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In silico, in vitro and *in vivo* toxicity assessment of the antitumoral peptide GK-1

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ABSTRACT

Peptide drugs have emerged as an attractive alternative for cancer treatment due to their potency, high specificity, general safety and low cost. GK-1 is a linear 18 amino acid peptide with proven immunomodulator, antitumor and antimetastatic capacity in animal models. Preclinical toxicity studies for its use as a vaccine adjuvant demonstrated its safety in various assay systems, but a comprehensive exploration of its toxicity profile is required to be used in cancer immunotherapy. Therefore, in the present work, the potential toxicity of GK-1 was predicted with ToxinPred 3.0 software, and its *in vitro* cytotoxicity, and single-dose and repeated-dose toxicity by subcutaneous route in mice were experimentally assessed. GK-1 peptide was predicted as a non-toxic and did not exhibit *in vitro* cytotoxicity for several non-tumor and tumor cell lines and primary cell cultures at concentrations up to 500 μM, reinforcing previous studies pointing that the antitumoral effect of GK-1 was not mediated by tumor cell cytotoxicity. The single-dose toxicity study did not evidence local or systemic toxicity up to the maximum tested dose of 1000 mg/kg. Moreover, no toxic effects were observed in the repeated-dose toxicity study based on four doses administered weekly at up to 300 mg/kg. Considering that GK-1 is effective in triple-negative breast cancer and melanoma models in mice at doses as low as 5 mg/kg, the present results support the safety of GK-1 as an antitumoral peptide candidate.

1. Introduction

Cancer is the second leading cause of death globally according to the World Health Organization [1]. Cancer caused 9.7 million deaths with 20 million new cases in 2022, and 35.3 million cancer cases worldwide are expected by 2050 [2]. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical and thyroid cancer are the most common among women [1].

Peptides have emerged as versatile tools in cancer research and drug development due to their unique properties, including high specificity, low toxicity, and ease of chemical modification with high yields [3–5]. Furthermore, the diversity of side chains and the high number of possible amino acid permutations provide a broad spectrum of potential targets. Over 80 peptides are in clinical use, more than 200 are in clinical trials and other 600 are in preclinical studies [6]. These short chains of amino acids serve as critical intermediates between small molecules and larger biologics, offering a bridge that leverages the advantages of both. In recent years, peptide identification from natural sources and the synthesis of bioactive peptides have opened new avenues for targeting cancer-specific pathways, enhancing drug delivery, and for modulating immune response [7].

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Peptides are particularly attractive in cancer therapy because of their ability to target molecular markers that are often overexpressed in tumor cells, such as receptors, enzymes, or signaling molecules [7,8]. Additionally, peptides play a pivotal role in drug delivery systems, such as the development of peptide-drug conjugates (PDCs) that combine the precision of peptides with the potency of cytotoxic agents [9,10].

Furthermore, advancements in peptide engineering have enabled the design of peptide vaccines and inhibitors that stimulate immune responses or block specific protein-protein interactions crucial for cancer progression, respectively [11]. Despite these advances, challenges remain, including the improvement of peptide stability, bioavailability, and resistance to proteolysis. Addressing these limitations through innovative strategies, such as cyclization, incorporation of non-natural amino acids, pegylation, lipidation and conjugation to larger proteins like albumin continues to expand the potential of peptides in oncology and other pharmaceutical applications [9,12,13].

From a toxicological perspective, peptides offer several advantages, including high specificity, minimal off-target effects, and typically lower immunogenicity. Peptides exhibit high specificity toward their target receptors, often leading to fewer adverse effects compared to conventional chemotherapeutics or other systemic drugs [9]. Their natural amino acid composition contributes to their favorable biocompatibility and low toxicity, as endogenous peptides are generally well tolerated by the human body. Moreover, metabolic interactions like cytochrome P450 inhibition are uncommon among peptide-derived drugs [7].

Despite these benefits, certain toxicological concerns require attention in peptide drug development. The rapid proteolytic degradation of peptides can lead to the generation of toxic metabolites in particular when modifications are introduced to enhance stability, such as cyclization or incorporation of non-natural amino acids, which may result in off-target effects. Accumulation in non-target tissues, especially for peptide-drug conjugates (PDCs), could also cause unexpected toxicities. Moreover, intrinsic toxicity of a number of peptides, depending on the amino acid composition and sequence, may lead to hemolysis, cytotoxicity, local necrosis, cardiotoxicity and neurotoxicity [14–17].

Computational toxicology tools have become essential for predicting and mitigating adverse effects early in the development pipeline. In general, several predicting models and computer programs have been developed in the last years to support the development of peptides as potential drugs. Rathore et al. [18], in their paper introducing ToxinPred 3.0, summarize recent advances in the field with reference to the most relevant computer programs developed so far and categorizes them in five main groups considering the properties that they predict: 1) therapeutic properties (half-life and structure); 2) cell-penetrating and tumor homing; 3) antimicrobial properties (antiviral, antifungal, and antibacterial peptides); 4) inhibitors other than antimicrobial peptides (anticancer and antihypertensive peptides); and 5) harmful effects (toxic and hemolytic activity). In this last group, models for the identification of toxic peptides, for the elucidation of specific mechanisms of toxicity and for the recognition of amino acid sequences responsible for toxicity have been developed.

ToxinPred 3.0 is a web-based server and standalone software package that classifies peptides as toxic or non-toxic molecules [18]. Based on a curated dataset of 5518 toxic and equal number of non-toxic peptides, and by using a group of alignment-based and alignment-free techniques, this software finds a final hybrid ensemble of models that predicts global toxicity of peptide sequences with high accuracy (93 %), specificity (93 %) sensitivity (92 %) and area under ROC (0.98), outperforming previous similar approaches.

GK-1 is a linear 18 amino acid peptide (GYYYPSDPNTFYAPPYSA) derived from the KETc7 recombinant protein, which was first isolated from a *Taenia crassiceps* cDNA library [19]. This has been shown immunomodulatory [20] antitumor and antimetastatic properties in several murine tumor models [21–24].

It has been recently reported that GK-1 is capable of entering bone marrow-derived dendritic cells through a clathrin-dependent mechanism, but it also has the ability to get into cells by passive diffusion [25]. Moreover, GK-1 peptide binds to TLR4 receptor at the same binding site as LPS [25], thus activating a MyD88-dependent pathway leading to the translocation of NfKB [26].

In terms of safety for its use as an immunomodulator, *in vitro* and *in vivo* studies demonstrated that GK-1 peptide is not genotoxic, non-sensitizing, non-pyrogenic and innocuous upon repeated administration in rats by intravenous or subcutaneous routes at doses of up to 12.5 mg/kg [27].

Although evidence compiled so far supports that the antitumor effect of GK-1 is due to its immunomodulatory properties, its cytotoxicity has not been systematically studied. Moreover, due to its low aqueous solubility, the in vivo toxicity of GK-1 peptide at high doses has not been thoroughly studied. Therefore, this study is aimed to further explore the safety profile of GK-1 peptide by using ToxinPred 3.0 software, testing its *in vitro* cytotoxicity as well as studying its toxicity by single and repeated dose administration in female BALB/c mice, the animal model in which GK-1 peptide has primarily demonstrated its immunomodulatory, antitumor and antimetastatic activity.

2. Material and methods

2.1. Toxicity prediction

The toxic potential of GK-1 peptide was predicted using ToxinPred 3.0 (https://webs.iiitd.edu.in/raghava/toxinpred3/prediction.php). The amino acid sequence of GK-1 [19] was uploaded as a FASTA file and the prediction was run with the hybrid deep-learning plus Motif-EmeRging and with Classes-Identification (DL-MERCI) method by setting the threshold value to 0.38 (default).

2.2. GK-1 peptide

GK-1 was synthesized by USV Private Limited (Mumbai, India) and supplied as a lyophilized powder with 97.01 % purity.

2.3. Cell lines and cultures

Tumor and non-tumor cell lines as well as primary cell cultures were used for cytotoxicity studies (Table 1). Tumor cell lines included 4T1 (CRL-2539, ATCC, Manassas, VA, USA) U87 MG (HTB-14, ATCC), B16-F10-OVA (MO4) (SCC420 Merck Millipore), Jurkat (CRL-2899, ATCC), THP-1 (TIB-202, ATCC) and MDA-MB-231(HTB-26, ATCC). Non-tumor cell lines used were NIH/3T3 (CRL-1658, ATCC), HEK 293 (CRL 1573, ATCC) and HEK 293/TLR4 (293-hmd2cd14, InvivoGen, San Diego, CA, USA). Mouse peritoneal macrophages, mouse peripheral blood mononuclear cells, bone-marrow derived dendritic cells were the primary cell cultures used.

Mouse peritoneal macrophages were collected as detailed elsewhere [28], mouse spleen lymphocytes were obtained by disaggregation and density gradient separation [29], and mouse bone-marrow derived dendritic cells were isolated, transformed and cultured as reported previously [25].

2.4. In vitro cytotoxicity test

Confluent cell cultures were detached by addition of a commercial mixture of trypsin (0.5 mg/mL and EDTA (0.5 %, Grand Island, NY, USA), centrifuged (1200 rpm, 5 min) and resuspended in Dulbecco's Modified Eagle Medium (DMEM, gibco, Grand Island, NY, USA) supplemented with 10 % heat-inactivated (56 °C, 45 min) fetal bovine serum (CORNING, Woodland, CA, USA) and antibiotics (200 μg/mL streptomycin and 200 IU/mL penicillin; gibco, Grand Island, NY, USA). After estimating the number of viable cells by blue trypan (Gibco, Grand Island, NY, USA) exclusion in Neubauer chamber, cell density was adjusted in supplemented DMEM to a previously optimized number of

Table 1
Cell lines used in the cytotoxicity assay.

Cell line	Species	Cell type	Culture type	Proliferative cell	Initial cell density (cells/mL)
4T1	Mouse	Epithelial	TCL (breast cancer)	Yes	20 000
B16-F10-OVA (MO4)	Mouse	Melanocyte	TCL (melanoma)	Yes	10 000
Jurkat	Human	Lymphocyte	TCL (acute lymphocyte leukemia)	Yes	20 000
THP−1	Human	Monocyte	TCL (acute monocyte leukemia)	Yes	20 000
MDA-MB−231	Human	Epithelial	TCL (breast cancer	Yes	20 000
U87 MG	Human	Epithelial	TCL (glioma)	Yes	20 000
NIH/3T3	Mouse	Fibroblast	nTCL (embryonic)	Yes	10 000
HEK−293	Human	Epithelial (kidney)	nTCL (embryonic)	Yes	20 000
HEK−293/TLR4	Human	Epithelial (kidney)	nTCL (embryonic)	Yes	20 000
Peritoneal macrophages	Mouse	Macrophage	PCC	No	500 000
Spleen lymphocytes	Mouse	Lymphocyte	PCC	No*	500 000
Bone-morrow derived dendritic cells	Mouse	Dendritic cell	PCC	No	100 000

TCL: tumor cell line; nTCL: non-tumor cell line; PCC: primary cell culture. * Inducible proliferation

cells/mL that eventually produced near 6000–8000 arbitrary units of fluorescence after 48 h incubation and the addition of resazurin (Table 1).

GK-1 was dissolved in supplemented DMEM, serially diluted (Six, 2-fold serial dilutions from 1000 μM to 32 μM, 100 μL/well) in a 96-well culture plate (Corning, Kennebunk, ME, USA) and mixed with appropriate suspensions of cells (100 μL/well). Final concentrations of GK-1 from 500 μM to 15.6 μM were tested. Positive controls consisted of cell cultures treated with 10 % (final concentration) DMSO, and negative controls were cultures of 100 μL + 100 μL mixtures of DMEM and cell suspensions. Each concentration was tested in triplicate. After 48 h incubation at 37 °C and 5 % CO_2, 20 μL of 2 mM resazurin (St Louis, MO, USA) were added and the plates were incubated overnight. Then, fluorescence was read in a CYTATION3 plate reader (BioTeck, Winooski Vermont, USA) at 550 nm (excitation)/590 nm (emission) and 50 % gain. Additionally, for mouse peritoneal macrophages and U87 MG cell line, the number of cells/mL was determined by counting in Neubauer chamber. The effect of GK-1 on cell proliferation was then assessed by plotting means ± standard deviation of fluorescence intensity - and cell number (cells/mL) for selected cell lines - *vs* GK-1 concentration.

2.5. Animals

Young-adult, female, 18–20 g, 6–8 weeks old BALB/c mice were supplied by the Unit of Biological Models of the Institute of Biomedical Research, National Autonomous University of Mexico. They were housed in T-2 polycarbonate cages with Sani-Chips® shredded wood bedding (Envigo, Mexico City, Mexico) sterilized in an autoclave. Climatic variables were maintained by injection of filtered air with heating as needed and an average of 16 air changes per hour. Room temperature (18–23 °C), relative humidity (40–60 %), light intensity (200–250 lx) and light cycle (12 h light - 12 h dark) were maintained during the experiment. Autoclavable food (TEKLAD-2018, Envigo, Mexico City, Mexico) and drinking water (acidified and autoclaved) were supplied for *ad libitum* ingestion; however, the consumption was measured as part of the study variables. A minimum five-day adaptation period was allowed before the studies started. Clinical observation was conducted during this time to ensure the health condition of the animals. Mice were humanely killed at the end of the experiments by either cervical dislocation (acute toxicity study) or sevoflurane anesthesia and total exsanguination (repeated dose toxicity study).

The experimental protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL 6338) which operates in accordance with national [30] and international standards [31].

2.6. Single dose toxicity test

The assay was conducted following the OECD Guideline for the Acute Toxic Class Method [32]. Dimethyl sulfoxide (DMSO) was used as a cosolvent to increase water solubility of GK-1 in order to attain a higher exposure of mice to GK-1 peptide. Since DMSO has very low systemic toxicity, with a median lethal dose by subcutaneous route in mice and rats of 13.9–20.5 g/kg [33], GK-1 was dissolved in DMSO and then in sterile isotonic saline solution (ISS) at a 1:19 (V:V) ratio for the GK-1 dose of 300 mg/kg and 1:14 for 1000 mg/kg. Three mice were treated subcutaneously with a starting dose of 300 mg/kg and subsequently, according to the mortality outcome at 48 h, similar groups of three mice were conceived to be treated with lower (50 mg/kg), equal or higher doses until the proper categorization of the substance, according to the Global Harmonized Classification System, was achieved (Annex 2c of reference [32]).

The acute toxicity test, according to the class method, does not include a control group in the design. However, mice awaiting dosing were used to monitor water and food consumption in untreated animals and served as a control for these variables.

Due to solubility issues, the volume in which the product was administered was 1 mL for the 300 mg/kg dose and 1.5 mL for the 1000 mg/kg dose. Considering the maximum recommendable volume through this route, the dose of 1000 mg/kg was divided into two applications with an interval of 4 h between administrations. The first application was made in the interscapular space and the second in the lumbar region.

Mice were clinically examined 30 min, 1 h, 2 h and 4 h after administration. Then, they were observed daily for fourteen days. The clinical observations were aimed at identifying changes in the skin and hair, the eyes and mucous membranes, the respiratory system, the circulatory system, the autonomic and central nervous system, somatomotor activity, behavior pattern, and any other alterations in other systems. Special attention was paid to the most frequent changes that mice present as indications of local or systemic toxicity, which included, but were not limited to repeated attention to the inoculation site, tearing, ocular occlusion or decreased eyelid opening, salivation, diarrhea, piloerection, tremors, convulsions, grouping, reduced mobility, excessive sleep, prostration, dyspnea, coma and death, among others [32].

Mice were weighed individually before dosing, and 3, 7 and 14 days after dosing. Body weight gain during the observation period (14 days) was calculated by difference respect the initial body weight. Water and food were administered for *ad libitum* consumption, however, both water and food were weighed and on the third or fourth day the remains were weighed. The consumption of each group was calculated by first subtracting the weight of the remainder from the weight of water or food provided. Subsequently, the average individual consumption was calculated by dividing the group consumption in the period by the number of animals [3] and the number of days elapsed (3 or 4) between weightings.

At the end of the 14 days of observation, animals were sacrificed by cervical dislocation and subjected to necropsy. It was conceived to take samples for histopathological studies of those organs that showed any

macroscopically visible change.

Statistical analysis: Body weights of mice treated with GK-1 at 300 mg/kg and 1000 mg/kg were compared by repeated measures analysis of variance (ANOVA). Water and food consumption were compared by one-way ANOVA. *P* values under 0.05 were considered significant. Statistical analyzes were performed in GraphPad PRISM (Version 8.0.2, 2019)

2.7. Repeated dose toxicity test

The design of the study took the "OECD Guideline for Testing of Chemicals 408: Repeated dose 90-day oral toxicity study in rodents" [34] as a reference, with some modifications considering the expected clinical scheme of administration as suggested by the ICH Guidance for Industry S9 "Nonclinical Evaluation for Anticancer Pharmaceuticals" [35].

Female BALB/c mice of 19.1 ± 0.8 g body weight at the moment of administration of the test substance were used. This way, weight variation of animals used was between −7.3–10.5 % of the mean weight, not exceeding the recommended ± 20 % [34].

A total of 30 mice were used, which were randomly allocated to five experimental groups: Three groups were treated with GK-1 at doses of 300 mg/kg, 200 mg/kg and 100 mg/kg, respectively, one was treated with 5 % DMSO (vehicle control) and one received no treatment at all. GK-1 was dissolved in DMSO and then in ISS (1:19, V:V), so that the final concentration of DMSO was 5 %. Treatment was administered every 7 days, four doses, 1 mL, by the subcutaneous route in the interscapular space.

Mice were examined clinically (as described above for the single-dose toxicity test) at 30 min, 1 h, 2 h, and 4 h after each administration. The remaining days, they were observed daily, including 10 days after the last dose. Mice were individually weighed at the end of the adaptation period, before each dosing, and seven days after the last dose was administered. Body weight gain was evaluated during the experimental period (30 days). Food and water intakes were assessed as described previously.

At the end of the 10 days of observation after the last dose, the mice were anesthetized by sevoflurane inhalation and blood was drawn by cardiac puncture. Blood samples for hematological studies (near 200 μL) were transferred to vials containing 20 μL of 10 % EDTA. The rest of the collected blood was allowed to clot for 30 min, centrifuged at 2300 rpm for 10 min, and serum was obtained for blood biochemistry analysis. Hematological parameters included: hematocrit, hemoglobin (Hb), erythrocytes, mean globular volume (MGV), mean globular hemoglobin concentration (MGHC), platelets, total solids, leukocytes, neutrophils (PMNN), lymphocytes, monocytes, eosinophils (PMNE) and basophils (PMNB). Blood biochemistry included the determination of glucose, urea, creatinine, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (ASP), alkaline phosphatase (ALP), creatine kinase (CK), total proteins and triglycerides.

After blood collection, mice were necropsied and samples of heart, lungs, thymus, liver, spleen and kidneys were taken for histopathological analysis.

Statistical analysis: Body weights were compared by repeated measures ANOVA. Weight variations from the beginning to the end of the experiment, water consumption and food consumption were compared by one-way ANOVA. Hematological and biochemical parameters that followed a normal distribution (Shapiro-Wilks test, $p > 0.05$) and had homogeneous variances (Brown-Forsythe test, $p > 0.05$) were compared by one-way ANOVA. In case of having a normal distribution, but unequal variances, an attempt was made to correct the inequality of the variances by means of logarithmic transformation of the values. If successful, the transformed values were compared by one-way ANOVA; otherwise, untransformed values were compared using the Kruskal-Wallis's test. *P* values under 0.5 were considered significant. Statistical analyses were performed in GraphPad PRISM (Version 8.0.2, 2019).

3. Results and discussion

3.1. Toxicity prediction

The hybrid DL-MERCI ensemble combines deep learning (DL) and Motif-EmeRging and with Classes-Identification (MERCI) as alignment-free and alignment-based methods to optimize the prediction of peptide toxicity. Final deep learning model included Long Short-Term Memory Artificial Neural Networks (ANN-LSTM) which identify distinctive recurrent amino acid patterns in toxic and non-toxic peptides, and MERCI method is based on matching the query sequence with unique sequences found in either the toxic or the non-toxic subsets of the peptide dataset [18].

DL-MERCI method resulted in a hybrid score of 0.28, a positive predictive value (probability of correct toxicity prediction) of 0.114; thus, 0.886 probability of been non-toxic. Considering that the default threshold of the program to consider a peptide sequence as toxic is 0.32, the final prediction of GK-1 sequence was as non-toxic. Moreover, MERCI scores were equal to zero, indicating that no match between the GK-1 amino acid sequence and any unique motifs in toxic or non-toxic peptides were found. Though an accurate classification method, the applicability domain of MERCI is limited to unique amino acid sequences represented in any of the peptide subsets. Thus, in this case DL-MERCI results relied on the DL component of the ensemble, which identified GK-1 sequence as a non-toxic peptide. Considering the high number of peptides (5518 toxic and 5518 non-toxic) used during DL model development, sufficient peptide structural diversity and a variety of toxicity mechanisms should have been covered. That, together with the high performance during the validation of the ensemble with an external dataset, support the accuracy of the prediction of GK-1 as a non-toxic peptide.

3.2. Cytotoxicity

Although methods of *in vitro* cytotoxicity based on the reduction of resazurin strictly measure the level of metabolic activity, they are indirectly sensitive to the number of cells and their viability, since only viable cells with an operative intermediary metabolism can maintain the appropriate reducing environment to transform resazurin [36]. The typical and expected aspect of fluorescence intensity versus drug concentration charts in cytotoxicity assays shows that fluorescence intensity decreases as the test compound concentration increases, normally describing an inverted sigmoid curve. In proliferating cells, fluorescence intensity decay can be a result of either viability reduction or inhibition of cell multiplication, or both. In contrast, in non-proliferating cell cultures (like differentiated primary cultures) it is an indicator of progressive loss of viability, since the number of cells does not vary.

Unexpectedly, in the cytotoxicity assays reported in the present paper, fluorescence intensity increased as the concentration of the GK-1 augmented. The same pattern was observed for tumor cell lines (Fig. 1), non-tumor cell lines (Fig. 2) and primary cell cultures (Fig. 3). As mentioned before, that could be a consequence of the stimulation of cell proliferation and/or the metabolic activation of cells. However, since BMDC, non-stimulated lymphocytes and mouse peritoneal macrophages are essentially non-proliferating cell cultures and they also evidenced the same profile of fluorescence intensity as the proliferating cell lines (tumor and non-tumor cells), it is reasonable to discard the idea that GK-1 peptide enhanced cell proliferation.

In fact, cell enumeration by using Neubauer's chamber conducted in parallel with the resazurin reduction assay in mouse peritoneal macrophages and U87 tumor cells (Fig. 4), showed the previously observed increase of fluorescence while the number of cells remained unchanged along the whole range (16 μM – 500 μM) of GK-1 peptide concentrations. A result that confirms the statement that GK-1 did not induce cell proliferation.

Test compounds may interfere with the assay system and even some

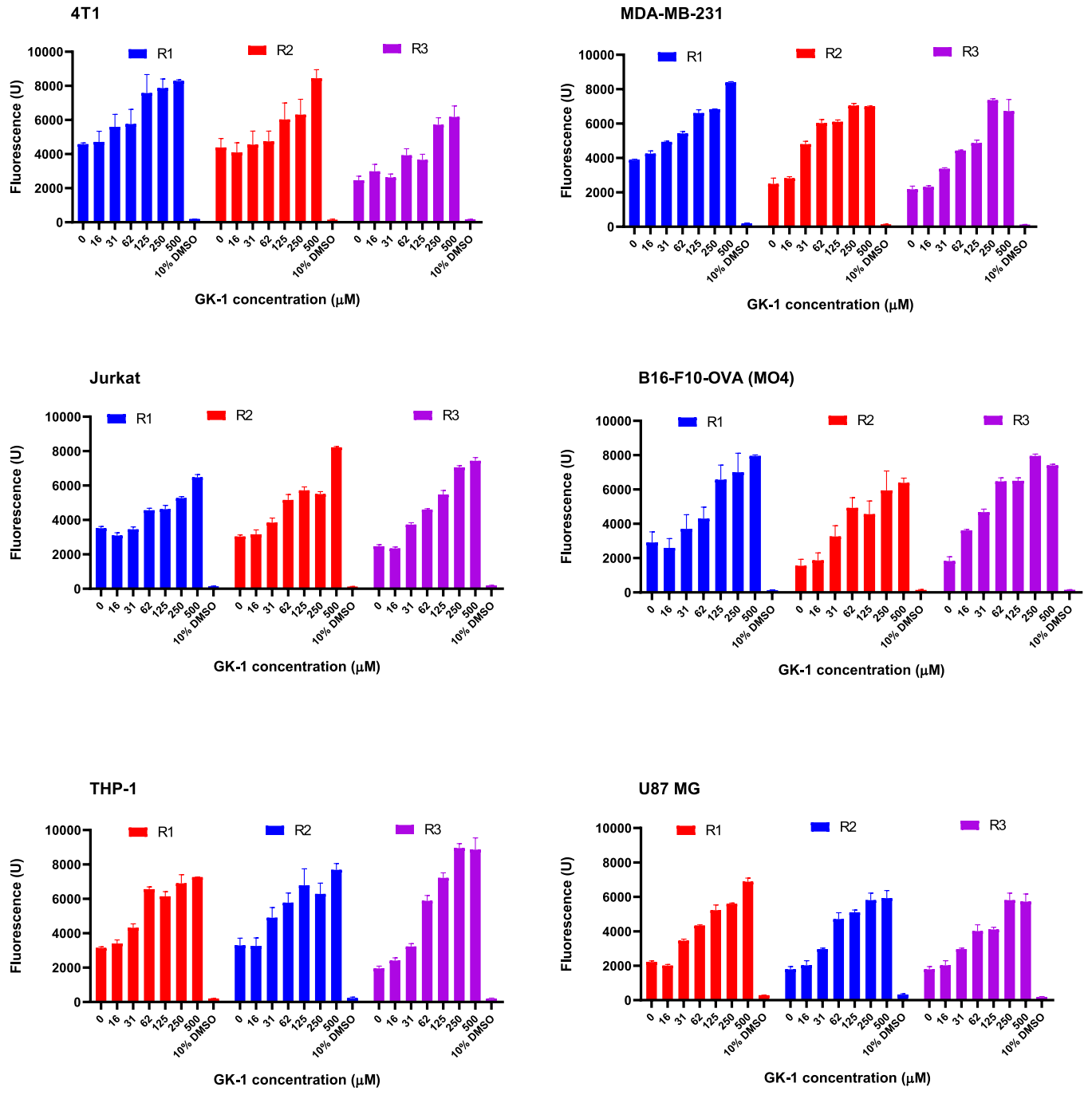

Fig. 1. *In vitro* cytotoxicity of GK-1 peptide in tumor cell lines.

compounds are capable of interfering in multiple assays, frequently resulting in false positives. These compounds are known as PAINS (Pan Assay Interference Substances) as a reference to their misleading impact in research projects [37]. Non-specific reducing agents like ascorbic acid (vitamin C), reduced glutathione (GSH) and N-acetylcysteine (NAC) can chemically reduce resazurin to resorufin without the need for enzymes [38]. A thorough exploration of small molecules that interfered in resazurin and MTT assays identified thiols and carboxylic acids as the most attributable sources of interference and found substantial increases of resazurin and MTT conversion in the presence of glutathione, D-penicillamine, L-cysteine, N-acetyl-D-penicill-amine, and mercapto-succinic acid [6].

Similarly, fluorescent compounds like the cytotoxic drugs doxorubicin, paclitaxel and bleomycin [39] have intrinsic fluorescence that can interfere with the reading of the assay, resulting in a fluorescence increase unrelated to the reduction of resazurin. Therefore, to rule out the possibility that GK-1 could cause interference by either causing direct resazurin reduction or as a result of autofluorescence, culture media (without cells) with 16 μM to 500 μM GK-1 peptide, incubated like cell cultures and with resazurin added, were assessed. As a result, very low fluorescence intensity values were recorded (Fig. 4, bottom panel), with an average value of 192 ± 66 U for all the tested GK-1 peptide concentrations. Thus, the hypothesis of GK-1 peptide as an assay interfering substance was disregarded.

In summary, experimental evidence supports that GK-1 was not cytotoxic but caused a concentration-dependent increase of resazurin reduction. The lack of cytotoxicity of GK-1 agrees with the recognized innocuity of peptides as a chemical class [3–5]. Although fluorescence

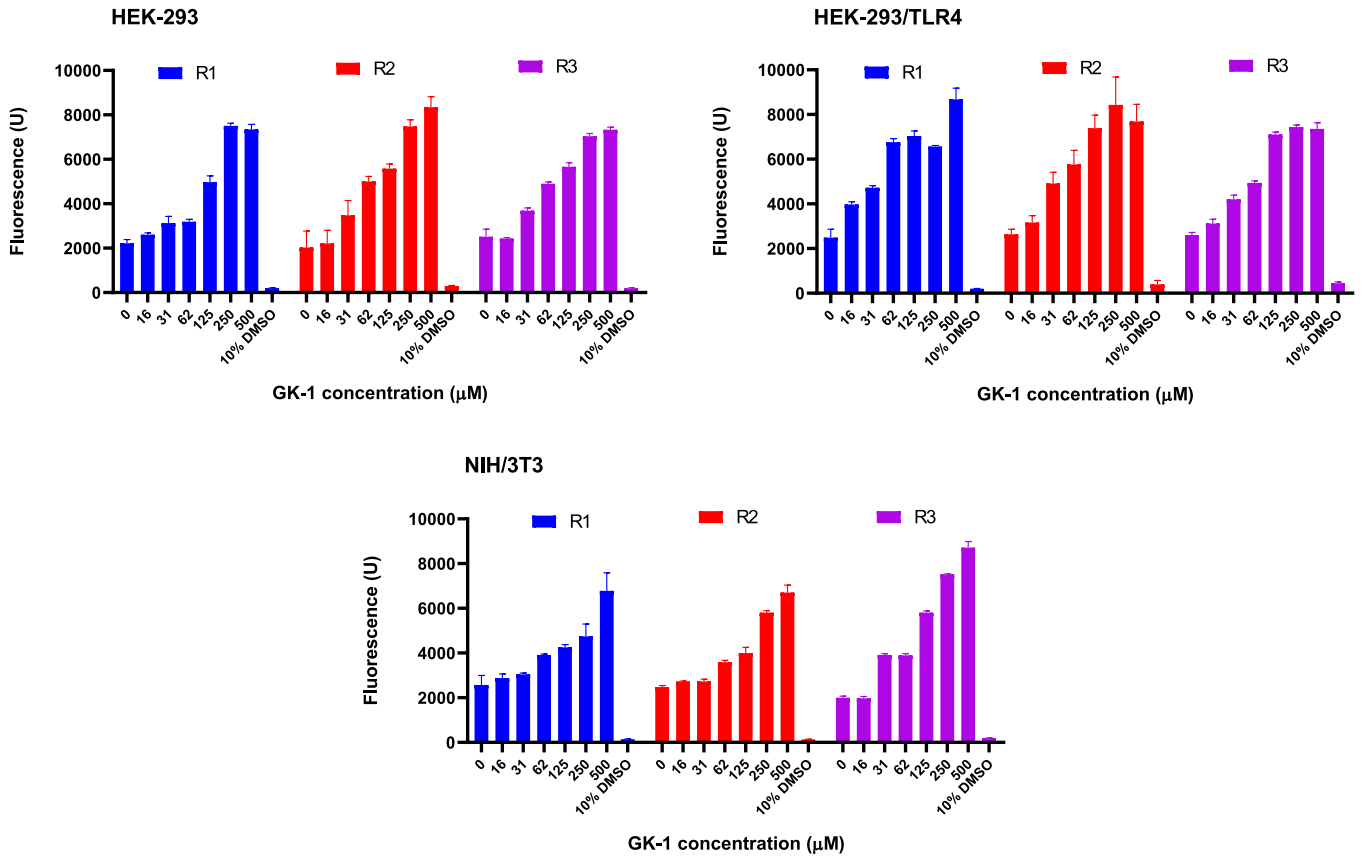

Fig. 2. *In vitro* cytotoxicity of GK-1 peptide in non-tumor cell lines.

increase was comparable in tumor, non-tumor and primary culture cells *in vitro*, the implications *in vivo* could be different, considering the distinctive microenvironment and predominant metabolic pathways in tumors and normal tissues [40,41]. These results warrant conducting metabolomic studies to elucidate cell changes induced by the exposure to GK-1 and their role in its mechanism of antitumor effect.

3.3. Single-dose toxicity

Although no longer considered mandatory as a stand-alone toxicity test [42], single-dose toxicity studies are not only essential to predict the toxicological risk associated with accidental overexposure to the test compound, but also to identify target organs of toxicity and for the proper design of repeated-dose toxicity studies.

Female BALB/c mice implanted with the 4T1 tumor cell line are a widely used murine model of triple negative breast cancer. 4T1 tumor cells were isolated from a breast tumor spontaneously developed in BALB/c mice and is specific for this mouse strain [43]. This is the primary animal model that has been used for the preclinical development of GK-1 as a candidate drug for human triple negative breast cancer [21,23,24]. For this reason, using BALB/c mice to further study the preclinical safety profile of GK-1 is more relevant than using other rodent species (such as rats) or non-isogenic mouse strains (such as OF1 or CD1) that are more frequently used in toxicological studies.

Likewise, female animals were used, firstly, since they are required for the 4T1 breast cancer model, and secondly, because females are recommended for acute toxicity studies [32] considering that conventional tests of mean lethal dose show that, although there could be little difference in sensitivity between the sexes, in those cases where differences are observed, females are generally more sensitive [44].

Since there was no previous information on the toxicity of GK-1 at doses over 12.5 mg/kg [27] a starting dose of 300 mg/kg was chosen. Subsequently, another group of mice were dosed with 300 mg/kg and two groups with 1000 mg/kg, following the dosing schedule recommended in Annex 2c of the Acute Toxic Class Method [32]. Although the recommended dose scalation after 300 mg/kg is 2000 mg/kg, only 1000 mg/kg were used due to solubility issues and because the selected dose offers a sufficient safety margin considering the pharmacologically active dose in mice that is near 5 mg/kg [22,24]. Moreover, a more recent ICH guideline on non-clinical safety studies considers appropriate a limit dose of 1000 mg/kg for rodents and non-rodents [42].

No deaths occurred and no animals showed signs of local or systemic toxicity during the 14 days following GK-1 subcutaneous administration at any of the dose levels tested. Once the dosing of six mice at 1000 mg/kg was completed without any deaths, it was concluded that the LD50 of GK-1 by subcutaneous route in female BALB/c mice was over 1000 mg/kg, which would classify GK-1, at least, as Globally Harmonized System of Classification and Labelling of Chemicals (GHS) Category 4 (>300 – 2000 mg/kg) [32].

The body weight of mice varied from 18.9 g to 21.6 g at the moment of administration. For all mice it was in the range of −8.3–7.6 % of the average weight of previously dosed animals, meeting the requirement of ± 20 % [32]. The body weight of mice treated with 300 mg/kg and 1000 mg/kg (Fig. 5**A**) was similar ($p > 0.1$, repeated measurements ANOVA) throughout the observation period. If there were a toxic effect of the product, a lower weight gain would be expected in mice treated with the highest dose. Moreover, all mice gained weight over the 14 days of observation. Although the increase was discrete, it corresponded to the body weight increase normally observed in healthy female BALB/c mice of this age [45,46].

The consumption of water and food did not show differences ($p > 0.05$) between mice treated with the two dose levels nor with respect to mice that were waiting to be treated, referred as “Control” in Fig. 5B.

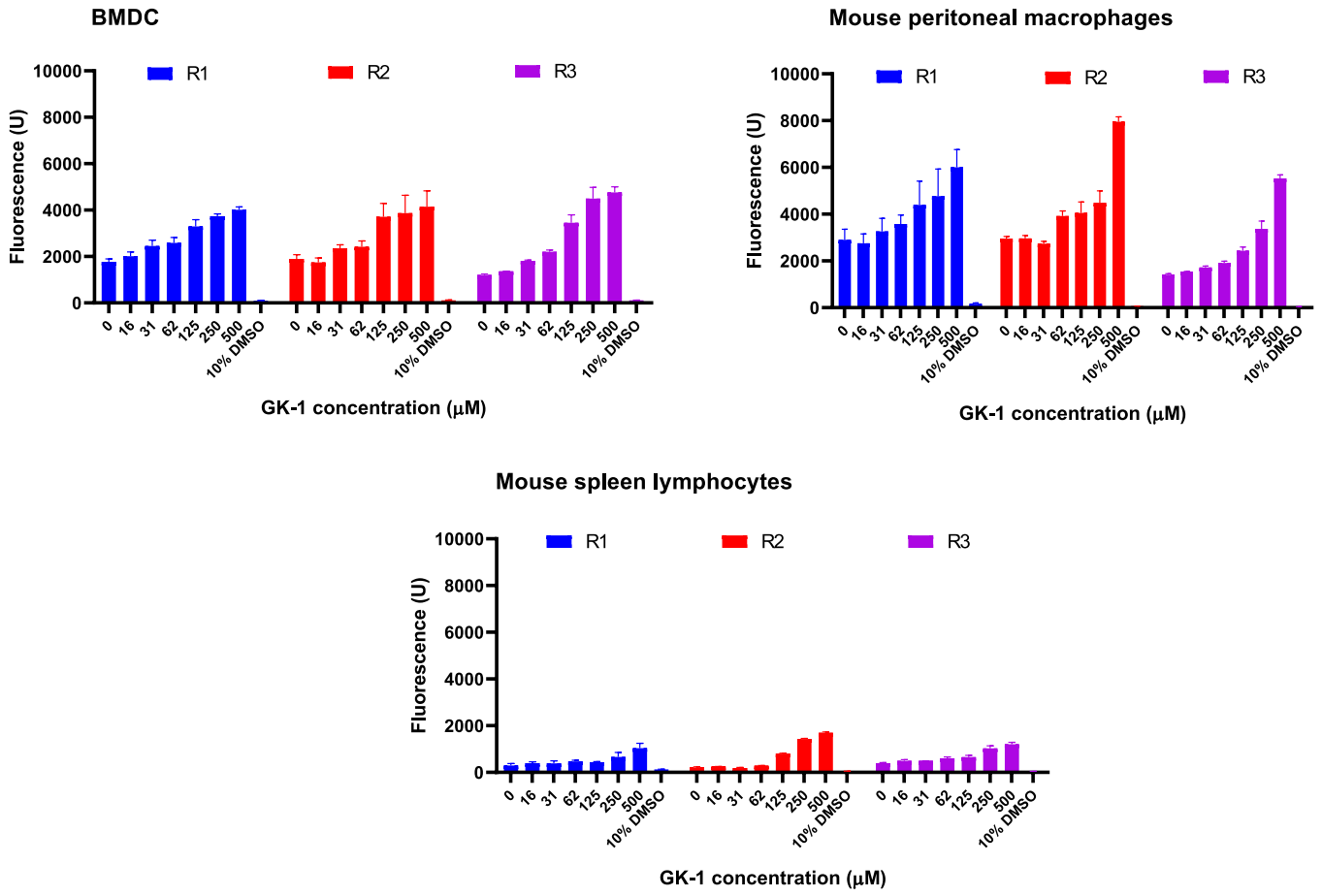

Fig. 3. *In vitro* cytotoxicity of GK-1 in primary cell cultures. BMDC: Bone-Marrow derived Dendritic Cells.

The anatomopathological study conducted two weeks after the administration of GK-1 did not show macroscopically visible changes in any of the treated animals. Therefore, no further histological studies were regulatorily required. In summary, the single-dose toxicity study classified GK-1 peptide, at least, as GHS Category 4 (>300 – 2000 mg/kg), no signs of local or systemic toxicity were found, and no target organs of toxicity were identified.

3.4. Repeated-dose toxicity

The same animal model used for the single-dose toxicity test was used for the repeated-dose toxicity test for the same reasons. Doses of 100 mg/kg - 300 mg/kg were administered to mice considering that an appropriate safety margin (60 times) would be covered. Antitumor and antimetastatic activity in murine models of breast cancer and melanoma has been demonstrated after subcutaneous administration at week intervals [22,47,48]. A similar schedule is expected to be used in clinical trials of GK-1 peptide as an anticancer drug. On that basis, four doses at week intervals were administered to mice in the present repeated-dose toxicity study since that schedule is considered appropriate to support clinical trials of anticancer pharmaceuticals planned to be administered weekly [35].

After four weekly doses of GK-1 from 100 mg/kg to 300 mg/kg, no deaths occurred, and no animals showed signs of either local or systemic toxicity during the dosing period and the 10 days of observation after the last dose.

Body weight curves (Fig. 6**A**), as well as the differences between the body weight at the beginning and end of the experiment (Fig. 6**B**) showed a similar ($p > 0.05$) behavior in the different experimental groups. Moreover, body weights corresponded to those normally observed in healthy female BALB/c mice of this age [45,46].

Water and food consumption also did not show differences ($p > 0.05$) among mice belonging to the different experimental groups (Fig. 7).

The anatomopathological study of mice 10 days after the administration of the last dose of GK-1 peptide did not show macroscopically visible changes in any of the animals in the experiment. Likewise, the relative weights of the organs did not show statistically significant differences ($p > 0.1$) among the groups (Fig. 8).

Histological studies identified focal renal fibrosis with fibrinoid degeneration

and vascular thrombosis in a control mouse, mild zonal epicardial mineralization in a DMSO-treated mouse, and focal mild hepatic fibrosis with bile duct hyperplasia in a mouse treated with 200 mg/kg GK-1 peptide.

Cardiac calcinosis is a spontaneous pathological condition of undetermined etiology described in BALB/c mouse sublines (BALB/cB and BALB/cJ), located mainly in the right ventricular epicardium, as in the case observed [49]. The rest of the changes found in the liver and kidney were mild, nonspecific and not related to the dose.

Hematology and blood biochemistry analysis carried out on blood samples taken 10 days after the fourth dose of GK-1 was administered did not show significant changes from a biological or statistical point of view (Table 2). Only a decrease in serum urea levels was observed in mice treated with the GK-1 at 200 mg/kg compared to controls ($p < 0.05$), but this finding lacks biological relevance, since in cases of nephrotoxicity would be expected not a decrease, but an increase in serum urea concentrations. Furthermore, no relationship was observed between serum urea levels and the dose of peptide administered.

Considering that no deaths or changes occurred in any of the

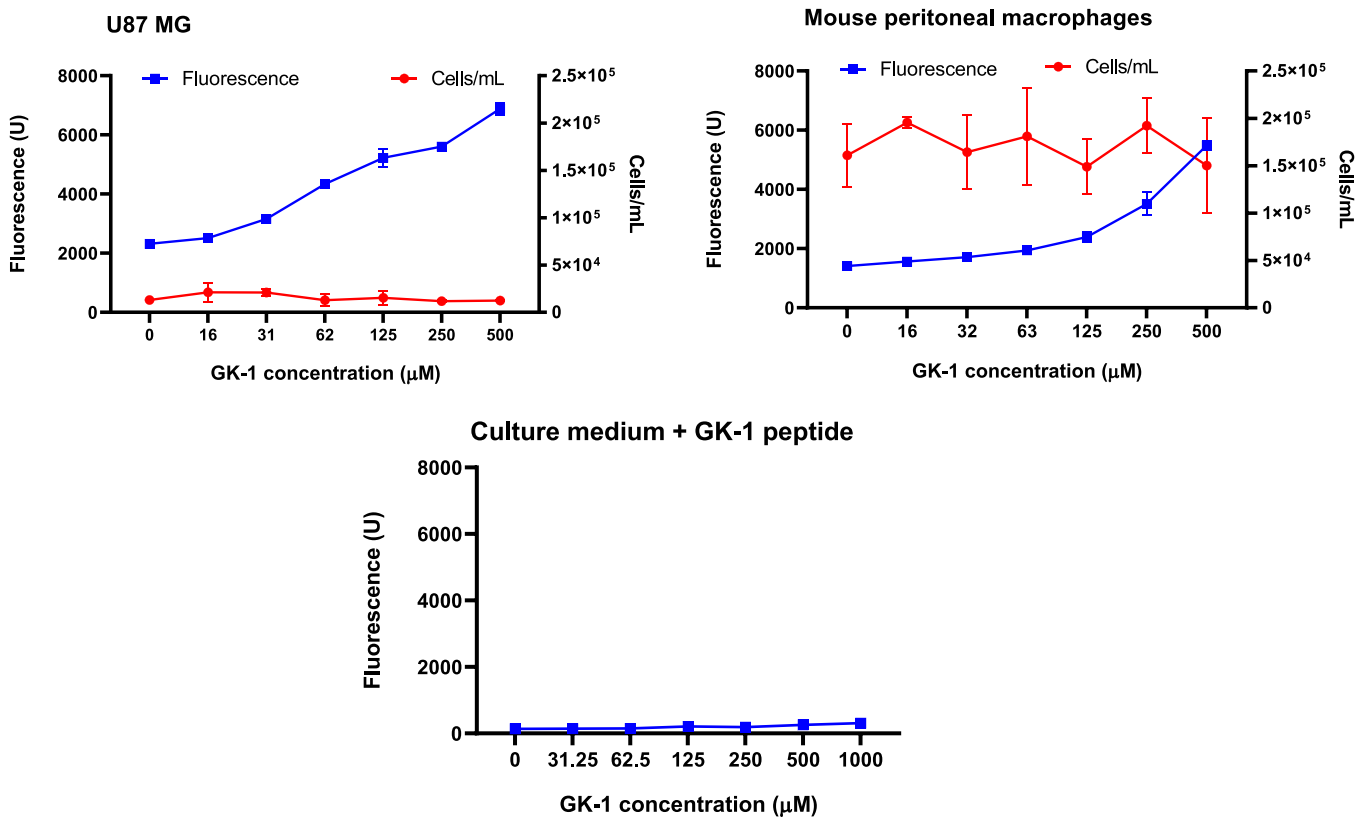

Fig. 4. Cell number and fluorescence intensity in a replicating tumor cell line (U87 MG) and in a non-replicating differentiated primary cell culture (mouse peritoneal macrophages) exposed to GK-1.

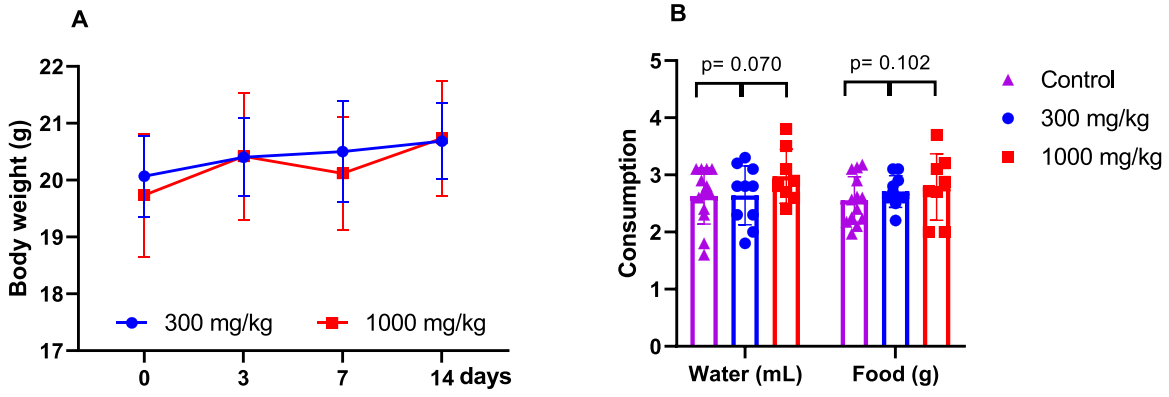

Fig. 5. Body weight (Panel A) and water and food consumption (Panel B) of BALB/c mice treated with GK-1 by the subcutaneous route.

biological variables measured, it was concluded that the no-observed-adverse-effect level (NOAEL) of exposure for GK-1 after four weekly subcutaneous doses in female BALB/c mice was over 300 mg/kg (maximum tested dose). Furthermore, no target organs of the GK-1 toxicity could be identified, since no clinical signs, pathological changes or modifications in hematology and blood biochemistry of the animals under study were observed.

In summary, GK-1 is an 18 amino acid peptide which was formerly tested as a vaccine antigen [19,50] and adjuvant [51] that is being eventually developed as an anticancer drug. It has proved efficacy in murine models of melanoma [48,52,53] and triple-negative breast cancer [21,23,24]. In the latter, GK-1 reduces tumor growth and the number of lung metastasis, primarily by inhibiting tumor angiogenesis and T-cell exhaustion [24]. Preclinical studies demonstrated that it is safe in rats after repeated intravenous or subcutaneous administration at doses up to 12.5 mg/kg (maximum tested dose), that it is non-mutagenic, as evidenced in three assay systems (Bacterial reverse mutation test, Mammalian erythrocyte micronucleus test and *In vitro* mammalian chromosomal aberration test), non-sensitizing in Guinea pigs (Buehler sensitization test) and non-pyrogenic in rabbits [27]. Moreover, in the present paper, GK-1 showed no cytotoxicity in a variety of cell cultures even at concentrations as high as 500 μM. In the single dose toxicity test it produced no signs of toxicity at the maximum tested dose of 1000 mg/kg and in the repeated-dose toxicity test it was also safe at doses between 100 mg/kg and 300 mg/kg. In conclusion, GK-1 is a promising preclinically safe peptide with antitumor and antimetastatic properties suitable to advance to clinical trials as a new potential drug to be used in cancer immunotherapy.

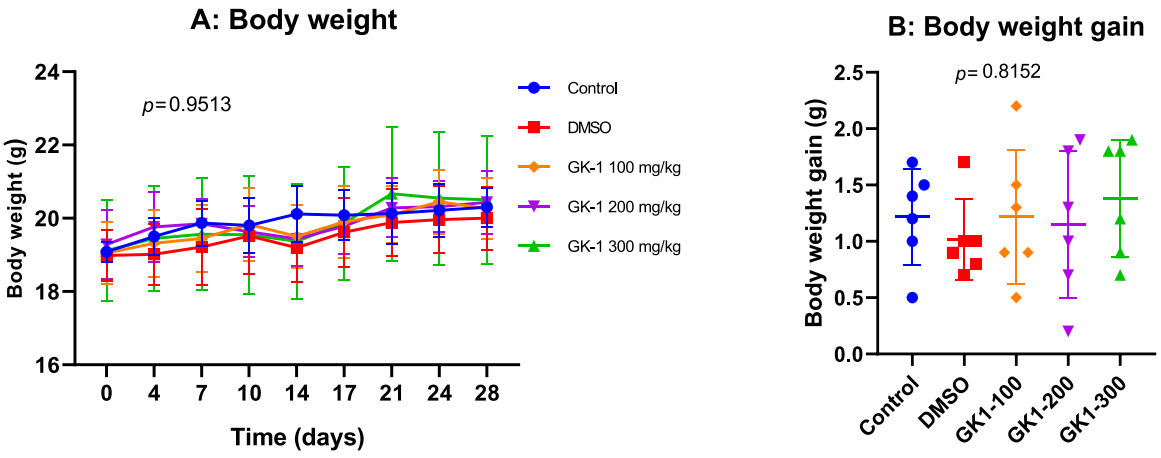

Fig. 6. Body weight of mice treated with repeated doses GK-1 peptide.

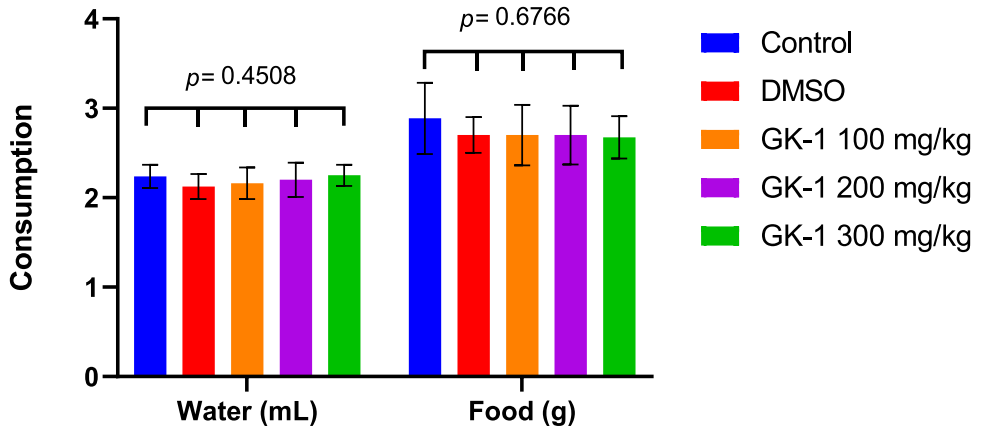

Fig. 7. Average daily water and food consumption after repeated doses of GK-1 peptide.

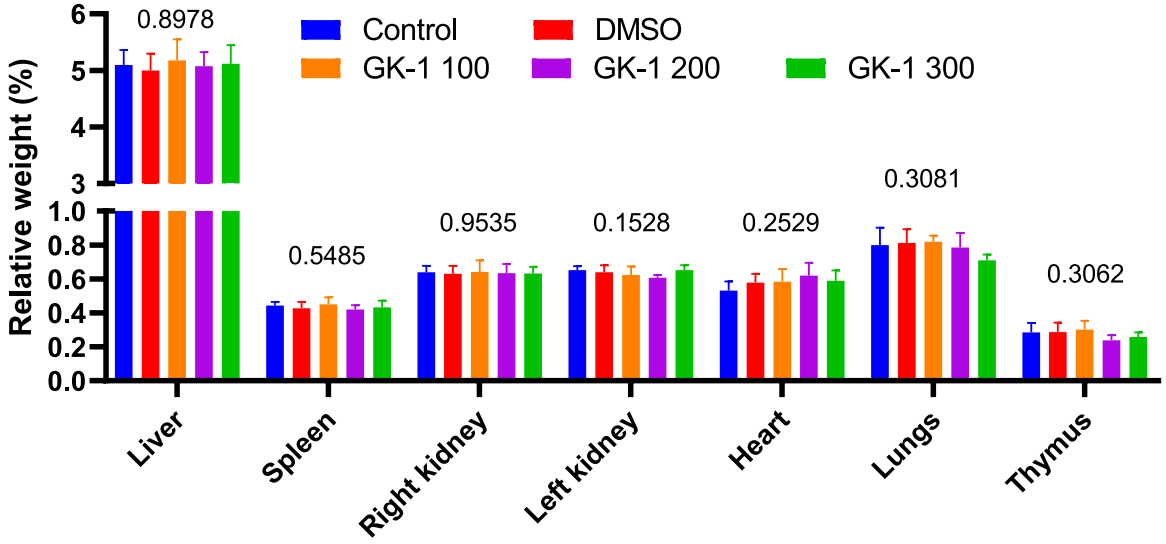

Fig. 8. Relative organ weights of mice treated with repeated doses of GK-1.

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Table 2
Hematology and blood biochemistry analysis of mice treated with repeated doses of GK-1 peptide.

	Control	DMSO	100 mg/kg	200 mg/kg	300 mg/kg	p
Hematocrit (L/L)	0.38 ± 0.02†	0.37 ± 0.02	0.37 ± 0.02	0.38 ± 0.03	0.37 ± 0.03	0.8370
Hb (g/L)	147 ± 6	139 ± 12	144 ± 12	147 ± 5	146 ± 8	0.6828
Erythrocytes ($\times 10^{12}$/L)	9.9 ± 0.4	9.3 ± 1	9.5 ± 0.8	9.9 ± 0.3	9.8 ± 0.7	0.7049
MGV (fL)	39 [38,39] ††	38 [37–41]	39 [37–40]	39 [38–40]	38 [37–39]	0.9722
MGHC (g/L)	386 [376−392]	386 [358−387]	388 [382−399]	379 [374−392]	389 [375−403]	0.7480
Platelets ($\times 10^{9}$/L)	605 [467−742]	508 [388−696]	716 [540−892]	980 [956–1004]	692 [508−876]	0.4416
Total solids (g/L)	54 [50–56]	52 [52]	52 [50–54]	54 [53,54]	54 [52–54]	0.6387
Leukocytes ($\times 10^{9}$/L)	4.4 ± 1.1	4.2 ± 0.8	4.9 ± 1.4	4.3 ± 0.9	4.4 ± 1.8	0.9396
PMNN ($\times 10^{9}$/L)	0.97 ± 0.48	0.98 ± 0.48	0.78 ± 0.31	0.87 ± 0.38	1.22 ± 0.57	0.6894
Lymphocytes ($\times 10^{9}$/L)	3.3 ± 0.7	3.1 ± 0.5	4 ± 1.3	3.3 ± 0.7	3 ± 1.2	0.9396
Monocytes ($\times 10^{9}$/L)	0.1 [0.1–0.1]	0.1 [0.0–0.1]	0.1 [0.08–0.13]	0.1 [0.08–0.1]	0.1 [0.0–0.1]	0.5177
PMNE ($\times 10^{9}$/L)	0.03 [0.0–0.1]	0.0 [0.0 – 0.0]	0.05 [0.0–0.1]	0.05 [0.03–0.08]	0.1 [0.0–0.1]	0.2313
PMNB ($\times 10^{9}$/L)	0 [0−0]	0 [0−0]	0 [0−0]	0 [0−0]	0 [0−0]	1.0000
Glucose (mmol/L)	11.5 ± 1.3	9.6 ± 3.7	12 ± 1.8	13.4 ± 1.8	12.9 ± 1.8	0.1429
Urea (mmol/L)	17 ± 2	17 ± 2	17 ± 2	14 ± 1 *	16 ± 2	0.0383
Creatinine (μmol/L)	36 [33–38]	35 [33–35]	31 [30–32]	35 [34–40]	36 [35–38]	0.2400
Cholesterol (mmol/L)	3.6 ± 0.5	3 ± 0.3	3.2 ± 1.1	3 ± 0.1	3.1 ± 0.2	0.4368
ALT (U/L)	51 [48–54]	67 [55–73]	87 [72−135]	71 [53−295]	74 [71−101]	0.4077
AST (U/L)	163 [125−227]	136 [130−174]	364 [204−404]	205 [159−402]	175 [163−191]	0.6363
ALP (U/L)	137 [134−143]	140 [135−160]	141 [125−144]	124 [109−133]	133 [113−144]	0.9680
CK (U/L)	750 [518–1812]	1288 [758–1976]	1276 [507–3798]	1200 [801–2519]	1022 [948–1378]	0.9680
Total proteins (g/L)	61 ± 10	54 ± 3	55 ± 8	53 ± 2	54 ± 5	0.2978
Triglycerides (mmol/L)	2.4 [2.1–2.5]	2 [1.8–2]	1.8 [1.6–2]	1.9 [1.8–2.1]	2.4 [2.1–2.5]	0.0613

† Results indicated as value ± value correspond to the mean ± standard deviation. These are data that followed a normal distribution and had homogeneous variances, so they were compared using one-way ANOVA. On the other hand, those data that are represented as value [value - value] ††, represent the median [quartile 1- quartile 3] and report data that did not meet the assumptions of normality and/or homogeneity of variance (even after their transformation) and that were compared using the Kruskal-Wallis test. * : Statistically significant differences compared to the control group ($p < 0.05$) according to ANOVA and Dunnett test. Hemoglobin (Hb), erythrocytes, mean globular volume (MGV), mean globular hemoglobin concentration (MGHC), leukocytes, neutrophils (PMNN), lymphocytes, monocytes, eosinophils (PMNE) and basophils (PMNB), alanine aminotransferase (ALT), aspartate aminotransferase (ASP), alkaline phosphatase (ALP), creatine kinase (CK).

Author statement

1) We have contributed substantially to this research and have approved the final version for submission to Toxicology Reports.
2) This study was conducted in compliance with ethical standards in animal use for biomedical research.
3) We have no conflicts of interest related to this research that could affect the results presented in the manuscript nor their interpretation.

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CRediT authorship contribution statement

Villalobos Nelly: Writing – review & editing, Methodology, Investigation. **Sciutto Edda:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Rocha Diego Moctezuma:** Writing – review & editing, Methodology, Investigation. **Pérez-Osorio Iván Nicolás:** Writing – review & editing, Methodology, Investigation. **Hernández-Aceves Juan Alberto:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Salas-Garrido Carlos Gerardo:** Writing – review & editing, Methodology, Investigation. **Sifontes Rodríguez Sergio:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fragoso Gladis:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gladis Fragoso reports financial support was provided by Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT). Gladis Fragoso and Edda Sciutto have patent #WO 2017/200369 A2 issued to Universidad Nacional Autónoma de México. Juan Alberto Hernández Aceves is a PhD student supported by CONAHCYT. The rest of the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data will be made available on request.

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