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Immunomodulatory effects of a set of amygdalin analogues on human keratinocyte cells

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Abstract: Peptide T (PT) is an octapeptide shown to resolve psoriatic lesions. Our previous investigations suggest that keratinocytes play an important role in conditioning the therapeutic effects of the PT in psoriasis. However, peptides are not good therapeutic agents, because they exhibit poor absorption, are easily metabolized and are immunogenic. Using computational methods, the natural product amygdalin was identified as peptidomimetic of PT. However, amygdalin exhibits a toxic profile due to its cyanide group. To overcome this deleterious effect, we synthesized analogues lacking the cyanide group. Human keratinocytes were treated with PT or with three different peptidomimetics of PT. To study its effects on the expression of HSP-70, TGF-β, α-v integrin, ICAM-1 and cytokines, we analysed the protein levels by Western blot and ELISA. Our results show that the different peptidomimetics of PT tested exhibit a similar biological behaviour in regard to the overexpression of HSP-70, TGF-β and α-v integrin than the native peptide. TNF-α is overexpressed by PT and SVT-03018; between the other two analogs, SVT-03016 do not produce any significant change in regard to the control, while SVT-03017 shows only a moderate increase in regard to control. SVT-03018 provokes a remarkable upregulation of IL-10, stronger than SVT-03016, SVT-03017 and PT. All the other three analogues reduce comparably to the PT, the expression of ICAM-1 and do not increase the release of proinflammatory cytokines. The results highlighted that the three analogues of amygdalin with the cyanide group removed exhibit the same biological effects of PT. Therefore, they can be considered peptidomimetics, suggesting their possible use in the treatment of psoriasis.

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Introduction

Peptide T (PT), an octapeptide fragment located in the V2 region of the HIV-1 gp120-coating protein, appears to be beneficial in the treatment of psoriasis (1–3). Various hypotheses have been proposed in connection to its mechanism of action (4,5). The clear clinical and histological improvement of the majority of patients studied by Talme et al. (3) suggests that PT may possibly become a new alternative in the treatment of psoriasis.

In spite of the promising properties exhibited by PT, peptides are not good therapeutic agents, because they exhibit poor absorption, are easily metabolized and are immunogenic. Accordingly, discovery of peptidomimetics represents a necessary step to design more useful molecules for testing their efficacy for the treatment of psoriasis.

Previous investigations demonstrate that PT induces IL-10 production in the human Th2 cell line and peripheral blood mononuclear cells, with a significant inhibition of IFN-γ production in this latter. Furthermore, PT significantly inhibits the monocyte and lymphocyte chemotactic activity of RANTES, being proposed that this antichemotactic activity as responsible of its therapeutic efficacy in psoriasis (6).

Our previous investigations using human keratinocyte cells treated with PT suggest that these cells play an important role in conditioning the therapeutic effects of the peptide in cutaneous psoriasis (7). Our studies show that PT induces a reduction of ICAM-1 as well as an overexpression of TGF-β, HSP70 and α-v integrin in human keratinocyte cells, and more specifically, the increase of HSP-70 is modulated by TGF-β, because anti-TGF-β antibodies reduce HSP-70 overexpression. Interestingly, TGF-β reversibly inhibits the growth of many cell types including keratinocytes (8), being also responsible of stimulating the differentiation of keratinocytes in culture (9). Overexpression of HSP-70 results in a dramatic change in the intermediate filaments (10). This is of particular interest in psoriasis, because the reduced adhesion of keratinocytes to the basement membrane was proposed as an initial alteration that leads to uncontrolled cellular proliferation. Thus, PT may act on keratinocytes, directly by stimulating the anti-inflammatory function, reducing the cellular hyperproliferation and favouring the regeneration of tissue.

In a previous study, from a structure-activity study using computational methods, the natural product amygdalin was identified as candidate peptidomimetic of PT (11). Subsequently, the molecule was tested for its chemotactic activity, showing a similar profile as PT. However, amygdalin exhibits a toxic profile due to the easy hydrolization of its cyanide group. To avoid this drawback in a subsequent study, guided by the pharmacophore hypothesis developed from the structure-activity studies previously described, different amygdalin analogues lacking the cyanide group were synthesized and tested. This study permitted us to identify new compounds with improved chemotactic activity (12). The aim of the present investigation was to determine the immunomodulating effects on keratinocytes of a subset of peptidomimetics of PT, selected for its higher chemotactic index. This series include compounds SVT0317, SVT0316 and SVT0318 shown in Fig. 1. For this purpose, we studied the effect of these analogues on the *in vitro* expression of TGF-β1, using human keratinocyte cells as an experimental model, with the aim to compare their biological effects with that of PT. Moreover, we analysed the modulation of ICAM-1, α-v integrin and HSP-70 on human keratinocyte cells treated or not, respectively, with them. Finally, we also analysed the expression of cytokines involved in immunomodulation of psoriasis, such as IL-6, IL-8, IL-10, TNF-β and IFN-γ.

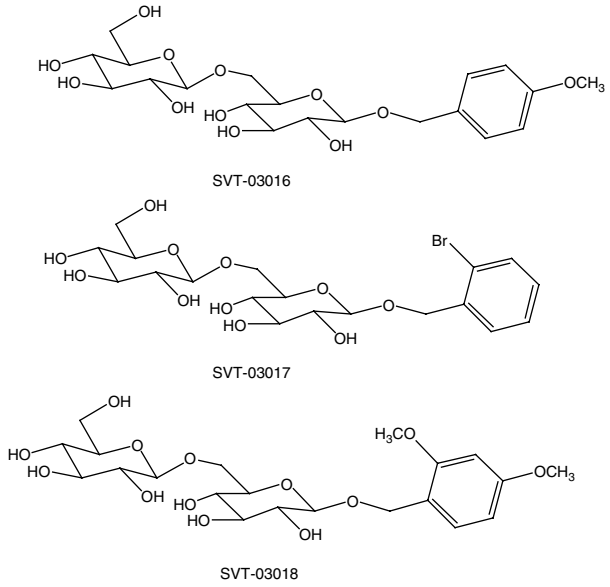

Figure 1. Structure of the three compounds studied in the present study.

Materials and methods

Cell culture and treatments

Human keratinocytes were obtained from surgical specimens of normal adult skin (following informed consent). The skin was split overnight in dispase (Sigma, Milan, Italy) at 4 °C. Epidermal sheets were removed from the dermis, and single-cell suspensions were obtained by placing epidermal sheets in 0.05% trypsin – 0.53 mM EDTA for 15′ at 37 °C. Following incubation, trypsin activity was stopped by adding 10 ml of soybean trypsin inhibitor (Life Technologies, Milan, Italy), and the keratinocyte cell suspensions were centrifuged. The resulting cell pellet was cultured in defined serum-free keratinocyte medium (Life Technologies) at a density of approximately 3×10^6 cells per flask in 15 ml of complete medium. Keratinocyte cultures were fluid changed with fresh complete medium every 2–3 days (13).

Where required, 5×10^5 cell aliquots were plated in multi-well plates (FALCON, Milan, Italy) and treated with PT (Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr, Sigma) for 24 and 48 h at 10^{-8} M as previously described (7) and the peptidomimetics of PT: SVT-03016 with a molecular weight of 462, SVT-03017 with a molecular weight of 511, SVT-03018 with a molecular weight of 492, at different times (24 and 48 h) and at various concentrations (10^{-8} and 10^{-6} M), as described in the different experiments carried out.

Protein extraction and Western blotting analysis

Human keratinocyte cells, 5×10^5 cells/well, were treated or not with PT, respectively, and with SVT-03016, SVT-03017 and SVT-03018 at various concentrations (10^{-8} and 10^{-6} M) and at different times (24 and 48 h). To study the role of TGF-β1 on HSP-70 induction, we stimulated human keratinocyte cells with peptidomimetics of PT, with or without antibody anti-TGF-β1 (1 and 5 μM) (Sigma), respectively, during 24 h. The cells were scraped with 1 ml of PBS, and the cell pellet was homogenized with 300 μl of ice-cold buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton, 1,5 mM $MgCl_2$ and 5 mM

EGTA) supplemented with 20 mM sodium pyrophosphate, 40 μg/ml of aprotinin, 4 mM PMSF, 10 mM sodium orthovanadate and 25 mM NaF. Total extracts were cleared by centrifugation for 30 min at 4 °C at 10000 *g* and assayed for the protein content by Bradford's method (14). Fifty microgram of proteins were electrophoresed through a polyacrylamide gel and transferred to nitrocellulose membranes, and the filters were stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. Blots were blocked overnight with 5% non-fat dry milk and then incubated with anti-HSP-70 (Stressgen, Milan, Italy), anti-TGF-β1 (Sigma), anti α-v (Chemicon, Milan, Italy), anti-ICAM-1 (Santa Cruz, Milan, Italy), anti-β-tubulin (Sigma) monoclonal antibodies and 1 μg/ml in tris-buffered saline (TBS) (150 mM NaCl, 20 mM Tris-HCl pH 8), for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, the filters were incubated with 1:2000 peroxidase-conjugated anti-mouse immunoglobulins for 1 h at 22°C. They were extensively washed and finally analysed using the ECL system (Amersham, Milan, Italy).

Release of cytokines IL-6, IL-8, IL-10, IFN-γ, and TNF-α from human keratinocytes

Cytokine release was evaluated on the harvested culture supernatants of human keratinocytes (2×10^6 cells/well) seeded in multiwell plates (Falcon Labware, Oxnard, CA, USA) and treated with PT and peptidomimetics of PT at previously indicated concentrations vs. the control. The incubation was performed for 24 and 48 h at 37 °C in 5% CO_2. Cell viability was then checked by the trypan blue exclusion test. All measurements were carried out using monoclonal antibodies. IL-6, IL-8, IL-10, IFN-γ and TNF-α were measured by immunoenzymatic methods (ELISA RD System Inc., Minneapolis, MN, USA).

Results

Peptidomimetics of peptide T modulate HSP70 expression in human keratinocyte cells

Semiconfluent human keratinocyte cells were treated or not with PT and with peptidomimetics of PT and at various concentrations (10^{-8} and 10^{-6} M) and for different times (24 and 48 h).

As can be seen in Fig. 2, Panel A, the induction of HSP70 compared with the control is greater at all the concentrations of PT analogues used, and it exhibits its largest values at concentrations 10^{-8} M, and consequently, it was used in our time experiments. Figure 2, Panel B, shows the induction of HSP70 at different incubation times. The increase in expression of these proteins is already evident after 24-h period of incubation with the different peptidomimetics of PT.

Peptidomimetics of peptide T induce TGF-β1 production in human keratinocyte cells

Semiconfluent monolayers of human keratinocyte cells were treated or not, respectively, with PT and with the different peptidomimetics of PT at a concentration 10^{-8} M during 24 and 48 h, respectively. In these periods, proteins were extracted and analysed by Western blot, as described in *Materials and methods* section, using an anti-TGF-β1 antibody. The results, shown in Fig. 3 Panel A, indicate that TGF-β1 is induced already at the 24 h.

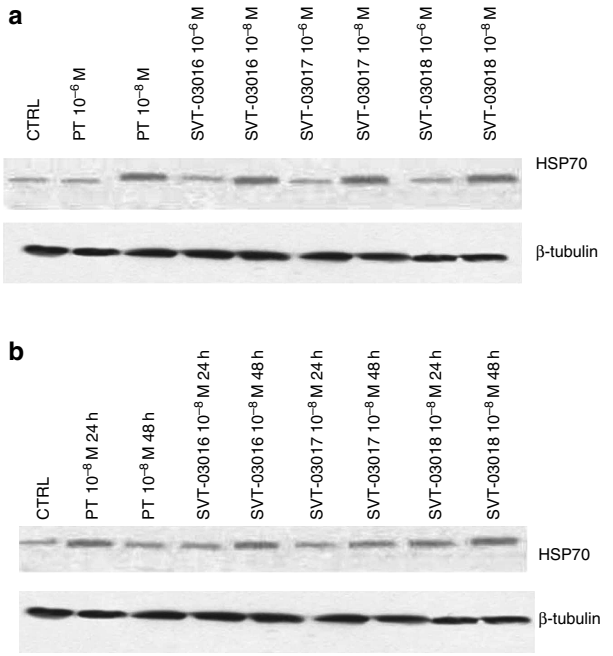

Figure 2. (a) HSP-70 induction at various concentrations (and 10^{-6} and 10^{-8} M), and (b) for different times (24 and 48 h), of peptidomimetics of peptide T (PT) and PT, in human keratinocyte cells. β-tubulin was used as internal control of protein loaded. The results are representative of three different experiments.

HSP70 induction by peptidomimetics of peptide T is TGF-β1 mediated

Human keratinocyte cells were treated or not with PT and the different peptidomimetics of PT at a concentration 10^{-8} M during 24 h in presence or not, respectively, of the anti-TGF-β1 blocker (1 and 5 μM). As can be seen in Fig. 3 Panel B, treatment with peptidomimetics of PT or PT in the presence of anti-TGF-β1 reduces the expression of HSP70. More specifically, the protein levels return to the baseline.

α-v integrins are early modulated by peptidomimetics of peptide T

To study the modulation of PT and its peptidomimetics on α-v integrins, we either treated or not human keratinocyte cells with PT and the different peptidomimetics of PT at a concentration of 10^{-8} M during 4, 12 and 24 h, respectively. At these times, proteins were extracted and

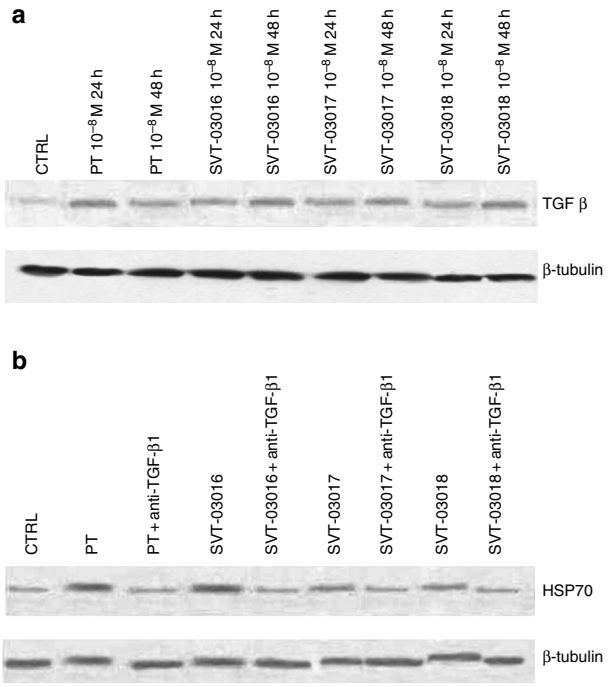

Figure 3. (a) TGF-β1 induction after 24 and 48 h of incubation with peptidomimetics of peptide T (PT) and PT 10^{-8} M in human keratinocyte cells. β-tubulin was used as internal control of protein loaded. The results are representative of three different experiments. (b) HSP-70 induction in human keratinocyte cells treated with peptidomimetics of PT and PT 10^{-8} M for 24 h in presence or not of the anti-TGF-β1 blocker (1 and 5 μM). β-tubulin was used as internal control of protein loaded. The results are representative of three different experiments.

analysed by Western-blot, as described in the *Materials and methods* section. The experiments show that the α-v integrin is modulated by peptidomimetics of PT at the different times studied. Specifically, Fig. 4 shows how SVT-0317 modulates the expression of the α-v integrin in an early stage (after 4 h), in a similar manner how PT does. On the contrary, SVT-0316 and SVT-0318 modulate the α-v integrin expression only after 12 h. At 24 h, the protein level returns to the baseline, independently of the treatment subjected.

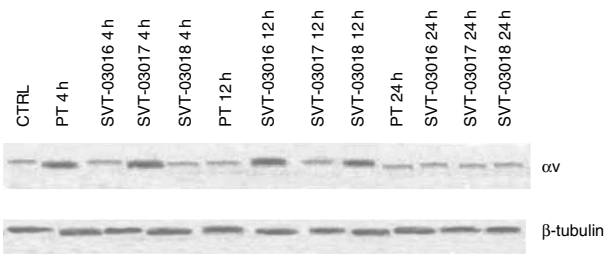

Figure 4. α-v integrin modulation in human keratinocyte cells treated or not with peptidomimetics of peptide T (PT) and PT 10^{-8} M for different times (4, 12 and 24 h). β-tubulin was used as internal control of protein loaded. The results are representative of three different experiments.

Peptidomimetics of peptide T downregulated ICAM-1 in human keratinocytes

Semiconfluent human keratinocyte cells were treated or not with PT and the different peptidomimetics of PT at a concentration of 10^{-8} M during periods of 24 and 48 h, respectively. As shown in Fig. 5, the different peptidomimetics of PT as well as PT itself downregulate ICAM-1 expression already 24 h after the treatment.

Release of cytokines IL-6, IL-8, IL-10, IFN-γ and TNF-α from human keratinocytes stimulated with of peptidomimetics peptide T

Cytokine release was evaluated on the harvested culture supernatants of human keratinocytes (2×10^6 cells/well) seeded in multiwell plates and treated with the different peptidomimetics of PT as well as PT, respectively, at the previously indicated concentrations. The incubation was performed for periods of 24 and 48 h, respectively, at 37 °C in 5% CO_2. All measurements were carried out using monoclonal antibodies. IL-6, IL-8, IL-10, IFN-γ and TNF-α were measured by ELISA.

IL-6, IL-8 and IFN-γ do not exhibit any difference in concentration on keratinocytes stimulated with the different peptidomimetics in comparison to the control keratinocytes or those treated with PT (data not shown). In contrast, IL-10 exhibits an increased concentration in the keratinocytes stimulated during 48 h by PT or the different peptidomimetics of PT. This increase is more evident after stimulation with SVT-03018 (Fig. 6, Panel A). Finally, TNF-α exhibits an overexpression in keratinocytes stimulated by

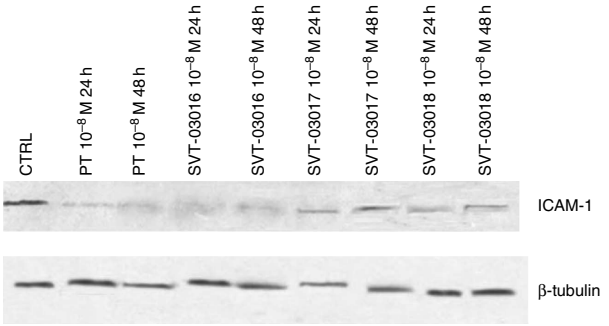

Figure 5. ICAM-1 reduction in human keratinocyte cells treated with peptide T (PT) and peptidomimetics of PT 10^{-8} M for 24 and 48 h. β-tubulin was used as internal control of protein loaded. The results are representative of three different experiments.

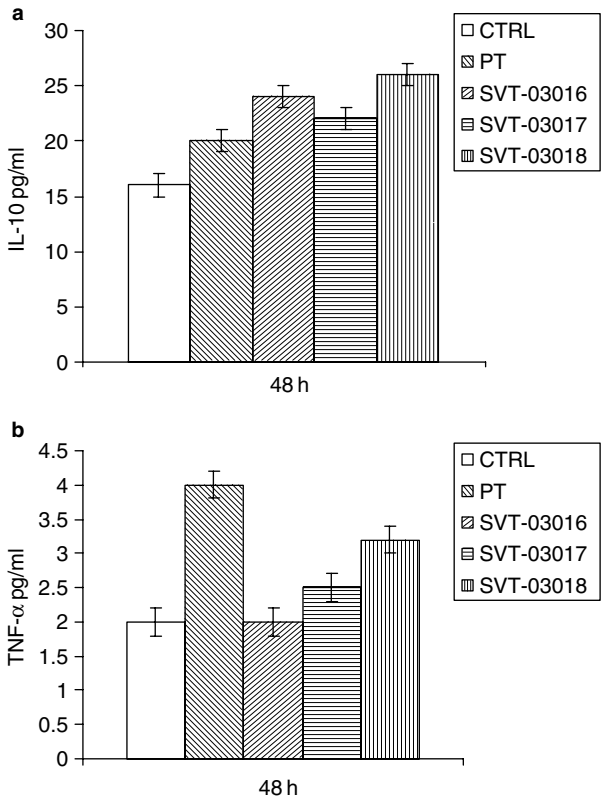

Figure 6. (a) Effect of peptide T (PT) and peptidomimetic of PT 10^{-8} M for 48 h on IL-10, and (b) TNF-α, release from human keratinocyte cells. The results are means ± standards errors of three different experiments. *, significantly higher ($P < 0.05$) than values obtained by control cells, as calculated by analysis of variance.

PT and SVT-03018 after 48 h at a concentration of 10^{-8} M; between the other two analogs, at the same time of stimulation, SVT 03016 (10^{-8} M) do not produce any significant change in regard to the control, while SVT 03017 (10^{-8} M) shows only a moderate increase of TNF-α expression in regard to the control (Fig. 6, Panel B).

Conclusions

Current hypotheses of the aetiology of psoriasis involve cell-mediated immune responses and cytokine expression that invokes an inflammatory response leading to psoriatic plaques. Selective immunomodulation treatment targets these specific immune responses with the goal of less-toxic side-effect profiles and serves as the next generation of therapy in controlling and possibly curing psoriasis. Four basic strategic approaches that focus on the steps involved in the immunopathology of psoriasis are elimination of the pathogenic T cells; inhibition of T-cell activation, proliferation and migration; immune deviation to downregulate the type 1 (TH1) response predominant in psoriasis and blockade of cytokine production (15).

Pioneering clinical trials dealing with immunotherapy include the use of PT (16). However, due to the poor pharmacological profile of peptides, it is sometimes difficult to prove their therapeutic benefit. Our previous studies evidenced the important role played by keratinocytes in justifying the therapeutic activity of PT. The best results were obtained with low concentration of PT, because many peptides at more high concentration show an self-aggregation, that prevents the correct interaction between the peptide and the specific receptor (7). For this reason, we decided to study the biological behaviour of different peptidomimetics of PT on human keratinocytes.

Our results show that the different peptidomimetics of PT tested exhibit a similar behaviour in regard to the overexpression of HSP-70 and TGF-β, as well as the modulation of the former by the latter. Moreover, present results show that SVT-0317 as well as PT modulates early (after 4 h) the expression of the α-v integrin, while the SVT-0316 and SVT-0318 modulate the α-v integrin expression only after 12 h.

Finally, the expression of TNF-α is clearly differentiated. TNF-α is overexpressed by PT and SVT-03018; between the other two analogs, SVT-03016 do not produce any significant change in regard to the control, while SVT-03017 shows only a moderate increase in regard to control. Several studies have demonstrated that TNF-α is implicated in the pathogenesis of psoriasis, and TNF-α levels have been shown to be increased in psoriatic plaques.

IL-10 exhibits an increase in its release from keratinocytes stimulated during 48 h by PT and the different peptidomimetics of PT, particularly by SVT-03018. Early data from patients with psoriasis treated with recombinant IL-10 suggest the therapeutic potential of this cytokine that has receptors in epidermal cells and appears reduced in psoriatic skin (17–20).

Finally, it is interesting to stress the similar behaviour of PT and the different analogues studied on the regulation of proinflammatory cytokines, such as IL-6, IL-8 and IFN-γ, as well as on the reduced ICAM-1 expression. The involvement of their proinflammatory role on psoriasis has been demonstrated, and specifically, the involvement of IFN-γ and ICAM-1 on the adhesion of lymphoblasts to epidermal keratinocytes has been demonstrated (21).

In conclusion, the results of the present work demonstrate that the three analogues of amygdalin with the cyanide group removed exhibit the

same functional properties of PT and can consequently be considered peptidomimetics, suggesting their possible use in the treatment of psoriasis.

The results obtained on HSP 70, TGF-β and α-v integrin expression demonstrate that the different analogues studied have the same effects of PT, with the property of provoking cell–cell contact detachment, inhibition of cellular proliferation, increase of migration of keratinocytes and epidermal regeneration of tissue.

SVT-03016 and SVT-03017 reduce the TNF-α release respect to PT and induce an increment of IL-10 expression comparable with that of PT. Finally, SVT-03018 provokes a remarkable upregulation of IL-10, stronger than SVT-03016, SVT-03017 and PT. Moreover, all the other three analogues reduce comparably with the PT, the expression of ICAM-1 and do not increase the release of proinflammatory cytokines.

Present results point out the differences in the pharmacological profile of the different analogous tested, suggesting further experiments in this direction also with different experimental cellular models.

Acknowledgements

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