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RESEARCH ARTICLE

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In vitro and *in vivo* characterization of an interleukin-15 antagonist peptide by metabolic stability, ^{99m}Tc-labeling, and biological activity assays

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Interleukin (IL)−15 is an inflammatory cytokine that constitutes a validated therapeutic target in some immunopathologies, including rheumatoid arthritis (RA). Previously, we identified an IL-15 antagonist peptide named [K6T]P8, with potential therapeutic application in RA. In the current work, the metabolic stability of this peptide in synovial fluids from RA patients was studied. Moreover, [K6T]P8 peptide was labeled with ^{99m}Tc to investigate its stability in human plasma and its biodistribution pattern in healthy rats. The biological activity of [K6T]P8 peptide and its dimer was evaluated in CTLL-2 cells, using 3 different additives to improve the solubility of these peptides. The half-life of [K6T]P8 in human synovial fluid was 5.88 ± 1.73 minutes, and the major chemical modifications included peptide dimerization, cysteinylation, and methionine oxidation. Radiolabeling of [K6T]P8 with ^{99m}Tc showed a yield of approximately 99.8%. The ^{99m}Tc-labeled peptide was stable in a 30-fold molar excess of cysteine and in human plasma, displaying a low affinity to plasma proteins. Preliminary biodistribution studies in healthy Wistar rats suggested a slow elimination of the peptide through the renal and hepatic pathways. Although citric acid, sucrose, and Tween 80 enhanced the solubility of [K6T]P8 peptide and its dimer, only the sucrose did not interfere with the *in vitro* proliferation assay used to assess their biological activity. The results here presented, reinforce nonclinical characterization of the [K6T]P8 peptide, a potential agent for the treatment of RA and other diseases associated with IL-15 overexpression.

KEYWORDS

^{99m}Tc, biological activity, interleukin-15, peptide, stability, sucrose

1 | INTRODUCTION

Interleukin (IL)−15 is a pleiotropic cytokine that exerts its effects through binding to a heterotrimeric receptor, composed by a private α chain (IL-15Rα), the IL-2Rβ, and the common γ chains.[1-4] Interleukin-15 binds to IL-15Rα forming a high-affinity complex that can present IL-15 in *trans* to neighboring cells expressing the IL-15Rβγ$_c$ receptor,[5] although the cis-presentation is also possible in a target cell expressing the trimeric receptor.[6]

Abbreviations: ^{99m}Tc, technetium-99m; Fmoc/tBu, 9-fluorenylmethyloxycarbonyl/tertbutyl; Fmoc-AM, *p*-[(R,S)-*a*-[1-(9*H*-fluoren-9-yl)methoxy-formamido]-2,4-dimethyl-benzyl]-phenoxyacetic acid; HSF, human synovial fluids; IC_{50}, half-maximal inhibitory concentration; IL, interleukin; IL-15R, interleukin-15 receptor; MBHA, 4-methylbenzhydrylamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; RP-HPLC, reversed-phase high-performance liquid chromatography; RPMI, Roswell Park Memorial Institute; TFA, trifluoroacetic acid; TNF-α, tumor necrosis factor alpha

Despite the constitutive expression of IL-15 mRNA in many cells and tissues, the protein expression is mainly regulated at the posttranscriptional level.[7,8] However, IL-15 overexpression has been associated with several autoimmune diseases like rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis, and ulcerative colitis.[9,10] Some therapeutic agents have been generated to treat these immunological disorders, for example, the soluble form of IL-15Rα, the mutant antagonist of IL-15, a humanized monoclonal antibody against the IL-2R/IL-15Rβ subunit, and a human IgG_1 anti–IL-15 monoclonal antibody.[11-15] Nevertheless, compared with monoclonal antibodies and recombinant proteins, the peptide-based therapy offers certain advantages, such as high biological activity, high specificity, small size, good tissue penetration, and low toxicity of the used peptides.[16] Furthermore, therapeutic peptides are typically associated with lower manufacturing complexity compared with protein-based biopharmaceuticals, and therefore, the Good Manufacturing Practice costs are also lower.[17]

Our previous studies focused on the identification of a peptide named P8 that comprises the region from amino acid 36 to 45 of the human IL-15 sequence (^{36}KVTAMKCFLL^{45}). We demonstrated that this peptide specifically recognized the IL-15Rα and displayed an antagonist effect on IL-15 activity in CTLL-2 cells.[18] Taking into account the high affinity of the interaction between the IL-15 and the IL-15Rα, and considering that the development of an IL-15 antagonist peptide with higher inhibitory effect allows the use of lower doses with less toxicity and adverse effects, the biological activity of P8 peptide was improved. Regarding to this, we identified a sequence termed [K6T]P8 peptide more active than P8 in the inhibition of IL-15–induced proliferation in CTLL-2 cells and TNF-α secretion by synovial cells from RA patients.[19] Despite the higher activity of this peptide, it exhibited lower solubility than P8, mostly at concentrations greater than 1 mg/mL. Then, the *in vitro* and *in vivo* characterization of the potential therapeutic [K6T]P8 peptide is mandatory for its use as a drug in humans. In the current work, we assessed the stability of [K6T]P8 peptide in human synovial fluids (HSF) from RA patients, and the major chemical modifications were identified. Also, this peptide was labeled with technetium-99m (^{99m}Tc) to evaluate its stability and affinity by *in vitro* tests and *in vivo* preliminary biodistribution studies in healthy rats. To solve the solubility issues presented by [K6T]P8 peptide and its dimer, the influence of some additives to Roswell Park Memorial Institute (RPMI) medium was studied and the biological activity of the mentioned peptides was evaluated by means of the CTLL-2 cells proliferation assay. The *in vitro* and *in vivo* studies shown in this work contributed to the nonclinical characterization of the [K6T]P8 peptide, as a new type of IL-15 antagonist for the treatment of diseases related to IL-15 overexpression.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

All reagents for peptide synthesis were of synthesis grade. Reagents for chromatography were of high-performance liquid chromatography (HPLC) grade. The RPMI medium 1640 was obtained from Thermo Fisher Scientific (USA). Sucrose, Tween 80, trifluoroacetic acid (TFA), trichloroacetic acid, acetonitrile, stannous fluoride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and methyl ethyl ketone were purchased from Sigma-Aldrich (USA). Recombinant human IL-15 was obtained from R&D Systems (USA). Phosphate-buffered saline (PBS) was obtained from Invitrogen (USA). Citric acid and NaCl were acquired from Merck (USA).

2.2 | Peptide synthesis, dimerization, and characterization

The peptides were synthesized on Fmoc-AM–MBHA resin by a solid phase method using the Fmoc/tBu chemistry.[20] The [K6T]P8 peptide was dimerized as previously described by Andreu et al.[21] All peptides were purified by reversed-phase (RP)–HPLC and identified by mass spectrometry, following the procedures described by Santos et al.[19] The obtained peptides, with more than 97% of purity, have the following sequences:

- [K6T]P8: KVTAMTCFLL-NH_2 and
- [K6T]P8 homodimer (C^7-C^7): KVTAMTCFLL-NH_2.

2.3 | Peptide stability assay in HSF

To obtain the HSF from knees of patients with RA, the written informed consent was acquired, and the study was conducted with the approval of the Ameijeiras Hospital Ethics Committee. After extracting the synovial fluid, it was incubated with hyaluronidase (10 μg/mL of fluid) for 60 minutes at 37°C. Synovial fluid cells were discarded by centrifugation at 1500 rpm (239 rcf) for 10 minutes, and the supernatant (HSF) was collected for peptide stability assays.

Peptide stability to proteolysis was assessed in HSF by triplicate. Briefly, a preheated HSF (37°C) was spiked with [K6T]P8 peptide to a final concentration of 100 μM. Degradation was studied up to 2 hours, sampling 100 μL of the reaction pool at t = 0, 5, 8, 10, 20, 30, 45, 60, 90, and 120 minutes. Human synovial fluids major proteins were precipitated treating each sample with 25 μL of acetonitrile and 25 μL of 200 g/L trichloroacetic acid solution. Samples were vortex mixed for 1 minute and centrifuged at 10 000 rpm (9300 rcf) for 5 minutes. Supernatant was separated and stored at −20°C, until its analysis by RP-HPLC UV (λ = 226 nm) on a WellChrom HPLC (KNAUER, Germany) using the EZChrom-Elite chromatography software ChromGate v3.1 from Agilent (USA). The [K6T]P8 recovery following this procedure was around 61.3%. The RP-HPLC mobile phase composition was (*a*) TFA/H_2O 0.1% and (*b*) TFA/acetonitrile 0.05%. An analytical reversed-phase C_{18} column (4.6 × 250 mm, 5 μm) (ZORBAX, USA) was used with a linear gradient 5% B to 95% B in 60 minutes. Intact peptide was estimated by chromatographic peak integration (retention time = 17.4 min), and degradation half-life was calculated as previously reported.[22] Main cleavage products generated from degradation were characterized by electrospray ionization–mass spectrometry in positive ion mode with a Z-spray ion source, using a hybrid quadrupole time-of-flight instrument (Waters,

Milford, Massachusetts). Mass spectra were acquired in the m/z range from 400 to 2000 Th.

2.4 | ^{99m}Tc-labeling of [K6T]P8 peptide

A 2.5-mg quantity of [K6T]P8 peptide was dissolved in 25 mL of 0.9% NaCl, previously saturated with N_2 (g) (to a final peptide concentration of 100 μg/mL). Two hundred microliters of the obtained solution (20 μg of [K6T]P8) was fractionated into vacuum vials and stored at −20°C. Labeling procedure was performed as follows: 500 μL of 0.1 M PBS (0.1 M NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4; pH 9) and 15 μg of stannous fluoride in 100 μL of 0.3 mM HCl were added to the 200 μL (20 μg) of the peptide stock solution. Then, 740 to 1110 MBq (20-30 mCi) of ^{99m}Tc from a freshly eluted ^{99}Mo/^{99m}Tc generator (GENTEC, Isotopes Center, Cuba) was added, and the mixture was incubated for 20 minutes at 25°C as previously described by Perera et al.[23]

2.5 | Quality control of the label by paper chromatography

Quality control of ^{99m}Tc-[K6T]P8 was carried out by paper chromatography using 0.5 cm × 8.0 cm strips of Whatman 3 MM as stationary phase and methyl ethyl ketone (R_f = 0.8-1.0 for $^{99m}TcO_4^-$) and 50% acetonitrile (R_f = 0.0 radiocolloids) as mobile phases. Counting rate was measured on an SR8 ratemeter (Nuclear Enterprise, UK). Labeling yield was calculated as follows:

$$\%^{99m}\text{Tc}-[\text{K6T}]\text{P8} = 100\% - \%\text{radiocolloids} - \%\,^{99m}TcO_4^-.$$

2.6 | Stability of ^{99m}Tc-[K6T]P8 in aqueous solution and human plasma

The stability study of the ^{99m}Tc-labeled peptide in aqueous solution and human plasma was performed as previously described.[23] For the stability assessment in aqueous solution, the samples were taken at 1, 3, and 24 hours and analyzed by RP-HPLC on a SHIMADZU chromatograph (Japan) coupled to in-line radiometric and UV detection set at λ = 205 nm. Radiochromatographic analysis was performed on a C_{18} RP column (3.9 mm × 150 mm, 5 μm; Delta Pak C-18-300A, Waters, USA), at a flow rate of 1 mL/min as described by Perera et al.[23] The solvent system was composed by (*a*) 0.1% TFA in water and (b) 0.1% TFA in acetonitrile. A separation gradient was used 0% B to 80% B in 20 minutes.

2.7 | Plasma protein binding assay

The assay was carried out as previously described by Perera et al.[23] Samples were analyzed by paper chromatography using 0.5 cm × 8.0 cm strips of Whatman 3 MM and 50% acetonitrile in water as a mobile phase. The percentage of peptide bound to plasma proteins was estimated from the percentage of the activity in the start of the strip.

2.8 | Scintigraphic study

Animal experiment was carried out according to the guidelines of the American Association for Accreditation of Laboratory Animal Care and was approved by the Ethical Committee for the Use of Experimental Animals at the Isotopes Center (CENTIS, Mayabeque, Cuba). Six healthy Wistar rats (3 females and 3 males), 8 weeks old, weighing between 190 and 210 g were included in the study. A dose of 740 MBq (20 mCi) of ^{99m}Tc-[K6T]P8, previously dissolved in 0.9% NaCl, was subcutaneously administered (0.2 mL) in the upper part of the left thigh. Planar scintigraphic images were acquired at 0, 1, 3, and 21 hours postinjection with a Diacam gamma camera (Siemens, Germany), using a pinhole collimator and a 256 × 256 matrix.

2.9 | Peptide solubility test

Solubility was evaluated by RP-HPLC UV (226 nm) in a Chromolith SpeedROD C_{18} column coupled to a SHIMADZU Prominence liquid chromatograph (SHIMADZU, Japan). The [K6T]P8 peptide and its dimer were added, at a final concentration of 2 mg/mL, to the RPMI medium with or without additives (20 mM citric acid, 10 mg/mL sucrose, and 0.1% Tween 80). Each sample was vortex mixed and centrifuged for 1 minute at 10 000 rpm (9300 rcf). Twenty microliters of this solution was mixed with water (80 μL), and 50 μL was injected into the chromatograph. Separation took place under a linear gradient 0% B to 100% B in 30 minutes, at a flow rate of 3.5 mL/min. In this case, mobile phase had the same composition as mentioned above in the peptide stability assay in HSF. The solubility, measured in terms of peak area from the unmodified peptides completely dissolved, was assessed by triplicate for RPMI with or without additives.

2.10 | Effect of 3 different additives on the activity of [K6T]P8 peptide and its dimer, using a CTLL-2 cell proliferation assay

The CTLL-2 cells were obtained from the American Type Culture Collection (TIB-214) and were grown following the manufacturer's specifications. To evaluate the biological activity of [K6T]P8 peptide and its dimer in the presence of 3 different additives, the CTLL-2 cell proliferation assay previously described by Santos et al[19] was carried out. Serial dilutions of the peptides (resuspended in RPMI medium with 20mM citric acid, 10 mg/mL sucrose, or 0.1% Tween 80) were incubated with 300 pg/mL of human IL-15 alone or with the additives mentioned above. Cell viability was measured using the MTT assay.[24]

3 | RESULTS

3.1 | Stability of [K6T]P8 peptide in HSF

In order to isolate potential metabolites of the [K6T]P8 peptide in HSF, the degradation reaction at 37°C was prolonged from t = 0 to t = 120 minutes. The [K6T]P8 peptide fraction was undetectable by RP-HPLC UV (λ = 226 nm) from 20 minutes of incubation time, exhibiting an average half-life of 5.88 ± 1.73 minutes with a coefficient of variation of 29.45% among 3 replicates. Main transformations of the peptide were peptide dimerization (through free cysteine residue by disulfide bond formation), cysteinylation (by a disulfide bond with free L-cysteine), and methionine oxidation to methionine sulfoxide or methionine sulfone (Table 1).

TABLE 1 Mass spectrometric characterization of main fractions collected from RP-HPLC UV (λ = 226 nm) chromatographic separation of the 3-min sample

Fraction	Rt, min	Monoisotopic Mass, Da		MS Identification
		Experimental	Theoretical	
1	16.05	1243.76	1243.61	Cysteinilation[a] (+119 Da)
2	17.46	1124.72	1124.61	[K6T]P8
		2279.46	2279.20	[K6T]P8 dimer/oxidized methionine[b] (+32 Da)
		1156.72	1156.61	[K6T]P8/oxidized methionine[c] (+32 Da)

MS, mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; Rt, RP-HPLC retention time (min).

[a]Cysteinylation.

[b]Sulfoxide methionine oxidation.

[c]Sulfone methionine oxidation.

3.2 | ^{99m}Tc-labeling of [K6T]P8 peptide

Labeling efficiency of the ^{99m}Tc-[K6T]P8 peptide was 99.79 ± 0.18% (0.03 ± 0.04% pertechnetate and 0.18 ± 0.16% radiocolloids).

3.3 | Stability of ^{99m}Tc-[K6T]P8 in aqueous solution and human plasma

The stability of ^{99m}Tc-[K6T]P8 in PBS and 30-fold molar excess of L-cys until 24 hours is shown in Figure 1. Without cysteine, the ^{99m}Tc-[K6T]P8 was stable with more than 90% of the activity remaining bound to the peptide at 24 hours. However, at 1 hour of incubation with 30-fold molar excess of L-cysteine, the activity of ^{99m}Tc-[K6T]P8 decreased to 83.22 ± 0.74%. Afterwards, 78.0 ± 1.0% of the activity remained bound to the peptide up to 24 hours.

Figure 2 displays the RP-HPLC chromatographic profile of ^{99m}Tc-[K6T]P8 incubated with human plasma during 0, 1, and 3 hours. No variation in the retention time of main peaks was observed, suggesting that [K6T]P8 peptide was stable to proteolytic degradation by the action of the peptidases during the studied time interval. The plasma protein binding of ^{99m}Tc-[K6T]P8 was 0.3 ± 0.5%.

3.4 | Scintigraphic study of ^{99m}Tc-[K6T]P8 in healthy Wistar rats

A similar distribution pattern of the labeled peptide was observed in rats regardless of sex. Figure 3 shows a set of images acquired from a healthy male Wistar rat. Up to 3 hours, the ^{99m}Tc-[K6T]P8 was located at the injection site and a kidneys uptake was observed. Then, activity in the intestinal tract and bladder is seemed at 21 hours. No uptake was detected either in stomach or in thyroid.

3.5 | Solubility of [K6T]P8 peptide and its dimer in RPMI medium with 3 different additives

In order to improve the solubility of [K6T]P8 peptide, we added citric acid, sucrose, or Tween 80 to RPMI medium. In this study, the [K6T]P8 dimer was also included, which was previously obtained in a completely dimeric form by oxidation of the thiol group with 20% DMSO.[19] Up to a concentration of 2 mg/mL, the solubility of both peptides was increased compared with RPMI medium without additives, where an extensive turbidity was observed. Table 2 summarizes the solubility test results, obtained from RP-HPLC analysis, in terms of the peak area corresponding to the amount of each peptide completely dissolved in RPMI medium with or without additives. The RP-HPLC chromatograms are presented in the Supporting Information.

3.6 | Influence of 3 different additives on the biological activity of [K6T]P8 and its dimer in CTLL-2 cells

Once citric acid, sucrose, and Tween 80 were identified as additives that improved the solubility of the [K6T]P8 and its dimer, the biological activity of these peptides was evaluated using a CLL-2 cells proliferation assay.[19,25] The maximum proliferation control (cells plus IL-15 at 300 pg/mL) with Tween 80 displayed lower absorbance values than the control without additive, being very close to the minimum of the assay (cells cultured in cytokine-free medium) as shown in Figure 4A.

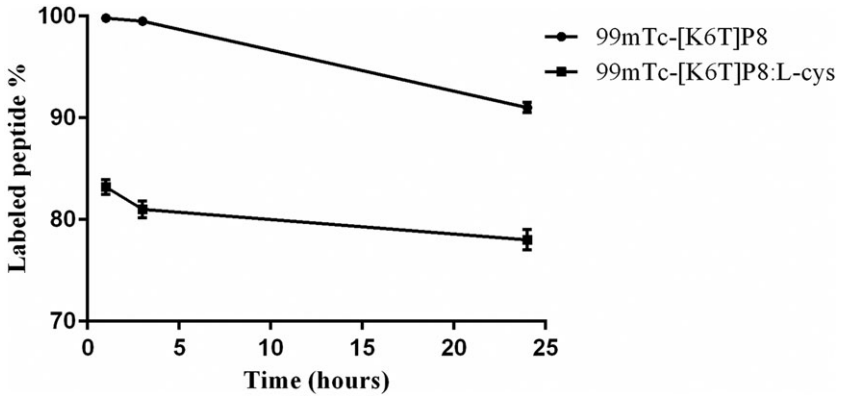

FIGURE 1 Stability of ^{99m}Tc-labeled [K6T]P8 peptide in aqueous solutions for 24 h. The curves represent the activity of labeled peptide (●) or labeled peptide in 30-fold molar excess of L-cys (■) after 1, 3, and 24 h of incubation. Data represent the mean values ± standard deviation from 3 independent experiments

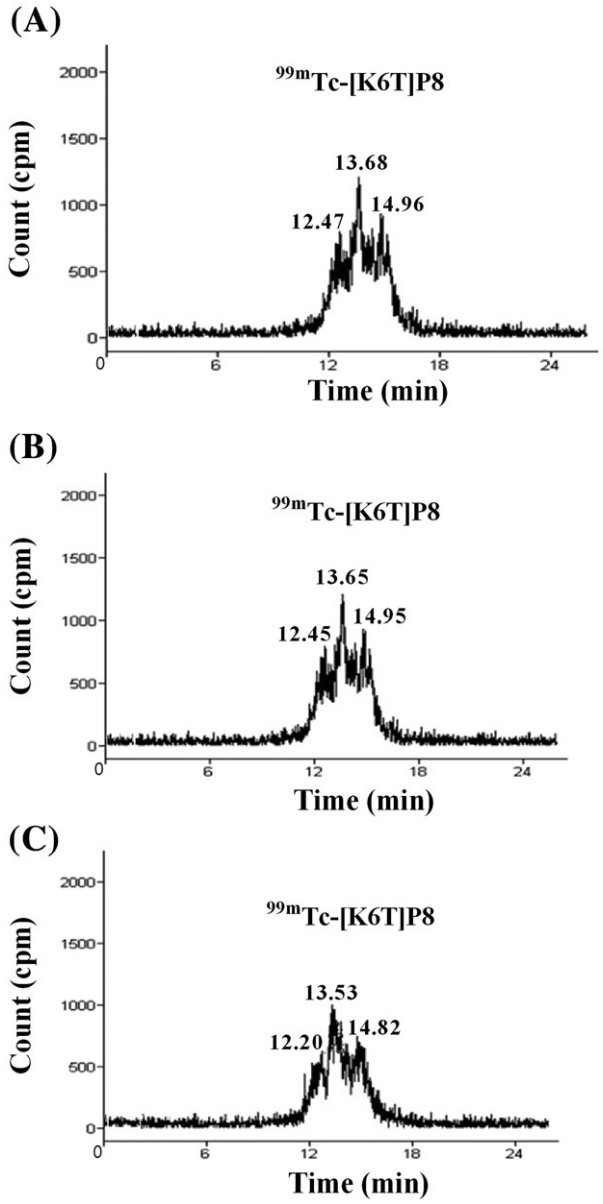

FIGURE 2 Radiochromatograms of ^{99m}Tc-labeled [K6T]P8 incubated in human plasma for (A) 0 h, (B) 1 h, and (C) 3 h. The retention times corresponding to main peaks of ^{99m}Tc-[K6T]P8 are indicated

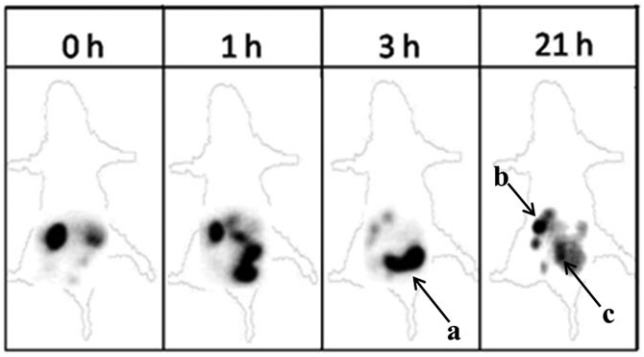

FIGURE 3 Gamma camera imaging of healthy Wistar rats after injection of ^{99m}Tc-labeled [K6T]P8 peptide. The biodistribution of ^{99m}Tc-[K6T]P8 at 1, 3, and 21 h after injection is shown. (A) Kidneys, (B) intestinal tract, and (C) bladder are indicated

Similar results were obtained, when citric acid was included in the assay control. In this case, the maximum cell proliferation showed optical density (OD) values below the minimum (Figure 4B). Although, an antagonist effect of peptides was observed on IL-15 activity, measured in this assay as the inhibition of cell proliferation in a dose-dependent manner, the control of maximal proliferation of the assay was diminished, suggesting an interference of these additives on the CTLL-2 cell proliferation assay.

Figure 4C depicts the effects of [K6T]P8 peptide and its dimer on CTLL-2 cells proliferation, when sucrose was used as an additive. Here, the sucrose does not affect the maximum cell proliferation, which showed absorbance values similar to this control without additives. The monomeric and dimeric form of [K6T]P8 peptide inhibited IL-15 biological activity with IC_{50} values of 27.7 μM and 11.7 μM, respectively, which correspond with results previously reported by Santos et al.[19] Taking into account these results, sucrose proved to be the additive of choice, because it increased the solubility of both peptides and did not interfere with the *in vitro* cell proliferation assay used to assess their biologic activity.

4 | DISCUSSION

Taking into account that IL-15Rα binds IL-15 with a high-affinity interaction, it is important to develop IL-15 antagonist peptides with enhanced inhibitory effect. We have previously reported that [K6T]P8 peptide was 5-fold more active than P8 in CTLL-2 cells,[19] but it was less soluble in aqueous solution, mainly at concentrations higher than 1 mg/mL. In the current work, the metabolic stability of [K6T]P8 and the preliminary biodistribution pattern of the ^{99m}Tc-labeled peptide in healthy rats were studied. Also, it was identified an additive that improved the solubility of [K6T]P8 and its dimer, which did not affect their biological activity in CTLL-2 cells.

The [K6T]P8 was synthesized as a linear 10 amino acids peptide that contains a free cysteine in its sequence.[19] Direct ^{99m}Tc-labeling of [K6T]P8 was performed, taking into consideration several existing reports about the use of that procedure in case of peptides with one or more cysteine in their sequences.[23,26,27] Final pH of the samples for labeling was adjusted to 9, taking into account that a basic pH favors the activation of the amino groups, which are essential in the formation of stable coordination bonds with reduced technetium. A satisfactory labeling yield (>95%) was achieved, which is consistent with the reports by other authors.[28-31] In aqueous solution, the percentage of ^{99m}Tc-[K6T]P8 was higher than 90%, in correspondence with the results previously reported.[23,26,28]

One of the most important factors that affect the *in vivo* stability of radiopharmaceuticals is the transchelation of the technetium to other molecules such as glutathione and cysteine in the blood or in different tissues. For this reason, a challenge against an excess of cysteine was performed to assess the stability of the ^{99m}Tc-[K6T]P8 complex. It is known that the stability of the ^{99m}Tc-complexes significantly depends on the chelating amino acid sequence. Stalteri et al reported percentages of cysteine translocation in the range of 10% to 100%, at 4 hours of incubation using a challenge against 10-fold molar excess of cysteine.[26] Thus, the outcomes of the present work

TABLE 2 Values of the peak area obtained from RP-HPLC chromatograms for [K6T]P8 peptide and its dimer dissolved in RPMI medium with or without citric acid, sucrose, and Tween 80

		Main Peak Area[a]			
Peptides		**RPMI Medium**	**RPMI Medium Plus Citric Acid**	**RPMI Medium Plus Sucrose**	**RPMI Medium Plus Tween 80**
[K6T]P8	Mean	72833	134148	415120	88511
	SD	16152	27431	165872	21221
	CV (%)	22.18	20.45	39.96	23.98
[K6T]P8 dimer	Mean	9686	63843	38822	16625
	SD	323	2338	1895	2283
	CV (%)	3.34	3.66	4.88	13.73

CV, coefficient of variation; RP-HPLC, reversed-phase high-performance liquid chromatography; RPMI, Roswell Park Memorial Institute; SD, standard deviation.

[a]Data correspond to the area of the main peak from 3 replicates.

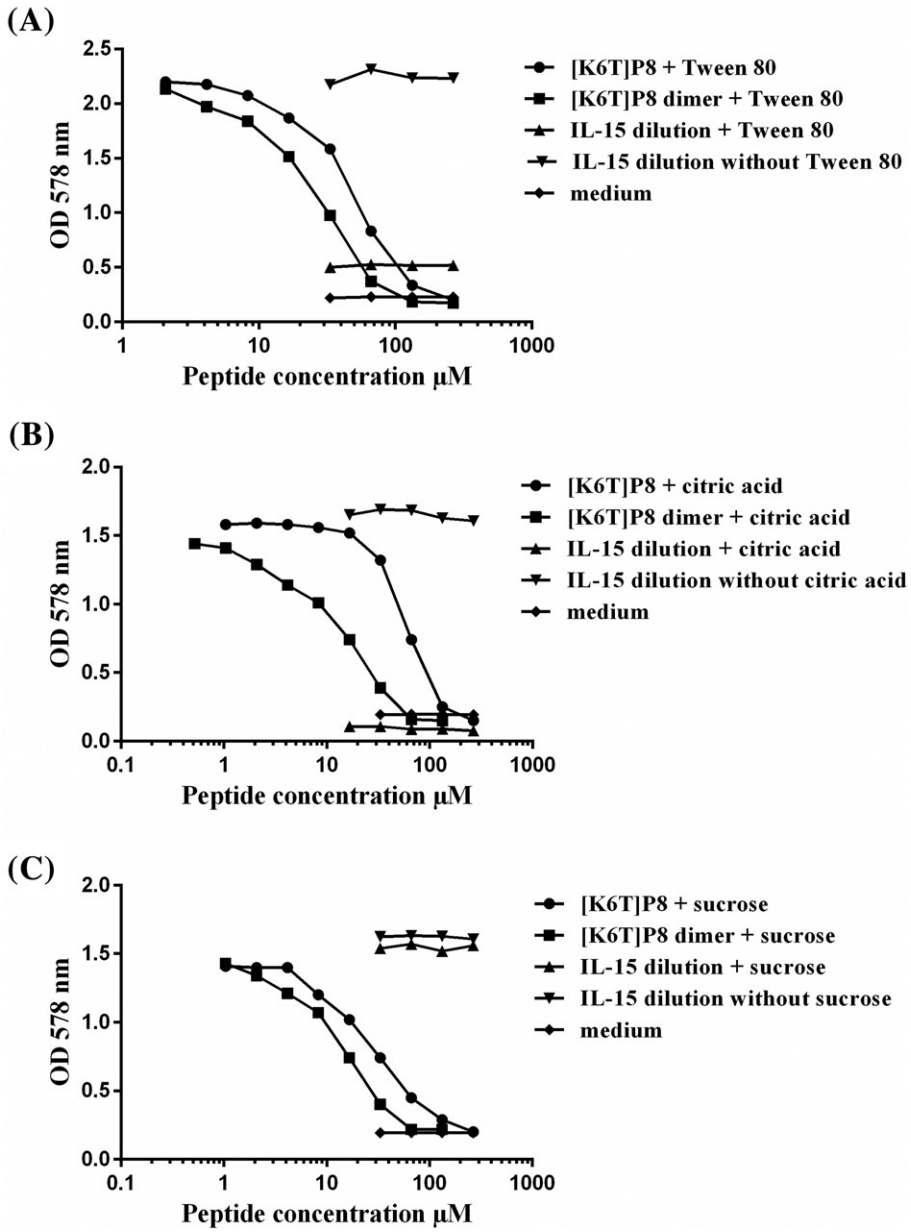

FIGURE 4 Effect on the proliferation of CTLL-2 cells of [K6T]P8 peptide and its dimer dissolved in Roswell Park Memorial Institute medium with (A) Tween 80, (B) citric acid, or (C) sucrose. The cells were cultured in the presence of 300 pg/mL of Interleukin (IL)–15 plus increasing concentrations of [K6T]P8 and its dimer with the additives mentioned above or medium. Controls of IL-15 dilution alone or with Tween, citric acid, or sucrose are shown. Cell proliferation was measured by MTT staining. Data were obtained from 2 independent experiments performed in triplicate

could be considered satisfactory, taking into account that higher molar excess of cysteine was used. However, other authors have reported better stability using other chelating amino acid sequences.[28,32] From the stability assay of ^{99m}Tc-[K6T]P8 in aqueous solution, it was found that after 1 hour of incubation with 30-fold molar excess of L-cysteine, the activity of ^{99m}Tc-[K6T]P8 decreased to an 83.22 ± 0.74%. This fact also supports our finding that cysteinilation and dimerization are the most frequent [K6T]P8 modifications after incubation with HSF. Both experiments pointed out that free cysteine residue is critical for peptide stability in any circumstances, considering that it is a reactive group sensitive to be quickly modified. Modified species have to be further investigated in terms of biological activity in respect of [K6T]P8. If they are at least as active as [K6T]P8, the high rate modification of this peptide would be irrelevant for the expected biological effect. The short half-life (5.88 ± 1.73 min) of [K6T]P8 peptide in HSF corresponds with the formation rate of the peptide modifications mentioned above. However, those molecules did no last much more in time, suggesting also the peptide degradation by proteolysis, despite it is very difficult to demonstrate because of the small size of the fragments that could be generated. The solubility issues of [K6T]P8 and its dimer precluded studying their metabolic stability in human serum.

Once in the bloodstream, peptides are susceptible to proteolytic degradation because of the action of the peptidases. Plasma stability assay allows the *in vitro* evaluation of this process, which is the main cause of *in vivo* instability of the radiolabeled peptides.[23,26] As a result of the action of the peptidases, the radiolabeled peptides are fragmented, giving more hydrophilic products with less retention time in the reversed-phase radiochromatogram.[23,26] In this study, no variation was observed in the retention times of the peaks corresponding to the labeled peptide, suggesting that ^{99m}Tc-[K6T]P8 should be stable to the action of the plasma peptidases in the studied time interval. In summary, stability studies in aqueous solution and human plasma suggested a satisfactory stability of radiolabeled complexes during the observation time.

Overall, the affinity for plasma proteins decisively influences the elimination pathway of the molecules. ^{99m}Tc-[K6T]P8 have low percentage of plasma protein binding, suggesting that this peptide should be eliminated by the renal route, mainly by glomerular filtration. Similar results have been reported by other authors for linear amino acid chains.[23] It has been reported that a low affinity for plasma proteins leads to filtration of the compound to urine through the glomerulus,[33,34] while an increase in the affinity leads to tubular secretion.[35,36] Imaging study showed similar biodistribution patterns of ^{99m}Tc-[K6T]P8 independently on the sex of rats and suggests a slow elimination of the product through the renal and hepatic pathways. No activity was detected in the stomach or thyroid, suggesting absence of free technetium, which is in correspondence with the results of labeled peptide. The apparently slow elimination of the compound could be related to the subcutaneously administration of the peptide. A more accurate biodistribution study in an animal model of adjuvant-induced arthritis will be necessary in the future to evaluate the uptake of the radiolabeled peptide in pathogenic tissues.

As mentioned above, [K6T]P8 peptide is an attractive agent to inhibit the inflammatory activity of IL-15 in some autoimmune diseases.[19] To develop this candidate, it is necessary to solve its solubility problems, mainly, when high concentrations are required. The insolubility of [K6T]P8 and its dimer is not surprising if one takes into account the hydrophobic nature of these peptides, where 50% of the residues that compose them are hydrophobic amino acids, eg, valine, methionine, phenylalanine, and leucine. As a solution to this trouble, different additives that improve the solubility such as sucrose, Tween 80, and citric acid were evaluated.[37] Both peptides were completely soluble in RPMI medium with the mentioned additives. According to *in vitro* studies, sucrose stabilizes the protein native conformation, increases the solubility, and reduces the aggregation extent.[38-40] On the other hand, nonionic surfactants like Tween 80 prevent surface adsorption and suppress aggregation.[41] Because of their dual hydrophobic/hydrophilic nature, surfactants in solution tend to orient themselves so that the exposure of the hydrophobic portion of the surfactant to the aqueous solution is minimized[42] and can improve drug solubility by providing regions for hydrophobic interactions in solution.[43]

Citric acid is another additive that increases the solubility of monomeric and dimeric form of [K6T]P8. In a citric acid solution at pH 2, these peptides acquire a positive net charge because the pH of the solution is below the theoretical isoelectric point estimated for these molecules: 8.22 for [K6T]P8 and 8.90 for [K6T]P8 dimer (www.expasy.org). This element can favor the solubility of these peptides by an increase in solute-solvent interactions.

Although the identification of additives that increase the solubility of these peptides is very important, it is necessary to determine the interference of these additives on available *in vitro* test to evaluate their biological activity. According to the results obtained in the CTLL-2 cell proliferation assay, peptides have an antagonist effect on the activity of IL-15 by decreasing cell proliferation in a dose-dependent manner. It should be noted that the control of maximal cell proliferation in the presence of Tween 80 showed a decrease by approximately 80% with respect to the control without additive, suggesting that Tween 80 exerts a negative effect on CTLL-2 cells proliferation. Similar results were obtained in *in vitro* experiments with murine bone marrow cells. In this study, different concentrations of Tween 80 from 0.005% to 0.01% were evaluated, obtaining a significant reduction in cells viability.[44]

A higher effect on cell proliferation was obtained, when citric acid was used as an additive. In this case, a marked decrease in cell growth with OD values below the minimum of the assay was produced. This can be explained by the pH value of the citric acid solution, which causes an acidification of the medium not favorable for cell growth, considering that the proper development of metabolic functions at the cellular level requires a physiologic range of pH (7.36-7.44). These results correspond with other studies, where the authors demonstrated that the use of a 47.6 mM citric acid solution at pH 2.3 results in the death of human fibroblast cells after 3 minutes of incubation. In other studies, citric acid concentrations of 11.9 mM, 23.8 mM, and 47.6 mM produce strong cytotoxicity (47-90% cell death) in the cultures of these cells, with a marked decrease in pH of the medium[45] from 7.5 to 3.8.

Similar to the results obtained with Tween 80, the citric acid effects decrease with the dilution resulting in a dose-response curve of [K6T]P8 peptide and its dimer in CTLL-2 cells. Nevertheless, these

results are not reliable because these additives are not compatible with the experimental conditions of the proliferation assay. In our study, sucrose proved to be the additive of choice because it permits complete solubilization of [K6T]P8 and [K6T]P8 dimer without affecting the viability of CTLL-2 cells. It is well known that the addition of sucrose has been shown to enhance the stability of peptide[46] considering it as one of the most commonly used excipients in peptide formulations.[47,48]

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