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Identification of a novel antitumor peptide based on the screening of an Ala-library derived from the $LALF_{32-51}$ region

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Novel therapeutic peptides are increasingly making their way into clinical application. The cationic and amphipathic properties of certain peptides allow them to cross biological membranes in a non-disruptive way without apparent toxicity increasing drug bioavailability. By modifying the primary structure of the *Limulus*-derived $LALF_{32-51}$ peptide we designed a novel peptide, L-2, with antineoplastic effect and cell-penetrating capacity. Interestingly, L-2 induced cellular cytotoxicity in a variety of tumor cell lines and systemic injection into immunocompetent and nude mice bearing established solid tumor, resulted in substantial regression of the tumor mass and apoptosis. To isolate the gene transcripts specifically regulated by L-2 in tumor cells, we conducted suppressive subtractive hybridization (SSH) analysis and identified a set of genes involved in biological processes relevant to cancer biology. Our findings describe a novel peptide that modifies the gene expression of the tumor cells and exhibits antitumor effect *in vivo*, indicating that peptide L-2 is a potential candidate for anticancer therapy.

Supporting information may be found in the online version of this article

Keywords: *Limulus* anti-LPS factor; cell-penetrating peptide; synthetic peptide; apoptosis; cytotoxic peptide

Introduction

The cyclic peptide $LALF_{31-52}$ is a small cationic and amphipathic peptide from the *Limulus* anti-LPS factor (LALF) [1]. The three-dimensional structure of a recombinant LALF suggests that an amphipathic loop on LALF represents an LPS-binding motif. However, previous investigations with cysteine and non-cysteine containing peptides derived from Tachypleus antilipopolysaccharide (LPS) factor (TALF), a protein highly homologous with LALF, showed little difference in LPS binding activity. In addition to their endotoxin binding and neutralization capabilities, Hoess [2] reveals that $LALF_{31-52}$ may be expected to show heparin-binding activity. The ability to neutralize properties associated with heparin, such as anticoagulation, angiogenesis, and tumor cell proliferation, was attributed to this peptide, although there are no experimental data supporting this statement. The above- mentioned LPS binding proteins are the source of cationic antimicrobial peptides (AMPs), which are toxic to bacteria but not to normal mammalian cells. A growing number of studies have shown that some of the AMPs exhibit cytotoxic activity against cancer cells. The amphipathic structure and high net positive charge of these peptides are believed to play a major role in the ability to kill cancer cells but the molecular mechanisms underlying the anticancer activity are not clear [3]. As we have reported in previous studies $LALF_{31-52}$ exhibits diverse biological properties with a greater therapeutic potential than that originally contemplated for an LPS-binding molecules [4,5]. In this work, we documented that analogous peptides from non-cyclic region 32–51 of the LALF possess the ability to inhibit tumor cell proliferation by a mechanism that does not imply to bind LPS. By screening a peptide library corresponding to the ala-scanning of LALF residues 32–51, a novel antitumor peptide (L-2) that lacks the ability to bind LPS and exhibits cytotoxic activity on tumor cells was identified. Interestingly peptide L-2 has a powerful *in vitro* cytotoxic effect on murine and human tumor

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cell lines and leads to regression when administered systemically to mice bearing solid tumors. The combined use of SSH and cDNA arrays was employed to identify a set of cancer-associated genes that are differentially expressed in tumor cells following treatment with the peptide, and that support its antitumoral activity. The originality of this work permitted us to gain inventive property on these newly discovered peptides and their application to cancer treatment. Furthermore, the results shown in this study suggest that peptide L-2 is efficiently internalized by cells and may be a potential agent in the treatment of solid tumors.

Materials and Methods

Peptide Synthesis

Peptides were synthesized on solid phase and purified by reverse-phase-high-performance liquid chromatography to >95% purity on an acetonitrile/H_2O-trifluoracetic acid gradient and confirmed by ion-spray mass spectrometry (Micromass, Manchester, UK). Lyophilized peptides were reconstituted in phosphate-buffered saline (PBS) to be used in *in vitro* and *in vivo*. The peptide HYRIKPTFRRLKWKYKGKFW was used to generate an alanine-scanning synthetic library. The analogous synthetic peptides used in this work contain a single alanine substitution in the original sequences (Figure 1b).

In vitro Selection of Peptides Devoid of Lipopolysaccharide (Lps)-Binding Capacity

This assay consists on a competition ELISA system [6]. Polystyrene plates (Costar, USA) were coated with LPS from *E. coli* 0111 : B4 (1 μg/ml) with 0.2% TCA. Plates were incubated overnight at 37 °C and washed ten times with PBS plus 0.1% tween-20 (washing solution). The binding of analogous peptides to the immobilized LPS was evaluated by competition with the biotinylated $LALF_{32-51}$ peptide at 0.2 μM. The analogous peptides were used at a fixed concentration of 0.5 μM, mixed with the biotinylated $LALF_{32-51}$ (0.2 μM) and then added to the ELISA plates coated with LPS. The biotinylated $LALF_{32-51}$ bound to LPS was detected by incubating for 45 min at 37 °C with a streptavidin-peroxidase conjugate at a 1 : 2000 dilution. Plates were washed five times with washing solution and the substrate solution was added (0.05 M citrate–phosphate buffer, pH 5.5, 1 tablet of 3,3′,5,5′-tetramethylbenzidine and 0.025% hydrogen peroxide). After 15 min incubation, the reaction was stopped by adding 2 mol/l sulphuric acid. The absorbance was quantified at 450 nm in a plate reader (Sensident Scan). The LPS-binding activity (%) was calculated using the following formula:

$$\{1 - (([OD]_{sample} - [O.D]_{min})/([OD]_{max} - [OD]_{min}))\} \times 100$$

$[OD]_{sample}$, optical density in the presence of analogous peptide; $[OD]_{min}$, background of the assay; $[O.D]_{max}$, optical density in the presence of $LALF_{32-51}$ peptide (cold peptide as a control).

Cell Viability Assay

The cell lines were maintained at 37 °C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) plus 30 μg/ml of gentamycin (Gibco). The cytotoxicity of the peptide was monitored by SRB [Sulforhodamine B, sodium salt] assay [7]. Peptide concentrations ranging from 0 to 200 μM were added to 10 000 cells/well and incubated for 72 h at 37 °C in 5% CO_2. Subsequently, 50 μl of 80% TCA solution was added to the cells and incubated for an hour at 4 °C. Finally, 100 μl of the SRB solution was added to the wells and absorbance was measured at λ 492 nm. Background corresponding to the absorbance of wells without cells was subtracted. Each sample point was done in triplicate, and experiments were carried out twice. IC50 values were obtained from the respective growth curves.

Analysis of Cell Cycle Progression

Cell cycle phase distribution was assayed by flow cytometry analysis [8]. Briefly, 1×10^6 human colon cancer cells (LS 174T) in RPMI 1640 (Hyclone) supplemented with 7% fetal bovine serum (HyClone, Logan, UT, USA) plus 30 μg/ml of gentamycin (Gibco) were seeded in 24-well flat plate (Costar) for 20 h at 37 °C in 5% CO_2. Subsequently, L-2 was added at 100 μM in 24 h. Meanwhile supplemented medium was added to the culture control group, and the cells were removed from the culture dishes by trypsinization and centrifugation. After washing with PBS they were suspended in PBS containing 0.1% Triton X-100 to prepare nuclei. The suspension was processed for labeling with 0.1% RNase and 50 ug/ml propidium iodide by using a Flow Cytometry kit for apoptosis (APO-DIRECT) obtained from SIGMA (St Louis, MO) as recommended by the manufacturer's protocol. DNA contents in stained nuclei were analyzed with FACStar (Partec, Flomax). The suspension of 1×10^6 cells was analyzed for each DNA histogram. The number of stained nuclei in each phase was measured according to the MultiCycle program in the FACStar. Flow cytometry was also used to show cells undergoing apoptosis, evident by the appearance of the sub-G1 peak.

Detection of Cell-penetrating Capacity

Cell-penetrating activity was monitored by fluorescence staining using the biotinylated peptide. Briefly, 270 μM of biotinylated peptide was added to 20×10^6 larynx tumor cells Hep-2 in DMEM (Hyclone) supplemented with 7% fetal bovine serum (HyClone, Logan, UT) plus 30 μg/ml of gentamycin (Gibco) and incubated for 10 min or 1 h at 37 °C in 5% CO_2. Subsequently, the cells were centrifuged at 8000 rpm for 10 min and the samples were immediately fixed with 4% paraformaldehyde in PBS at 4 °C and then mounted on gelatine-coated glass slides and stored for 2 days at −20 °C. Mounted samples were hydrated for 10 min in PBS. The samples were incubated with fluorescein isothiocyanate (FITC)-labeled avidin (1 : 100 dilution in PBS-T, Vector laboratories, Inc., Burlingame, CA, USA) for 1 h at room temperature (RT). After three washes with PBS-T the sections from all samples were counterstained with propidium iodide (1 : 1000 dilution, Vector laboratories, Inc. Burlingame CA, USA) in 5–10 min, followed by extensive washing with PBS-T. Samples were viewed with a 60× objective on a Nikon microscope with attached laser confocal scanning system MRC 600 (Bio Rad, Watfod). Ten to twelve fields were examined for each sample. Four to fifteen serial optical z-sections (0.2–0.5 μm thick) were collected from each observed field using the dual channel imaging with a 554 nm filter for propidium iodide excitation and a 494 nm filter for the fluorescein channel. Each series of confocal optical sections was scanned through a total of 25 μm. The resulting optical sections were fully projected onto two-dimensional planes using the imaging processing system of the microscope (Camos package).

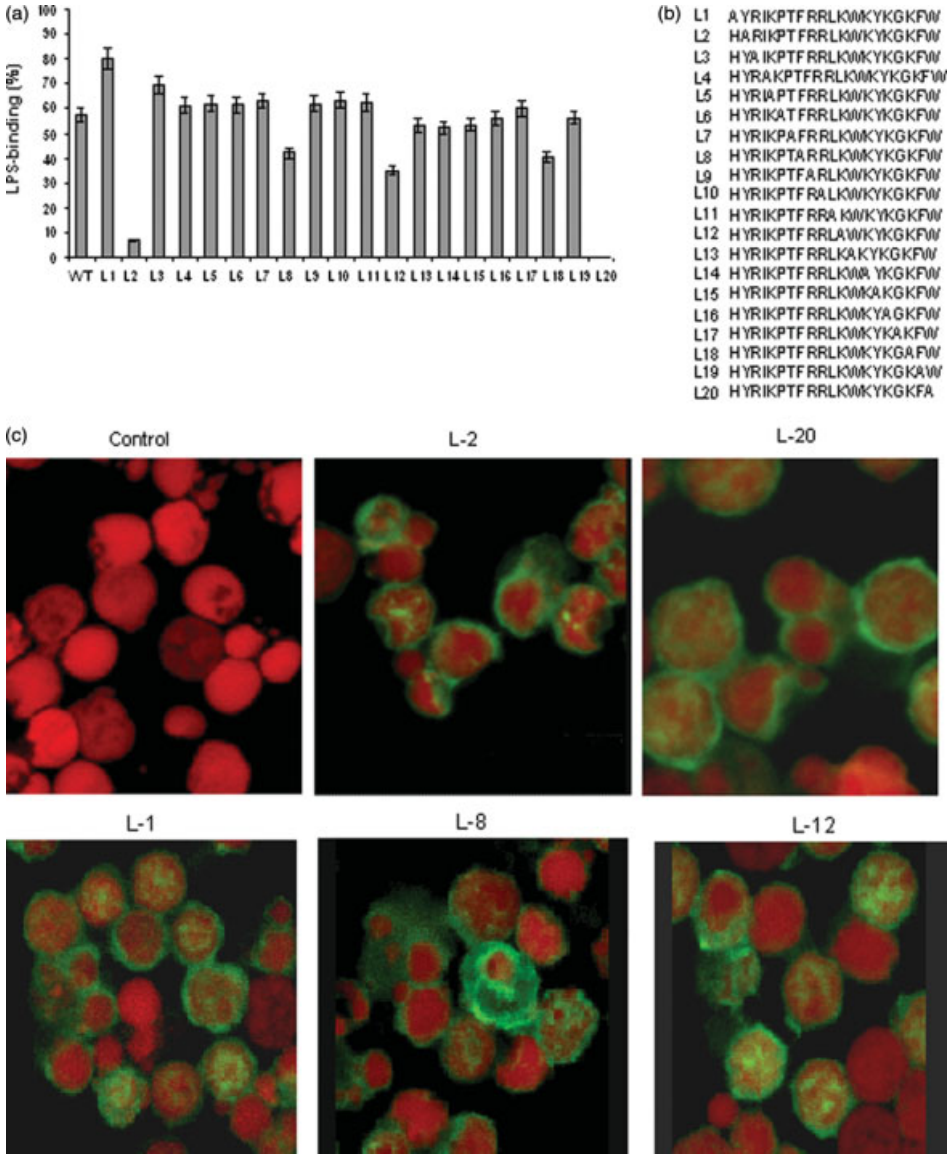

Figure 1. (a) Displacement of the binding of the biotinylated $LALF_{32-51}$ to LPS adsorbed to the solid surface using synthetic analogous peptide from the $LALF_{32-51}$ restricted ala-scanning library. In this experiment analogous peptides at 0.5 μM were mixed with biotinylated $LALF_{32-51}$ at 0.2 μM and then added to wells pre-coated with LPS from *E. coli* 0111 : B4 (1 μg/ml). The biotinylated $LALF_{32-51}$ bound to LPS was detected by incubating with a streptavidin-peroxidase conjugate. The graphic represents the capacity of analogous peptides to displace the binding of the biotinylated $LALF_{32-51}$ from LPS adsorbed to the solid surface, according to their capacity to bind the LPS. Results are expressed as LPS- binding (%) and represent the mean values ± SD from three independent experiments. (b) Sequences of the analogous synthetic peptides. (c) Cell-penetrating capacity of the analogous peptides in the ala-scanning library. The cells (20×10^6) were incubated with 270 μM of biotinylated peptides (L-1, L-2, L-8, L-12, and L-20) for 10 min. The samples were incubated with FITC-labeled avidin diluted 1 : 100 in PBS-T and the sections from all samples were counterstained with propidium iodide diluted 1 : 1000 (Vector laboratories, Inc. Burlingame CA, USA) for 5–10 min, followed by extensive washing in PBS-T at room temperature. Finally, slides were observed with a 60× objective on a Nikon microscope with attached laser confocal scanning system MRC 600 (BioRad, Watfod). Green fluorescent staining indicates peptide localization and red staining corresponds to propidium iodide-labeled nuclei.

In vivo Therapy of Solid Tumors in Mice

C57BL/6 mice were challenged with 50 000 TC-1 murine lung epithelial tumor cells in the left back foot. After 16 days, tumor-bearing mice ($n = 10$) were injected subcutaneously on the right flank, 1 dose per week, with 2 mg/kg of L-2 or L-20 in a volume of 200 μl in PBS, or with only the vehicle PBS as a control. For human tumor xenografts, pathogen-free female athymic nu/nu (nude) mice (Harlan, Germany/CNEA, Buenos Aires) were used at 6–8 weeks of age. Mice were subcutaneously injected with 4×10^6 ATCC-LS 174T human colon cancer cells in 300 μl of PBS in the right flank. Tumor-bearing animals ($n = 6$) with 30 mm^3 received subcutaneous injections in the left flank, at the timepoint indicated, with 1 or 2 mg/kg of L-2 or vehicle PBS. 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) × length (mm)/2. In both animal models, survival was registered until tumor volume reached approximately 1000–1200 mm^3 and animals were then sacrificed by cervical dislocation because of impairments in life quality.

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Apoptosis Analysis

To verify the induction of apoptosis *in vivo*, two TC-1 tumor-bearing C57/BL6 mice from the L-2 (2 mg/kg) group and two from the PBS group were analyzed. One week after the last injection, animals were sacrificed and the tumor tissues were excised, paraffin-embedded and sectioned into 5 μM for TUNEL and immunostaining analysis. *In situ* DNA fragmentation on tumor sections was detected using a DeadEndTM Flourometric TUNEL System (Promega, WI, USA). Protocol was done as suggested by the manufacturer and finally counterstained with propidium iodide. Green fluorescence (fragmented DNA) and red fluorescence (intact DNA) were easily differentiated. Digital images were obtained with a Zeiss Axioscop (40FL) microscope equipped with a UV source and a digital color camera (Cannon PC1089) interfaced with a computer. The images were taken at 20× and 40× for presentation using Adobe Photoshop.

SSH experiment

Larynx tumor cells Hep-2 were grown at 37 °C in 5% of CO_2 in DMEM (Hyclone) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) plus 30 μg/ml of gentamycin (Gibco). Cell density was maintained at 1×10^6 cell/ml in P-100 culture plate (Costar) containing 10 ml of culture medium for 24 h. Then, cells were incubated with 150 μM of L-2 for 2 h. Total RNA was isolated from treated and untreated cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The integrity of RNA and mRNA was checked on a 1% agarose gel. cDNAs were synthesized from 5 μg of total RNA using 200 U SuperScript II RT (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 μl. Subtractive libraries from treated and untreated RNA were constructed using the Clontech PCR-Select™ cDNA Subtraction kit (Clontech, Palo Alto, CA, USA). The amplified products from both subtraction samples were independently ligated into a pGEM-Teasy vector (Promega Co., WI, USA) and transformed into *E. coli* strain DH10B. From each library 94 white colonies were randomly selected and inoculated into 96 deep well plates. A blue colony and an empty well were used as controls. The DNA was purified and sequenced. The 94 identified genes are listed in Table S1.

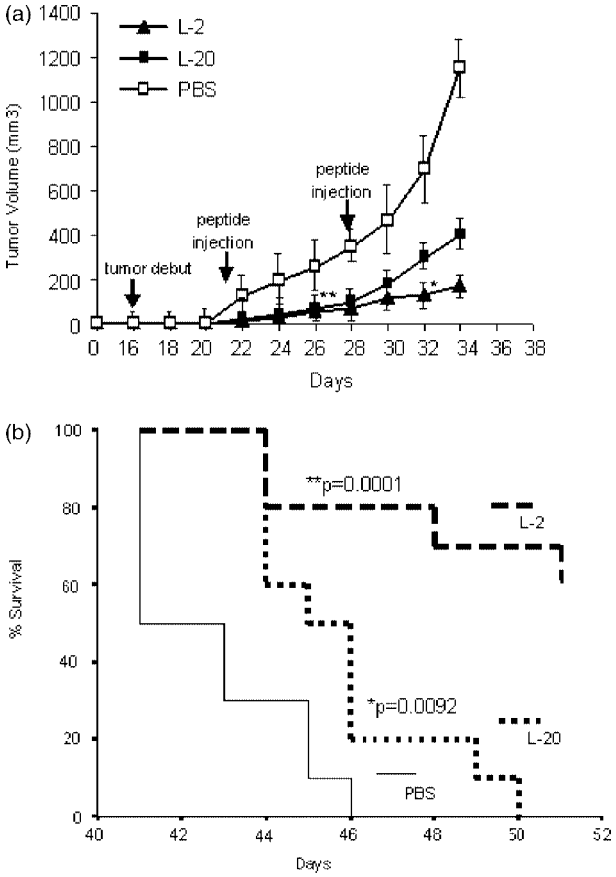

Figure 2. *In vivo* administration of L-2 elicits antitumor effect in immunocompetent mice. After tumor debut, C57BL/6 mice were injected systemically with 40 μg of L-2 or L-20 or PBS (represented by arrows), as detailed in the Section on Material and Methods. (a) Represents the mean of tumor mass volume and the respective standard deviation from the murine TC-1/C57/BL6. (b) Represents the Kaplan Meier curves of survival registered until day 50, when animals were sacrificed. Tumor growth on each day was analyzed by using the Tukey's test. $*p = 0.001$ (L-2 *vs* L-20); $**p = 0.0001$ (L-2 and L-20 *vs* PBS). The survival was analyzed by the log rank test. $*p = 0.0092$; $**p = 0.0001$.

Data and Statistic Analysis

The anti-tumor activity (as measured by differences in tumor mass) was expressed as mean and standard deviations. *In vivo* data were analyzed using SPSS 11.0 (SPSS, Inc.). The statistical significances of differences in survival rates were determined by log rank test. Statistical significances of differences in tumor volume were determined by one-way ANOVA, followed by Tukey's test for three or more groups, a value of $p < 0.05$ was considered significant. The normal distribution of the data was analyzed by Shapiro-Wilk test.

Results and Discussion

In this study, a peptide-based approach was used to identify a novel antitumor peptide, L-2, with cell-penetrating capacity. Peptide L-2 has advantages over other current anticancer peptide strategies that need a carrier to deliver the active peptide into tumor cells [9].

To identify peptides devoid of LPS-binding capacity, a peptide library corresponding to the ala-scanning of LALF residues 32–51 was screened. Figure 1a shows the percentages of analogous peptides inhibition, at a fixed concentration of 0.5 μM, according to their ability to displace binding of the biotinylated $LALF_{32-51}$ (0.2 μM) from LPS adsorbed to the solid surface. Peptides L-2 and L-20 demonstrated a limited ability to displace the parental $LALF_{32-51}$ from LPS, consistent with their diminished capacity to bind the LPS. The region comprising amino acids 31–52 was first proposed as the LPS-binding domain of LALF, based on the similarities of the amphipathicity pattern of this region to that of other human LPS-binding proteins such as Lipopolysaccharide binding protein (LBP) and Bactericidal/permeability increasing protein (BPI) [10]. Subsequent studies have demonstrated the apparent importance of single residues for synthetic peptide corresponding to these putative LPS-binding sites [11,12]. On the other hand, several studies have documented a correlation between anti-LPS activity and nanostructure formation for LPS-neutralizing peptides derived from LALF and BPI [13,14].Our findings demonstrate that alanine substitutions at positions Tyr33 and Trp51 in the non-cyclic $LALF_{32-51}$ sequence, abolished its ability to bind LPS, maybe due to the loss of the amphipathicity pattern and/or a suitable conformation. Further analysis of

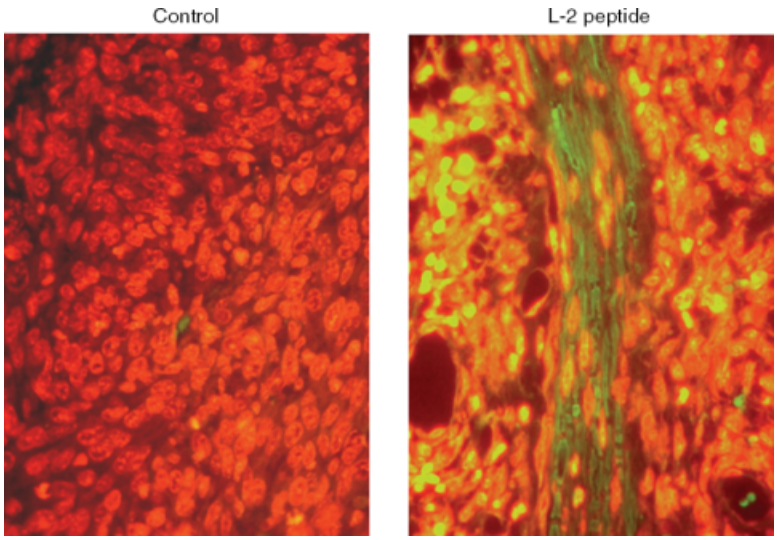

Figure 3. Apoptosis analysis. For the analysis of apoptosis *in vivo*, TC-1 tumor-bearing animals were treated with 2 mg/kg of L-2 during 2 weeks (one dose/week) by subcutaneous administration or with PBS vehicle. Two days after the last administration, tumors were excised and the respective sections were prepared for TUNEL analysis. Tumor apoptosis was detected using a DeadEnd Fluorometric TUNEL System with subsequent counterstaining with propidium iodide as recommended by the manufacturer. Microphotographs in one representative tumor from the PBS and the L-2-treated groups are presented.

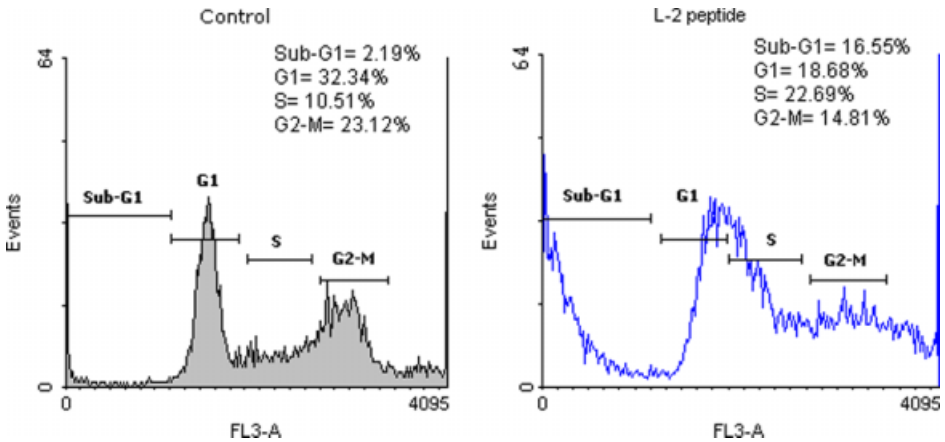

Figure 4. DNA flow cytometry analysis. LS 174T cells were treated with supplemented medium or L-2 (100 μM) for 24 h and analyzed by flow cytometry. Percentages of cells in sub-G1, G1, S and G2-M phase were calculated using MultiCycle program in the FACStar and are represented within the histograms. Data shown here are from a representative experiment repeated two times with similar results. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

comparative structure-activity relationships among close-related analogues should be conducted in order to clarify this query.

Since our interest in this study is focused in the peptides that lose capacity to bind LPS, the effect of the newly designed peptides, L-2 and L-20, on the proliferation of various tumor cell lines of different origins was determined. Importantly, peptide L-2 exhibited differential cytotoxic effects on various tumor cell lines as determined by the IC_{50} values (Table 1). Human colon and pancreas cancer cells had the highest sensitivity to the citotoxic effect of L-2. Interestingly, L-20 showed diminished cytotoxic effect with IC_{50} values higher than 100 μM for all cell lines tested. These results suggest that single amino acid residues (Tyr33 and Trp51) are essential for an effective cytotoxic activity, and provide preliminary data on the structural features of these peptides that seem to be important for their antitumor activity.

The analogues peptides in the Ala-scanning library share features present in all cell-penetrating peptides (CPP) such as high content of basic amino acids, amphipathicity and net positive charge [15]. In a second experiment, the analogous peptides were analyzed for their cell-penetrating capacity. To investigate this possibility some of the peptides in the Ala-scanning library (L-1, L-2, L-8, L-12, and L-20), were biotinylated and incubated with larynx tumor cells Hep-2 for 10 min and 1 h, and cellular localization was visualized by fluorescence staining and laser confocal microscopy. As early as 10 min after incubation, the peptides were clearly detected inside the cells. They were localized around the nucleus, as evidenced by the green fluorescent peptide, while the red fluorescence was indicative of nuclei (Figure 1c). A similar result was observed for 1-h incubation time. A subset of each cell population was analyzed by trypan-blue vital staining to confirm the viability of the cells. The cell lines CasKi, SiHa (human cervical carcinoma), human mononuclear cells and TC-1 (murine lung epithelial tumor cell) have also been employed to demonstrate the penetrability of these peptides (data not shown). In this

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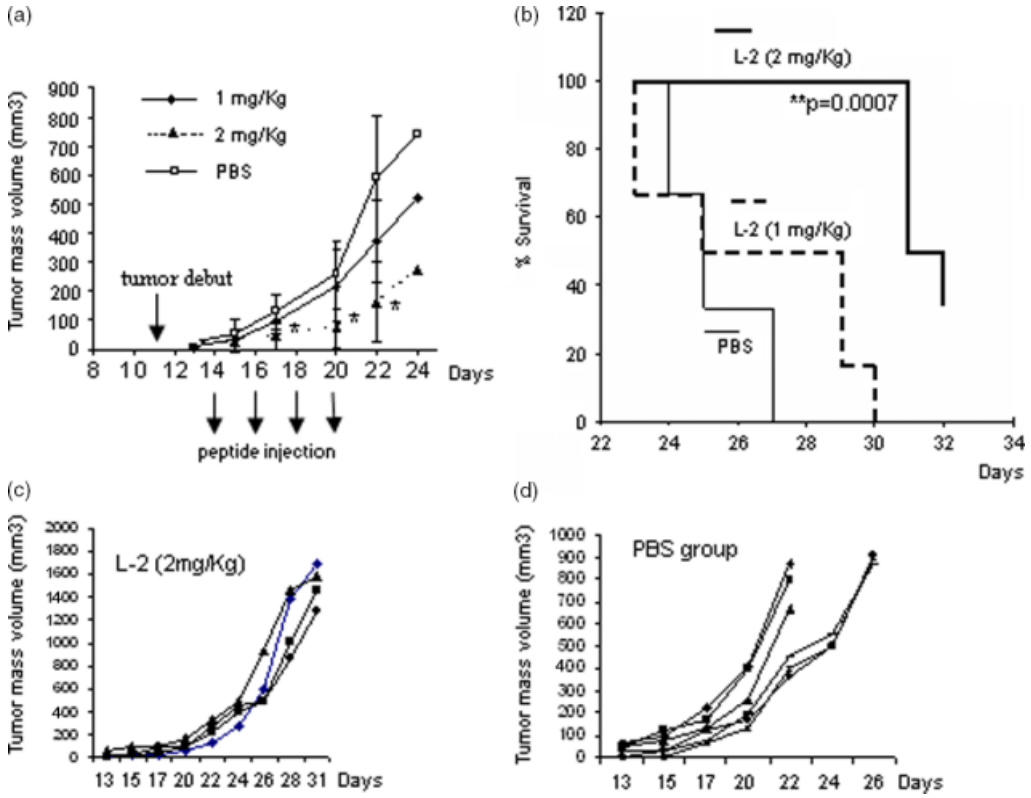

Figure 5. Systemic administration of L-2 in human colon tumor xenografted in nude mice. After tumor debut, mice were injected systemically with different doses of L-2 or PBS vehicle (represented by arrows), as detailed in the Section on Material and Methods. (a) Represents the mean of tumor mass volume and the respective standard deviation from the human tumors xenografted in nude mice. Tumor growth on each day was analyzed by using the Tukey's test, $*p < 0.05$. (b) Represents the Kaplan Meier curves of survival registered until day 32, when animals were sacrificed. (c,d) Represent the plotted individual tumor sizes in the groups treated with L-2 (2 mg/kg) or PBS. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Table 1. Effect of L-2 and L-20 on cell viability in different tumor cell lines

Tumor cell line	Origin	L-2 IC_{50} (μM)[a]	L-20 IC_{50} (μM)[a]
TC-1	Mouse lung HPV16-transformed cell	84 ± 6	120 ± 9
H-125	Human non-small-cell lung cancer	60 ± 9	238 ± 15
Hep-2	Human larynx carcinoma	76 ± 9	120 ± 10
Colo-205	Human colon adenocarcinoma	37 ± 10	125 ± 10
LS174T	Human colon adenocarcinoma	51 ± 3	135 ± 6
HT-29	Human colon adenocarcinoma (cisplatin-resistant cell)	42 ± 3	150 ± 9
CRL-1682	Human pancreas adenocarcinoma	65 ± 3	162 ± 9
MRC-5R	Human lung fibroblast	155 ± 6	159 ± 6
PBMC	Human mononuclear cells	234 ± 9	249 ± 6

The peptides were added to 10 000 cells in a range of concentrations from 0 to 200 μM. After 48 h of incubation, cell viability was determined by SRB [Sulforhodamine B, sodium salt] assay. Finally, absorbance was measured at 492 nm, and the IC_{50} values were calculated from the growth curves.
[a] Mean ± SD of three determinations. Data were obtained from two different experiments.

experiment a similar cell-penetrating capacity was demonstrated for all Ala-scanning peptides tested, and it might be expected in view of their structural similarity. This result demonstrates that L-2 is an antitumor peptide with cell-penetrating capacity but both bioactivities should be kept separate.

A TC-1 tumor model in C57/BL6 mice was used to evaluate the systemic antitumor activity of the peptides (L-2 and L-20). The administration of L-2 to tumor-bearing mice led to a significant reduction of tumor growth compared with the groups treated with either PBS or L-20 (Figure 2a). By day 26 following the first peptide-injection, mice inoculated with peptides L-2 and L-20 exhibited significantly less tumor volume than those receiving PBS ($**p = 0.0001$), as determined by the Tukey's test (95% confidence intervals). However, by day 32, a difference statistically significant in the reduction of tumor growth was observed between the two peptide-treated groups ($*p = 0.001$).Interestingly, this difference was maintained after the cessation of treatment and until the end of the assay. In line with the reduction observed for tumor mass volume in this model, L-2 significantly increased the survival time in animals compared with PBS or L-20, as revealed by the log rank analysis (Figure 2b). The mean survival was 51 days for mice treated with L-2 ($**p = 0.0001$) *versus* 42 days for the PBS

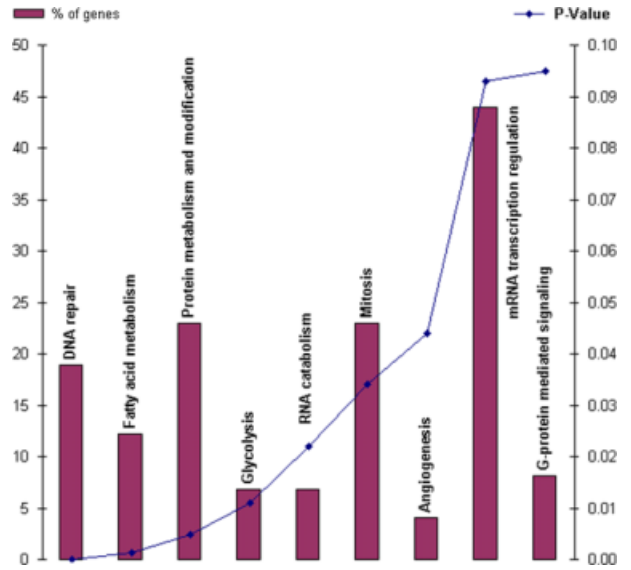

Figure 6. Functional classification of 94 genes differentially expressed in Hep-2 tumor cells treated with L-2 (Supplementary Information, Table S1). The classification was conducted using the annotation of biological processes from http://www.pantherdb.org/ using the tools from DAVID. The significance (*p*-value) of the functional classification of genes was determined by the EASE Score/*p*-value (http://david.abcc.ncifcrf.gov/). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

group and 46 days for the L-20 group ($*p = 0.0092$). Consistent with the results obtained with the tumor cell lines, L-2 showed a potent antitumor effect compared to L-20 when systemically administered in the TC-1 model.

To gain insights into the mechanism by which L-2 reduces the tumor growth *in vivo*, the type of cell death was determined by analysis of *in situ* DNA fragmentation along the *in vivo* study of the TC-1 model. An increase in the apoptotic cell fraction was shown in data from TUNEL analysis, as evidenced by the green fluorescent nuclei (fragmented DNA), while red fluorescent nuclei were indicative of living cells with intact DNA (Figure 3). The TUNEL-positive population is probably not the single fraction driving to apoptosis in the tumor, as those in which steps upstream to DNA fragmentation were occurring are not detected with the TUNEL system. On the other hand, apoptotic cells were almost undetectable in tumors corresponding to PBS-treated mice. Therefore, the data obtained from the TUNEL analysis in this work suggest that the systemic administration of L-2 elicits antitumor effect by inducing apoptosis in the tumor mass.

Since apoptosis often occurs as a consequence of a cell cycle block, we checked whether the cytotoxicity of L-2 was mediated by an alteration of the cell division cycle. Analyses by flow cytometry of the tumor cells treated with L-2, showed the absence of the G2/M peak and the accumulations of the cells in S phase (Figure 4). Furthermore, cells undergoing apoptosis after a 24-h treatment were observed by a significant increase in the sub-G1 peak. Here we show that in proliferative LS 174T cells, L-2 induces a cell cycle arrest, followed by cell death. The exact mechanism(s) of apoptosis and cell cycle deregulation by L-2 needs a further exploration of genetic and signal transduction pathways.

To prove the anticancer effect of L-2 under *in vivo* conditions, a human colon tumor xenografted in nude mice was used. As shown in Figure 5a, a significant antitumor effect was observed when L-2 was systemically inoculated at a dose of 2 mg/kg in LS 174T human colon tumors xenografted in nude mice. By day 17, mice inoculated with L-2 (2 mg/kg) exhibited significantly less tumor volume than those receiving PBS ($*p = 0.028$) and this difference was maintained after the cessation of treatment and until the end of the assay. However, at a dose of 1 mg/kg, no statistically significant difference was observed with the PBS group. Moreover, survival was also significantly increased in the L-2 (2 mg/kg)-treated group with respect to the PBS group (Figure 5b). The survival time was 32 days for L-2 (2 mg/kg) *versus* 25 days for the PBS group ($**p = 0.0007$). By day 31, two animals of the L-2 (2 mg/kg) group remained tumor-free. Interestingly, a plot of individual tumor sizes (Figure 5c and d) indicated that in the LS 174T/nude mice model, tumors in the surviving animals in the L-2 group (2 mg/kg) reached a volume of up to 1600 mm^3 without showing impairments in quality of life. The animals from the PBS group were sacrificed when the tumor reached a volume of approximately 1000 mm^3 and signs of distress were evident. These data verified that L-2 at 2 mg/kg reduced the tumor growth at similar extents in both models, and that no major differences in the survival time were observed using this dose. Taken together, our findings demonstrate that L-2 could behave as a cytotoxic peptide targeting tumor cells and leading to a significant antitumor response in mice, mediated by apoptosis of the cancer cells.

Given the antiproliferative activity of L-2 in several human tumor cell lines and its antitumor effect on human tumors xenografted in nude mice, the molecular mechanism by which L-2 induces its cytotoxic effect was analyzed. To examine this question, the SSH approach was used to identify populations of mRNA that could distinguish a Hep-2 human cell line treated for 2 h with L-2 from an untreated one. As a result, two subtracted cDNA collections were obtained: one consisting of cDNA from the treated cell line subtracted from a cDNA of untreated cells which represent the up-regulated genes; and a second library, referred to as down-regulated genes, and obtained by reverse subtraction. A total of 94 clones from each library were sequenced and annotated (Table S1). With a list of gene names derived from our results, a classification based on gene function by Gene Ontology Annotation was created [16]. L-2 was shown to inhibit important biological processes in cancer cells (Figure 6). Based on our experimental data and on the information obtained from the pathway analysis, we hypothesized that several specific pathways may contribute to the antitumor activity of L-2. Interestingly, the reduction on the expression of three genes in the Glycolysis pathway (PDGK1, PGM and ENO1) predicts a decline of Glycolysis, which would explain the cell cycle arrest and apoptosis of the tumor cells treated with L-2 [17]. Furthermore, a decrease in the transcription of genes related to protein biosynthesis such as EEF1G, EEF1A1 and RPS6 will surely explain a decrease in the levels of certain proteins that may be involved in cancer formation [18,19]. Future experiments should be conducted to test which of the genes identified in our experiments make a major contribution to the antitumor activity of L-2.

In summary, this study provided a proof-of-concept that peptide L-2 is an antineoplastic agent with cell-penetrating capacity, but both properties are not associated. The differential expression of a unique set of genes in tumor cells treated with L-2 may be one of the mechanisms by which this peptide exerts its antitumor activity. Thus, this is the first report describing a novel peptide with specific cytotoxic activity against tumor cells and selected from a peptide-based approach (ala-scanning library). L-2 may serve as the prototype of a peptide-based drug with the potential to reduce

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tumor burden alone or when coadministered with conventional chemotherapy.

Supporting information

Supporting information may be found in the online version of this article.

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