RESEARCH

Identification of an interleukin-15 antagonist peptide that binds to IL-15Rα

✉ Alicia Santos[1], Ania Cabrales[2], Osvaldo Reyes[2], Haydee Gerónimo[3], Yunier Rodríguez[1], Hilda Garay[2], Celia Aurora-Arrieta[1], Ricardo Silva[1], Gerardo Guillén[1]

[1]Pharmaceutical Division
[2]Chemistry and Physics Division
[3]Quality Control Division, Center for Genetic Engineering and Biotechnology
Ave 31 e/ 158 and 190, Playa, PO Box 6162, Havana 10 600
E mail: alicia.santos@cigb.edu.cu

ABSTRACT

Interleukin-15 (IL-15) is a pro-inflammatory cytokine that is expressed in several autoimmune and inflammatory diseases. We have identified the 36-45 sequence KVTAMKCFLL on human IL-15 that is recognized by a soluble form of recombinant hIL-15Rα-Fc fusion protein. This sequence synthesized as a 10 aa (amino acids) synthetic peptide is bound to the IL-15Rα while it blocked the biological activity of IL-15 in two IL-15 dependent cells lines. Furthermore, this peptide inhibits IL-15Rα induced IL-6 transcription in a human prostate carcinoma cell line; a reverse signal described for membrane-bound IL-15. We also examined the dimer formation of this peptide and found that the antagonist effect is abrogated when Cys were replaced by Ala or Ser and the dimers formed through free Cys are more active than monomers. This peptide is the first IL-15 antagonist peptide described until now and could be a potential therapeutic agent to treat diseases related to IL-15 expression.

Keywords: antagonist, cytokine, IL-15, peptide, receptor alpha

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RESUMEN

Identificación de un péptido antagonista de la interleucina-15 que se une a la IL-15Rα. La interleucina-15 (IL-15) es una citocina pro-inflamatoria que se expresa en muchas enfermedades autoinmunes e inflamatorias. Nosotros hemos identificado la secuencia 36-45 KVTAMKCFLL de la IL-15 humana, que es reconocida por la proteína de fusión hIL-15Rα-Fc soluble. Esta secuencia sintetizada como un péptido de 10 aa , se une a la IL-15Rα y bloquea la actividad biológica de la IL-15 en dos líneas celulares dependientes de IL-15. Además, este péptido inhibe la trascripción de la IL-6 inducida por IL-15Rα en una línea celular humana de carcinoma de próstata; una señalización inversa descrita para la IL-15 unida a la membrana. Nosotros también examinamos la formación de dimeros de este péptido y encontramos que cuando la Cys fue remplazada por Ala o Ser se pierde el efecto antagonista del péptido y que el dímero formado a través de la Cys libre es más activo que el monómero. Este péptido es el primer antagonista peptídico de la IL-15 descrito hasta el momento y puede ser un potencial agente terapéutico para el tratamiento de enfermedades relacionadas con la expresión de la IL-15.

Palabras clave: antagonista, citocina, IL-15, péptido, receptor alfa

*I*ntroduction

Interleukin-15 (IL-15) is a member of the four α helix bundle cytokine family. The pleiotropic expression of IL-15 and its receptor may indicate the broad role of IL-15 in multiple cell types and tissues, with various important contributions to activation and homeostasis of T lymphocytes [1]. However, numerous data support a pro-inflammatory role for IL-15 in immunopathologies, such as rheumatoid arthritis, inflammatory bowel disease, sarcoidosis, and multiple sclerosis, related with the uncontrolled expression of IL-15 [2-5]. The neutralization of IL-15 has been proposed as a strategy in the therapy of these diseases.

The effects of IL-15 are mediated by a trimeric membrane receptor comprising β and γ subunits of the IL-2 receptor and α different a subunit [6, 7]. Cells bearing the IL-15Rα can bind with IL-15 with a high affinity and present the cytokine to $\beta\gamma^+$ cells such as T and NK cells [8]. Moreover IL-15Rα exists not only in the anchored membrane but also in a soluble form [9].

Furthermore membrane-bound IL-15 is constitutively expressed on the cell surface of the PC-3 human prostate carcinoma cell line and IFN-γ-activated human monocytes and mediates reverse signaling events in both cell types upon its stimulation with recombinant sIL-15Rα or anti-IL-15 Abs, functioning as a receptor molecule. This signaling scenario involves the phosphorylation of members of the MAPK family. Moreover, reverse signaling through transmembrane IL-15 upregulated the transcription level of IL-6 and IL-8 and dramatically enhanced the migratory properties of prostate cancer cells [10].

Here, we have identified a small P8 peptide corresponding to the 36-45 IL-15 sequence KVTAMKCFLL which binds to IL-15Rα and shows inhibitory effects on IL-15. This is the first description of an IL-15 antagonistic peptide, potentially allowing for an effective future treatment of IL-15 related diseases with a potential use in the treatment of IL-15 related diseases.

*M*aterials and methods

Synthesis of IL-15 peptides on α cellulose support

To identify the IL-15 regions involved in the binding to IL-15Rα, a peptide spot synthesis approach was

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✉ Corresponding author

used as previously described [11]. The derivatization of Whatman 540 paper was carried out esterifying the first anchor component, Fmoc-beta-Ala-OH, using N,N´-di-isopropyl-carbodiimide (DIC) and N-methylimidazole (NMI) in dry N,N-dimethylformamide (DMF). The spot array on the cellulose membrane was defined anchoring the Fmoc-beta-Ala-OH on the previously marked positions, according to the required number of 10-mer peptides (22 peptides, overlapping in 5 amino acid residues of the IL-15 sequence). As controls, a non-related 10 aa (amino acids) peptide was synthesized on the spot 23, and spot 24 only had the first anchor component Fmoc-beta-Ala-OH; for the assembly of all these molecules; the N-terminus and side chains of all peptides were deprotected by stan-dard Fmoc-/tBu chemistry after the final cycle for the synthesis of the assembly.

Binding of IL-15RαFc to peptides synthesized on a cellulose support

A cellulose sheet was soaked in ethanol to prevent possible hydrophobic interactions among the peptides on it. Ethanol was exchanged for Tris-buffer saline (TBS) (150 mM NaCl, 10 mM Tris, pH 7.6) by sequential washing. Non-specific binding was blocked by incubating the membrane overnight in 10 mL of TBS blocking buffer (5% dry milk in TBS). The sheet was subsequently incubated for 3 h with IL-15Rα-Fc, diluted in 10 mL of the T-TBS sample buffer (5% dry milk, 0.5% Tween-20 in TBS) at 5 µg/mL, in the same buffer solution. The cellulose sheet was washed four times with the T-TBS buffer. Then an alkaline phosphatase conjugate anti-IgG (Fc specific) (Sigma), diluted in the T-TBS sample buffer, was added for 1 h. The cellulose sheet was washed four times again, with T-TBS and the detection of the bound peptides was achieved by incubating the membrane with 0.5 mg/mL of 5-Bromine 4-Chlorine 3-Indolyl Phosphate (BCIP) (Sigma), in buffer substrate (100 mM NaCl, 2 mM $MgCl_2$, 100 mM Tris, pH 8.9). Positive spots developed a blue/violet color. Washing with PBS stopped the staining. The cellulose sheet was finally regenerated for others assays.

Peptide synthesis

The peptides were synthesized by the Fmoc/tBu strategy, using Fmoc-AM-MBHA resin at 0.54 mmol/γ and synthesis protocols with mechanical shaking. After a trifluoroacetic acid treatment, the peptide was lyophilized and tested by HPLC-MS.

Cell culture conditions

PC-3 human prostate carcinoma cell lines were maintained in an RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin For each assay, 5 x 10^6 cells/mL were stimulated with sIL-15Rα (1 ng/mL) or anti-IL-15 Abs MAB647 (R&D) (100 ng/mL) for 8 h at 37 °C.

Effect of described peptides on CTLL2 cell line proliferation

To evaluate the neutralizing capacity of the peptide, serial dilutions of peptides were made in 96 well plates (Costar, USA) with a volume of 25 µL of the RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Washed CTLL-2 cells were added at a rate of 5 x 10^3 cells/well and further incubated for 30 min at 5% CO_2 and 37 °C. Then, 300 pg of IL-15 were added to each well and the plate was incubated for 72 h under the same conditions. To measure proliferation, the mitochondrial staining assay using 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide was used[12].

Enzyme-linked immunoassay (ELISA) to measure the binding to peptide/IL-15Rα

The 96-well microtiter plates (Corning Costar) were treated with 2% Glutardialdehyde solution for 2 h at 37 °C. After two washes with water, plates were coated with 10 µg/mL of P8 peptide/well and the plates were incubated at 4 °C overnight. After three washes, non-specific binding sites were blocked by incubation for 1 h at 37 °C with PBS containing 1% BSA. The blocking solution was replaced by 50 µL of human IL-15 at different concentrations in PBS, containing 0.01% BSA and 0.05% Tween 20 and 50 µL of IL-15Rα-Fc at 0.125 µg/mL in the same buffer dilution. All the samples were in triplicate. Following incubation at 37 °C for 60 min and three washes, the bound IL-15Rα-Fc was detected with HRP-conjugated goat anti-human IgG (Sigma) at 37 °C for 1 h, followed by washes. The reaction was visualized by adding the substrate solution [(3,3',5,5'-tetramethylbenzidine (TMB)] and absorbance at 450 nm was measured by an ELISA plate reader (Biotrak).

Measurement of peptide-IL-15Rα binding with IL-15 as a coating protein

ELISA plates were coated with 1 µg/mL of IL-15 / well in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with PBS containing 1% BSA, 50 µL of serial dilutions of peptide and 50 µL of IL-15Rα-Fc at 0.125 µg/mL were added and incubated at 37 °C for 1 h, followed by washes with PBS containing 0.1% Tween 20. Bound IL-15Rα-Fc was detected as described above.

RT-PCR and real-time PCR

RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Real time RT-PCR was used to measure IL-6 transcripts. Specific oligos (forward 5´-CAGGAATTGAATGGGTTTGC-3´ and reverse 5´-AGCAGACTAGGGTTGCCAGA-3´ were used.

A 25 µL real-time PCR reaction included Brilliant SYBR Green QRT-PCR Master Mix according to the manufacturer's instructions (Qiagen). Real-time quantitations were performed on the Rotor-Gene 3000™. The fluorescence threshold value was calculated using Rotor-Gene Series software. The calculation of relative change in mRNA was performed using the delta-delta method [13], with corrections for the housekeeping ubiquitin C gene.

Results

Identification of a binding sequence to IL-15Rα-Fc

The peptide spot synthesis approach was used to identify regions of IL-15 involved in the binding to IL-15Rα. The cellulose sheet display 22 peptides of

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the IL-15 sequence were incubated with IL-15Rα fused to Fc of human IgG_1 as described in Materials and methods. We observed a strong signal on spot 8 corresponding to the 36-45 sequence KVTAMKCFLL on mature IL-15. No positive spots were observed when cellulose was incubated with human antibody IgG_1, ruling out the possibility that IL-15Rα-Fc occurred on binding through its Fc region (Figure 1).

P8 competitively inhibits the binding of IL-15 to IL-15Rα

The sequence corresponding to spot 8 (KVTAMKCFLL) was synthesized as a soluble linear 10 aa peptide (named P8). To test the specificity of binding of the P8 peptide to IL-15Rα we performed competitive ELISAs. IL-15Rα and different concentration of P8 were co-incubated with IL-15 immobilized in the plate. The results showed that the P8 peptide displaces the binding of IL-15Rα to hIL-15 in a dose-dependent way (Figure 2A). We also demonstrated that IL-15Rα recognized the P8 peptide immobilized in the plate and IL-15 displaces the binding of the P8 peptide/IL-15Rα in a dose- dependent way (Figure 2B).

Inhibition of IL-15 dependent proliferation by the P8 peptide

For a biological evaluation the P8 peptide was tested for it ability to inhibit the proliferation of two IL-15 dependent cell lines, CTLL-2, a murine cell line and Kit225 a human cell line that expresses the trimeric receptor IL-15Rαβγ. The P8 peptide inhibited IL-15-induced proliferation in a dose-dependent manner at plateau concentrations of 300 pg/mL of IL-15 in the Kit225 cell line (Figure 3). The inhibitory effect of the P8 peptide was also dependent on IL-15 concentration in the CTLL-2 cell line (Figure 4). Additionally, the P8 peptide alone was unable to affect the proliferation of these cells induced by IL-2 (data not shown).

Inhibition of IL-6 mRNA induced by IL-15Rα in human prostate carcinoma cell line

Stimulation of the human carcinoma cell line PC-3 with sIL-15Rα or anti-IL-15 Abs enhanced the transcription level of IL-6. Real time PCR RT-PCR analysis of IL-6 mRNA expression showed that the stimulation of PC-3 cells for 8 h with sIL-15Rα or anti-IL-15 Abs enhanced the transcription level of IL-6 and the treatment with the P8 peptide completely inhibited the IL-6 transcription induced by the treatment with IL-15Rα (Figure 5).

Dimer formation of the P8 peptide

In order to evaluate the role of the free Cys on the P8 sequence and the possibility of forming dimers through it, we studied the biological activity of the Cys mutant change this aa for Ala or Ser. Our results show that its mutants completely lost the inhibitory activity on IL-15 (Figure 6) and the binding capacity to IL-15Rα (Figure 7).

The mass spectrometry analysis revealed that at 72 h after the dilution of the P8 peptide in water, every peptide molecule was forming dimers (data not shown). The dimers were chemically synthetized and the results showed that the dimers had a greater activity inhibiting IL-15-dependent CTLL-2 proliferation

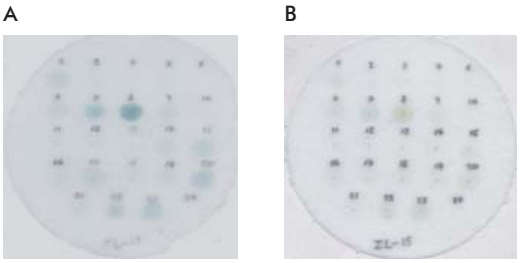

Figure 1. Analysis of the IL-15Rα-Fc binding to IL-15 10-mer peptides. 10-mer peptides spanning the entire sequence of human IL-15 were synthesized in cellulose sheet and tested for their reactivity with IL-15Rα-Fc at 5 mg/mL (A) or a human monoclonal antibody IgG_1 at 5 μg/mL (B) and their binding was revealed with alkaline phosphatase conjugate anti-human IgG (Fc specific).

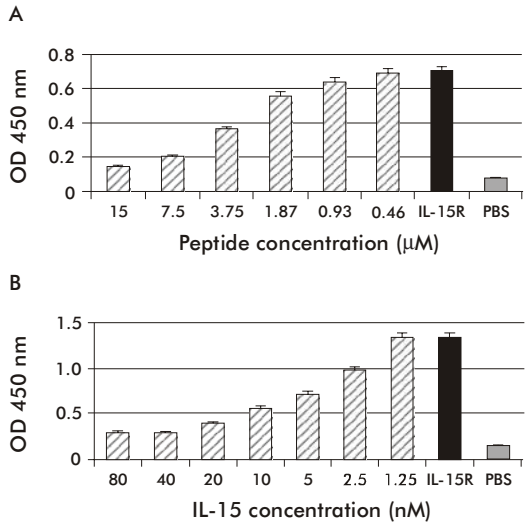

Figure 2. Measurement of the displacement of hIL-15 binding to IL-15Rα by ELISA. (A) P8 displaces the IL-15 binding to IL-15Rα. IL-15 immobilized on the plate was co-incubated with IL-15Rα at 15nM and P8 at different concentrations (15, 7.5, 3.75, 1.87, 0.93, 0.46 μM) (B) IL-15 displaces the P8 peptide binding to IL-15Rα. The P8 immobilized on the plate was co-incubated with IL-15Rα at 15 nM and IL-15 at different concentrations (80, 40, 20, 10, 5, 2.5, 1.25 nM).

than a fresh solution of a mixture, mainly formed by monomers (Figure 8).

*D*iscussion

This study reports the identification of the IL-15 antagonist peptide and also provides substantial evidence on the inhibitory effect of this peptide on soluble and membrane bound IL-15 activity. Here we used the spot synthesis technique, a valuable tool to study molecular recognition and to biologically identify ac-tive peptides [14]. The greatest signal observed after incubation with IL-15Rα-Fc corresponded to sequence 8 containing the 36-45 *aa*. KVTAMKCFLL of human IL-15, which suggests a binding sequence to IL-15Rα. Interestingly, this sequence comprises the end of the loop connecting helix A and helix B and the beginning of the helix B as published elsewhere [15], and does not include amino acids described as involved in the interaction with IL-15Rα. To test the interaction

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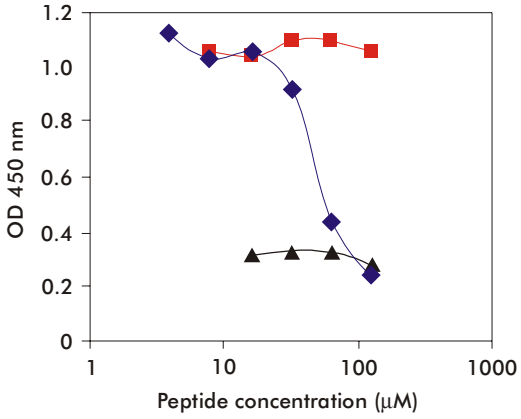

Figure 3. Effect of the P8 peptide on IL-15 proliferative activity in KiT225 cells. KiT225 were cultured in the presence of 300 pg/mL of IL-15 (■), 300 pg/mL of IL-15 plus increasing concentrations of P8 (◆), or medium (▲). Cell proliferation was evaluated by MTT staining.

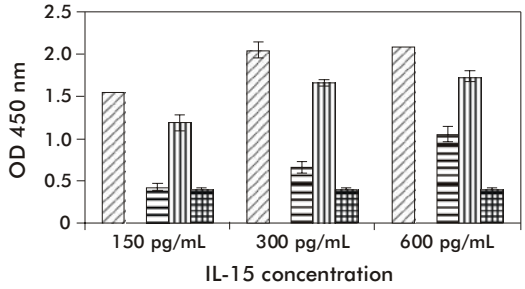

Figure 4. The P8 peptide inhibits IL-15 proliferative activity in CTLL-2 cells. They were cultured in presence of 15 μg/mL of the P8 peptide (▤) or a non-related peptide plus different concentrations of IL-15 (□). Control IL-15 (▨) and medium (▤) conditions were included. Cell proliferation was evaluated by MTT staining.

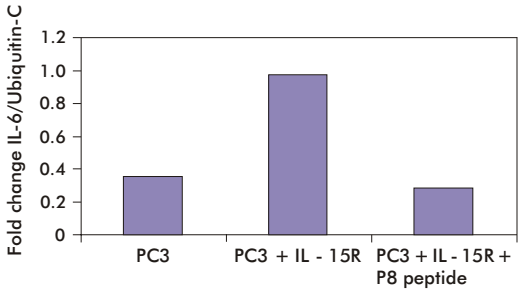

Figure 5. Real time PCR results were normalized to the housekeeping ubiquitin-C gene and shown as a 3-fold change compared with non treated control cells.

between this sequence and IL-15Rα, competitive ELISA experiments were performed with soluble synthetic peptide P8 and we found that this peptide displaces the binding of IL-15Rα to hIL-15 in a dose-dependent way, confirming that the P8 peptide could bind to IL-15Rα specifically and interfere with the IL-15/IL-15Rα binding.

IL-15 binding to its trimeric receptor promotes the proliferation of CTLL-2 and KiT225 cell lines, two IL-15-dependent cell lines. Since the β chain is also

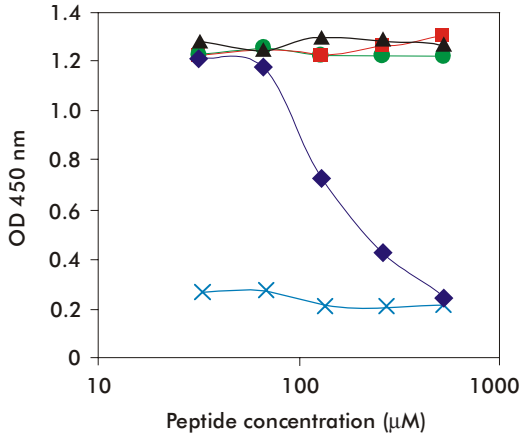

Figure 6. Effect of P8 and P8 mutant peptides on the IL-15 proliferative activity in CTLL-2 cells. CTLL-2 were cultured in the presence of 300 pg/mL of IL-15 (■) or 300 pg/ mL of IL-15 plus increasing concentrations of the P8 peptide (◆), mutants Cys-Ser (●), Cys-Ala (▲), or the medium (X). Cell proliferation was evaluated by MTT staining.

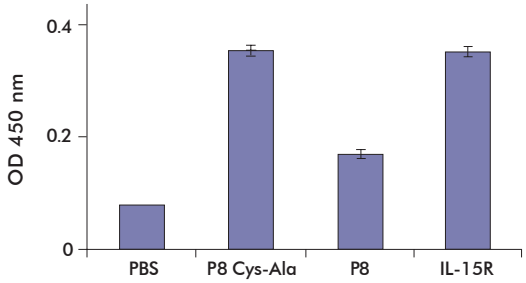

Figure 7. Evaluation of displacing the binding of peptides to IL-15Rα by ELISA. IL-15 immobilized on the plate was co-incubated with L-15Rα-Fc at 0.125 μg/mL and peptides at 20 μg/mL and the binding was revealed with an anti-human IgG-alkaline phosphatase conjugate.

shared with IL-2 and the γ chain is common for several cytokines IL-4, IL-7, IL-9, IL-21, chain α of IL-15R confers cytokine specificity and enhances the affinity of cytokine binding [16]. Moreover, the binding of IL-15 to the cell-surface IL-15Rα may effectively prolong the half-life of IL-15, thereby allowing for a continual acquisition of cytokine by lymphoid cells without the need of a continuous biosynthesis of IL-15, whose half-life *in vivo* is extremely short [17]. The expression of IL-15 has been previously observed across a range of autoimmune and inflammatory processes and hematological malignancies suggesting that the de-regulation of IL-15 may result in deleterious effects for the host. This peptide was tested for the inhibition of IL-15 induced proliferation. Results showed that the P8 peptide inhibits the IL-15 induced proliferation on CTLL-2 cells and also that the P8 inhibits the IL-15 induced proliferation on the Kit225 cell line derived from a patient with T-cell chronic lymphocytic leukemia, indicating that the P8 peptide is capable of inhibiting IL-15 activity. However, this peptide had a half inhibitory concentration (IC50) of 130 μM and showed a weak affinity to the target protein IL-15Rα compared to the high affinity IL-15/IL-15Rα complex. This is predictable because short pep-

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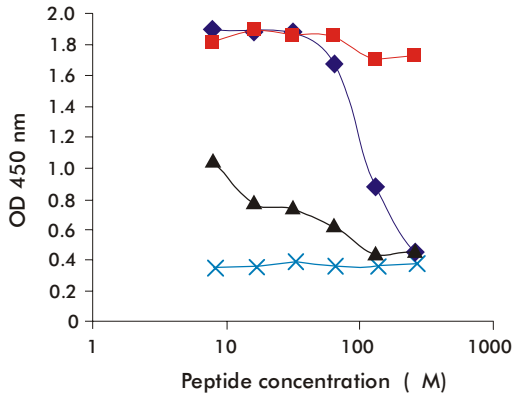

Figure 8. Effect of the P8 peptide dimer on IL-15 proliferative activity in CTLL-2 cells. CTLL-2 were cultured in the presence of 300 pg/mL of IL-15 (■), 300 pg/mL of IL-15 plus increasing concentrations of the P8 freshly prepared(◆) or the dimer P8 (▲), or the medium (✕). Cell proliferation was evaluated by MTT staining.

tides usually cannot hold the conformation needed for high-affinity biomolecular interaction. Furthermore, we also show evidence that the P8 peptide might play a role in inhibiting reverse signaling induced through membrane bound IL-15 by soluble IL-15Rα in the human prostate carcinoma cell line PC-3, which is another mechanism of action described for this cytokine. The over-expression of IL-15 and IL-15Rα has been reported in benign hyperplasic prostate suggesting a role of IL-15 in prostatic inflammation [18]. Recently, the existence of a natural sIL-15Rα was described that could justify in this context the existence of a reverse signaling activating mechanism [19]. Here, we demonstrated that the P8 peptide is able to inhibit IL-6 transcription induced by IL-15Rα in PC-3 cells. IL-6 is known to have a role in regulating androgen receptor expression in an androgen-independent manner. As a result, it may be involved in making prostate tumors resistant to endocrine therapy [20]. In this sense, it has been shown that blocking the IL-6 action potentiates the sensitivity of the prostate carcinoma cell line PC-3 to the cytotoxic effects of etoposide and cisplatin [21]. This IL-15 antagonist peptide could be useful in inhibiting IL-15 reverse signaling in tumor cells to impair soluble IL-15Rα binding to IL-15 on the membranes.

A series of IL-15 antagonists such as neutralizing antibodies directed against IL-15 itself or alternatively, against the IL-2R/IL-15Rα, have been developed [22] but, a neutralizing antibody exerts an effect similar to IL-15Rα on membrane bound IL-15 enhancing IL-6 transcription level and it may not be useful in this context.

The identification of an IL-15 antagonist peptide that specifically binds to the IL-15Rα receptor could have important applications in inhibiting the IL-15/IL-15R complex activities in the chronic inflammatory context, where soluble or membrane-bound IL-15 have been detected.

In order to improve the activity of this peptide, we also studied the spontaneous formation of peptide dimers in the presence of free Cys and we observed that a dimer molecular form is more active than monomeric peptide with an IC_{50} of 24 μM. We will continue studies for the optimization of the peptide antagonist activity.

This is the first description of an IL-15 antagonist peptide which could be prepared synthetically and administered at high concentrations instead of large macromolecules that present limitations in relation to high production cost and immunogenicity with repeated use.

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