamente se implantó tumores malignos TC-1 de células epiteliales de pulmón. Los péptidos incrementaron significativamente la supervivencia de los animales en comparación con el LALF<sub>32-51</sub> ( $p < 0.05$ ). La sustitución por Ala en la posición 2 incrementó el efecto citotóxico, y en sustitución de los residuos Phe8, Lys12 y Trp20, evidenció la importancia de estos para la actividad antitumoral del péptido. La administración del L-2 redujo significativamente el crecimiento tumoral respecto al tratamiento con PBS o con L-20 en células TC-1 de ratones C57Bl/6. Este péptido desreguló el ciclo celular e indujo apoptosis en las células tumorales, mediante mecanismos que afectaron a las rutas de la glicólisis y la biosíntesis de proteínas, e incrementó significativamente a 32 días la supervivencia de ratones desnudos xenotrasplantados con células LS-174T de cáncer de colon humano. El péptido L-2 puede ser un prototipo de fármaco peptídico con potencial para reducir la carga tumoral o que se pueda coadministrar con la quimioterapia convencional. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2013.

Palabras clave: péptido antitumoral, biblioteca peptídica química, *Limulus*, LALF32-51, barrido de alaninas

### Introduction

Cancer remains as the second mortality cause in humans, only surpassed by cardiovascular diseases. Every year about 19 000 people die of malignant tumors and 29 000 new cases are diagnosed. In Cuba, cancer is the first cause of death in ages 15-49 (rate 32.5 every 100 000 inhabitants) and 50-64 (rate 290

every 100 000 inhabitants), according to the Cuban Health Statistics Yearbook, 2010 [1].

For these reasons, the development of new drugs, more selective and effective, of low toxicity and able to be used in combination with standard therapies is still a challenge. Therapies against molecular targets

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relevant for tumor cell survival, proliferation and spread are reshaping the paradigm of cancer treatment and would probably be used in most patients in the next ten years.

In this context, peptides are emerging as therapeutic alternatives for small molecules. There have been three traditional sources to obtain therapeutic peptides: 1) bioactive peptides produced by plants or animals; 2) peptides isolated from recombinant libraries and 3) peptides isolated from chemical synthesis libraries [2, 3]. Peptides are advantageous as compared to other types of molecules for cancer therapeutics. The systemic toxicity effect is minimized since peptide degradation byproducts are amino acids; therefore, peptides are well tolerated, highly specific and selective for tumor cells, as compared to most chemotherapeutic treatments. Additionally, they are small molecules of simple structures which make them less immunogenic and increase their penetrating capacity than their protein counterparts or even antibodies. Their relatively simpler structures also make them easy to synthesize and modify [4].

Most antitumor peptides tested so far were originally isolated from natural sources. The “peptidomes” of plants and animals have also been recognized as sources for the identification of novel antitumor agents. Another significant source comprises the use of combinatorial peptide libraries, either chemical or recombinant, with affinity for tumor signaling pathways ligands and able to block their functions such as: receptor binding, cell adhesion and metastasis [5].

Previously, we were able to identify the peptide comprising amino acids 32-51 on the antimicrobial protein limulus anti-LPS factor (LALF) from *Limulus polyphemus*, based on its capacity to bind to the bacterial lipopolysaccharide (LPS). In fact, cationic antibacterial peptides are toxic for bacterial but for normal mammalian cells, and display a wide spectrum of cytotoxic activity against cancer cells, as is the case of LALF<sub>32-51</sub> [6, 7]. In this work, we designed a chemical peptide library by alanine scanning from the sequence of the LALF<sub>32-51</sub> peptide. The results obtained are encouraging for the use of a chemical library for the rational design of novel cytotoxic peptides with therapeutic potential against cancer. This research granted the 2013 Award of the Cuban National Academy of Sciences.

**Results**

**Selection of new peptides lacking LPS-binding capacity**

New peptide variants were designed by a single alanine residue scanning for every amino acid position on the sequence of the *L. polyphemus* LALF<sub>32-51</sub> peptide. Peptide variants lacking LPS-binding capacity were identified by a competitive ELISA test [8]. Briefly, polystyrene plates were coated with *E. coli* 0111:B4 LPS (1 µg/mL) and all the peptides were tested for its affinity to LPS by direct competition against 0.2 µM biotin-labeled LALF<sub>32-51</sub>, which achieved 90 % maximum binding. Inhibition curves were estimated at peptide concentrations ranging 10 to 0.01 µM, and using LALF<sub>32-51</sub> as control. The LPS-bound biotin-labeled LALF<sub>32-51</sub> peptide was detected by incubating the plates with peroxidase-conjugated streptavidin, and its

absorbance quantified at 450 nm by using a microplate reader (Sensident Scan).

Results are shown in figure 1. Peptides denominated L-2 and L-20 completely lost their LPS-binding capacity, while peptides L-8, L-12 and L-18 showed partial binding capacity. In contrast, peptide L-1 increased its binding strength to LPS.

These peptides resembled properties of the high penetration peptides, due to their high content of cationic and amphipathic amino acids and their net positive charge; therefore, their cell penetrating capacity was investigated. For that purpose, Hep-2 cells were incubated with biotinylated peptides L-1, L-2, L-8, L-12 and L-20 at 270 µM each for 10 min. The samples were incubated with FITC-avidin and propidium iodide for 5-10 min, followed by an extensive wash in PBS-Tween 20 at room temperature. Subsequently, slides were observed under a Nikon confocal MRC 600 microscope. The new peptides retained their cell penetrating activity and were clearly detected into the cell nucleus [9]. A similar result was observed after 1-h incubation, and in other cell lines tested. A sub-population of the cells was analyzed by trypan blue staining to confirm cellular viability on each case.

**Antitumor effect of the new peptides L-2, L-8, L-12 and L-20 in the TC-1 tumor model**

Ten female C57Bl/6 mice, 8-to-10 weeks-old, were used per experimental group in the assays. Tumors were implanted by using malignant TC-1 lung epithelial cells from the C57Bl/6 strain. An amount of 50 000 cells was inoculated in 200 µL in mice by subcutaneous route in the posterior right leg. The first inoculation was administered when tumors had grown to 100 mm<sup>3</sup> and the second ten days later. The assay tested a dose of 4 mg of peptide per kg of body weight (each mice weighed 80 g) and the parameters

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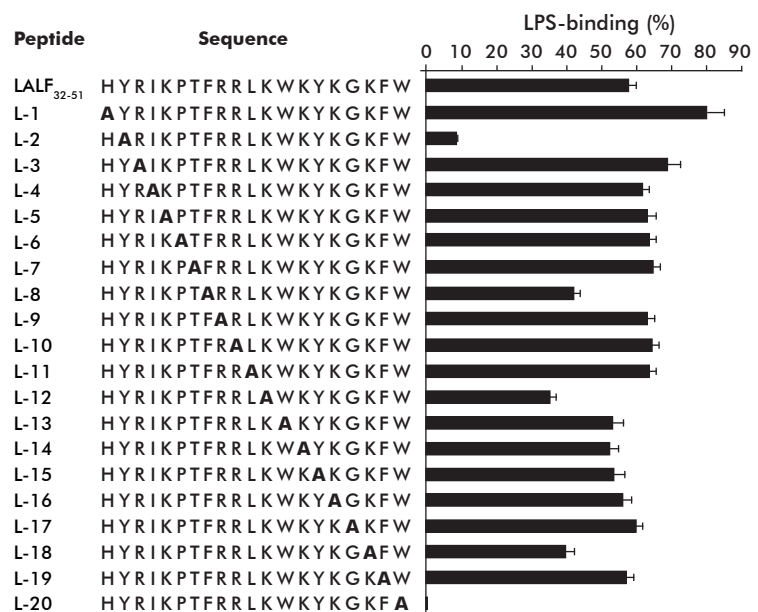


Figure 1. Percentage of inhibition of the LPS-binding capacity of peptides analogous to the LALF32-51 peptide. Peptides were obtained by the alanine scanning technique from a chemical library. The respective sequences are also shown.

under evaluation to measure the antitumor effects of the peptides were animal survival and tumor volume. As shown in figures 2A and B, peptides L-2, L-8, L-12 and L-20 were capable of inhibiting tumor progression and increased animal survival, respectively. These results evidenced the antitumor efficacy of the analogous peptides in the TC-1 solid tumor model. Significant statistical differences were determined by the Log-Rank test, with the four peptides significantly increasing animal survival ( $p < 0.05$ ) as compared to LALF<sub>32-51</sub>.

#### Effect of the treatment with peptides L-2 and L-20 on the viability of tumor cells

The proliferation capacity of different tumor cells of varied origin was evaluated. Cells were seeded in 96-well plates and challenged with increasing amounts of peptides. Their cytotoxic effects were estimated by development with sulforhodamine and the mean inhibitory concentration (IC50) was calculated. Peptide L-2 displayed the highest cytotoxic effect (IC50) against the tumor cell lines tested, with IC50 in 8 cell lines from  $37 \pm 10$  to  $84 \pm 6 \mu\text{M}$ , in 7 out of 9 tumor cell lines [9]. Human colon cancer and pancreas cancer cells showed the highest sensitivity to such effect. Peptide L-20 did the same to lower level, with IC50 values from  $120 \pm 9$  to  $159 \pm 6 \mu\text{M}$  in 8 out of 9 cell lines tested [9].

The alanine scanning experiment evidenced that the LPS-binding activity of the LALF<sub>32-51</sub> peptide was not related to its antitumor effect. Moreover, it allowed identifying the substitution of Tyr residue to Ala at position 2 in the peptide sequence as enhancing for the cytotoxic effect, while residues Phe8, Lys12 and Trp20 were found essential for the antitumor activity. These findings supported the rational design of this new L-2 peptide of improved antitumor effect in murine tumor models and increased cell penetrating activity.

#### The administration of the L-2 peptide significantly reduced tumor growth in comparison to PBS- or L-20 peptide-treated lung TC-1 cells in C57Bl/6 mice

A 0.2 mg/kg dose was subcutaneously administered in this schedule. Treatment started once the tumors had reached 80 mm<sup>3</sup>. After 26 days, tumor growth was

very highly significantly reduced as compared to those in PBS-administered mice when treated with L-2 and L-20 peptides ( $p = 0.0001$ ; Tukey's test, 95 % confidence interval). On day 32, there was a highly significant difference in tumor growth between animals' groups administered with each peptide ( $p = 0.01$ ), a difference maintained until the end of the experiment [9]. In agreement with the decrease observed in tumor volume, the L-2 peptide significantly increased animal survival in respect to animals treated with PBS or the L-20 peptide, as detected by the Log-Rank test. A very highly significant mean survival of 51 days was achieved in mice treated with L-2 ( $p = 0.0001$ ) and significant of 46 in animals inoculated with the L-20 peptide ( $p = 0.0092$ ), both compared to the 42 days of survival in the PBS-treated group. In agreement with results obtained in tumor cell line experiments, L-2 displayed the most potent antitumor effect, higher than that achieved with L-20, when systemically administered in the TC-1 model.

In order to unravel the mechanism by which the L-2 peptide reduced tumor growth, the DNA laddering analysis by the TUNEL assay was done. Data obtained suggested that the systemic administration of this peptide exerts an antitumor effect by the induction of tumor cell apoptosis. DNA flow cytometry analyses of LS-174T tumor cells treated with the L-2 peptide revealed the absence of the G2/M subpopulation and the accumulation of DNA in cells in S phase. Moreover, cells undergoing apoptosis after 24-h incubation with L-2 showed a significant increase in the pre-G1 phase DNA [9]. This indicated that L-2 induces the cell cycle arrest in these tumor cells followed by apoptosis. The signaling pathways by which the L-2 peptide deregulates the tumor cell cycle and induces apoptosis remain to be characterized.

#### L-2 peptide-associated differential expression profiles

The differential expression profile assays are essential to understand the oncogenic mechanisms in cancer, to discover new targets, to develop new drugs and to identify biomarkers for personalized treatments. In our case, this type of assay was required to identify the network of genes involved in the early response to the treatment with the L-2 peptide, due to its anti-proliferative effect in several human tumor cell lines

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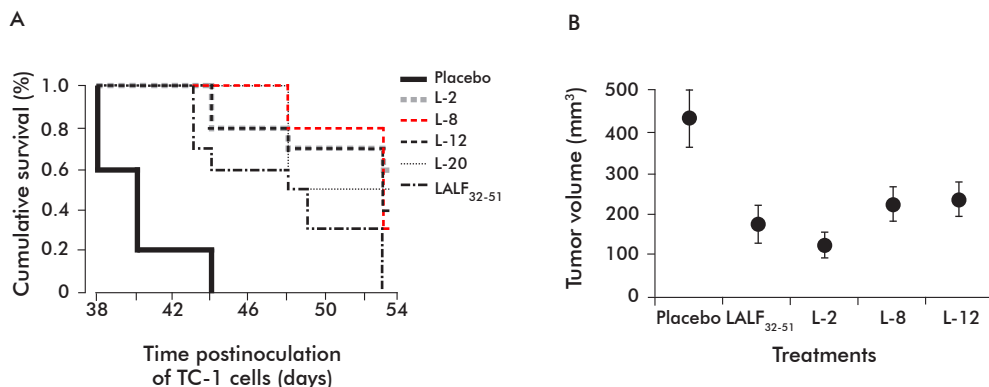


Figure 2. Antitumor effect of the new peptides L-2, L-8, L-12 and L-20 on the TC-1 tumor model in C57Bl/6 mice ( $n = 10$ ). A) Cumulative survival. B) Tumor volume reduction.

and antitumor activity in mice tumor models. For this purpose, the suppression subtractive hybridization technique was used, resulting in the identification of 74 genes differentially expressed in Hep-2 human tumor cells. Based on the analysis of their respective biological categories and signaling pathways, it was evidenced that the L-2 peptide acts through the modulation of major biological processes in cancer cells [9].

Based on experimental data and the information obtained, we hypothesize that the L-2 peptide activates its antitumor activity through multiple pathways. One is glycolysis, since the expression of three genes of this signaling pathway (PDGK1, PGM and ENO1) was decreased, supporting the assumption that this pathway becomes less active after treatment and also explaining the halt in cell cycle progression and apoptosis of treated tumor cells. Additionally, reduced transcription of genes involved in protein biosynthesis, such as EEF1G, EEF1A1 and RPS6, could affect this fundamental cellular process [10, 11]. Further experiments would elucidate which of the identified genes are major contributors to the L-2 antitumor activity.

#### **Antitumor effect of the L-2 peptide in the human colon tumor model in xenotransplanted nude mice**

A statistically significant antitumor effect was achieved by the systemic administration of a 2 mg per kg of body weight in human colon cancer LS-174T xenotransplanted nude mice. On day 17 postinoculation, mice administered with the L-2 peptide showed a significant reduction in tumor volume compared to that of tumor in mice treated with PBS ( $p = 0.028$ ), a difference which remained throughout the rest of the study, and coincided with a statistically significant increase in animal survival. A very highly significant survival of 32 days was achieved in animals administered with 2 mg/kg of body weight as compared to 25 days in the PBS group ( $p = 0.0007$ ). Notably, on day 31 two animals in the group treated with that dose of L-2 were found tumor free. Nevertheless, the 1 mg per kg of body weight dose showed no statistical differences [9].

Our data corroborate that tumor growth was reduced in the animals treated with L-2 at the optimal dose

of 2 mg per kg of body weight. Altogether, our results show that the L-2 peptide behaves as a cytotoxic peptide, could attack tumor cells and mount a significant apoptosis-mediated antitumor effect in cancer cells. The L-2 peptide could serve as peptide-based prototype drug with potential to reduce the tumor load or coadministered with conventional chemotherapy.

Our results support the use of a chemical peptide library for the rational design of new cytotoxic peptides with potentialities for cancer treatment.

#### **Relevance of the study**

This was the first report on the use of a chemical library to identify a proapoptotic cytotoxic peptide with potential application in cancer. The study of peptide sequences derived from the LALF<sub>32-51</sub> peptide by alanine scanning evidenced that their capacity to bind LPS was unrelated to the antitumor effects of the peptides. Besides, the substitution of the Tyr residue in position 2 to Ala increased the cytotoxic effect and the Phe8, Lys12 and Trp20 residues were essential for the antitumor activity. These findings supported the rational design of a new peptide analogue, L-2, with increased antitumor effect in murine tumor models and with cell penetrating capacity. Additionally, a set of genes involved in the action of the new peptide was identified by differential expression studies. These results granted a patent entitled *Immunomodulatory and Anti-Tumour Peptides* in Europe (EP 1992638 B1), Mexico (282158 B1), Russia (2403-154287 RU/253), USA (US 8283324), and in Australia, China and India. These results have also been presented in international scientific congresses. On practical grounds, our results support the use of a chemical peptide library for the rational design of new cytotoxic peptides with potentialities for cancer treatment.

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