Immunostimulation by a Partially Modified *retro-inverso*-Tuftsin Analogue Containing Thr$^1\Psi$[NHCO](*R*,*S*)Lys^2 Modification[†,‡]

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The tuftsin retro–inverso analogue H-ThrΨ[NHCO](*R*,*S*)Lys-Pro-Arg-OH was synthesized through a novel procedure for the high-yield incorporation of isolated retro-inverso bonds into peptide chains and the use of the new Meldrum's acid derivative $(CH_3)_2C(OCO)_2CH(CH_2)_4NHCOCF_3$ followed by its efficient coupling in solution to trimethylsilylated H-D-Thr(*t*-Bu)NH_2. Closely related peptide impurities were eliminated both from the crude final peptide and the fully protected tetrapeptide amide precursor via ion-exchange and reversed-phase displacement chromatography, respectively. The tuftsin retro-inverso analogue proved to be completely resistant to enzymatic degradation in vitro, either against isolated aminopeptidases or human plasma proteolytic enzymes. When administered either orally or intravenously, it was significantly more active than normal tuftsin in increasing the number of specific antibody secreting cells in spleen of mice immunized with sheep erythrocytes. Furthermore, the analogue exerted an enhanced stimulatory effect on the cytotoxic activity of splenocytes against YAC-1 tumor cells. Finally, *retro-inverso*-tuftsin was about 10-fold more potent than the native peptide in reducing rat adjuvant arthritis. The resistance of the retro-inverso analogue to peptidases might explain the increased in vivo activities and allows its further immunopharmacological characterization.

Introduction

Tuftsin, the tetrapeptide threonyllysylprolylarginine (H-Thr-Lys-Pro-Arg-OH), which represents residues 289–292 of the heavy chain of leukokinin, is enzymatically released from the protein in its active state first by splenic tuftsin-endocarboxypeptidase upon cleavage at Arg^{292}-Ser^{293} and then by phagocytic cell membrane leukokininase which sets tuftsin free upon hydrolysis at Lys^{288}-Thr^{289}. Tuftsin potentiates polymorphonuclear granulocytes and tissue macrophage functions such as phagocytosis, motility, pinocytosis, chemotaxis,[1-7] and antibody-dependent cellular cytotoxicity (ADCC)[8,9] as well as bactericidal[10,11] and tumoricidal activity.[11-14] The tetrapeptide modulates also cyclic nucleotides and Ca^{2+} concentration inside the cells.[15] Tuftsin is also able to increase natural killer (NK) activity and, depending on the schedule of administration, the antibody response to both T helper-dependent and T helper-independent antigens.[8,16,17] Moreover, tuftsin was shown to potentiate the antigen-specific macrophage-dependent education of T lymphocytes[17] and, when conjugated to the antigen, to increase interleukin 1 (IL-1) production by macrophages as well as the expression of cell-surface Ia antigens.[18] It is known that these molecules are necessary for T lymphocyte recognition and proliferation[19,20] through the induction of interleukin 2 (IL-2) secretion and IL-2 receptor expression.[21,22]

Despite these pleiotropic activities observed in the experimental system and the encouraging results obtained in cancer therapy,[13] the instability of tuftsin in plasma may strongly reduce its stimulating effect on immunocompetent cells. The tetrapeptide degradation at both chain terminals by carboxy- and aminopeptidase(s) may produce in vivo the two biologically active tripeptides, H-Thr-Lys-Pro-OH and H-Lys-Pro-Arg-OH, which can inhibit tuftsin activity. In fact, apart from H-Lys-Pro-Arg-OH, which inhibits tuftsin after cleavage of the threonine residue by a highly active cytoplasmic aminopeptidase,[23] it has been

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† Abbreviations used in this paper: FPLC, fast protein liquid chromatography; RP-HPLC, reversed-phase high-performance displacement chromatography; HPDC, high-performance displacement chromatography; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; Bzl, benzyl; *t*-Bu, *tert*-butyl; TMSAc, *N,O*-bis(trimethylsilyl)acetamide; TIB, *I,I*-bis(trifluoroacetoxy)iodobenzene; g, geminal (The amino acid residue converted to *gem*-diaminoalkyl derivative is designated by a "g" immediately before the designation for the amino acid); m, malonyl (The malonic acid derivative is designated by a "m" before the analogous amino acid); NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectrometry; Boc, *tert*-butyloxycarbonyl; Me, methyl; TFA, trifluoroacetic acid; NK, natural killer; CFA, complete Freund adjuvant; IFN, interferon, PBS, phosphate-buffered saline.

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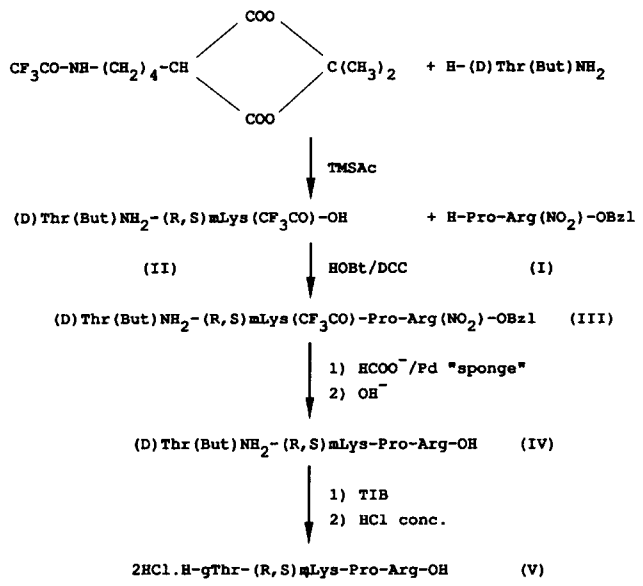

Figure 1. Synthesis of 2HCl·H-gThr-(*R,S*)mLys-Pro-Arg-OH.

recently found that the tripeptide originating from the hydrolysis of the prolylarginine bond is able to inhibit phagocytosis, cell locomotion, and IL-1 production as well as ADCC, β-glucuronidase release, and superoxide anion production.[24,25] Therefore, to protect labile peptide bonds against enzymatic hydrolysis, several tuftsin analogues with amino acid substitutions, protected N- and C-terminals, elongated chains, and derivatized sugar moieties have been prepared so far.[26]

In order to contribute to the solution of this problem we have prepared by chemical synthesis an analogue of tuftsin (*retro-inverso*-tuftsin) in which the bond between Thr and Lys, representing the most vulnerable site of the molecule, has been stabilized against enzymatic hydrolysis by inversion of its direction, i.e. from –CONH– to –NHCO–.

This paper describes the synthesis of the partially modified retro-inverso analogue of tuftsin H-ThrΨ-[NHCO](*R,S*)Lys-Pro-Arg-OH and the control of its chemical and metabolic stability in human plasma and against isolated aminopeptidases in vitro, as well as a preliminary immunopharmacological characterization in experimental models.[27-29]

Chemistry

The synthesis of the tuftsin retro-inverso analogue is schematically depicted in Figure 1. The malonyl residue has been easily inserted in the peptide chain first upon condensation of the new Meldrum's acid derivative 2,2-dimethyl-5-[4-(trifluoroacetamido)butyl]-1,3-dioxane-4,6-dione to H-D-Thr(But)NH_2 in the presence of N,O-bis-(trimethylsilyl)acetamide, and then by coupling the fully protected pseudodipeptide with H-Pro-Arg(NO_2)-OBzl.[30] The trimethylsilylation of D-threonine α-NH_2 is necessary to shift the Meldrum's acid derivative keto–enol equilibrium towards the "reactive" keto form at the expense of the "unreactive" enol form, which predominates in the presence of basic free amino groups.[31]

The scheme was designed to avoid the use of the malonic acid derivative HOOCCH[$(CH_2)_4$NHBoc]COOEt, which can be prepared only in very low amounts following a lengthy and difficult procedure.[32]

The terminal *gem*-diaminoalkyl residue was obtained from the fully protected pseudotetrapeptideamide IV by reaction with TIB, following a well-established procedure.[33-35]

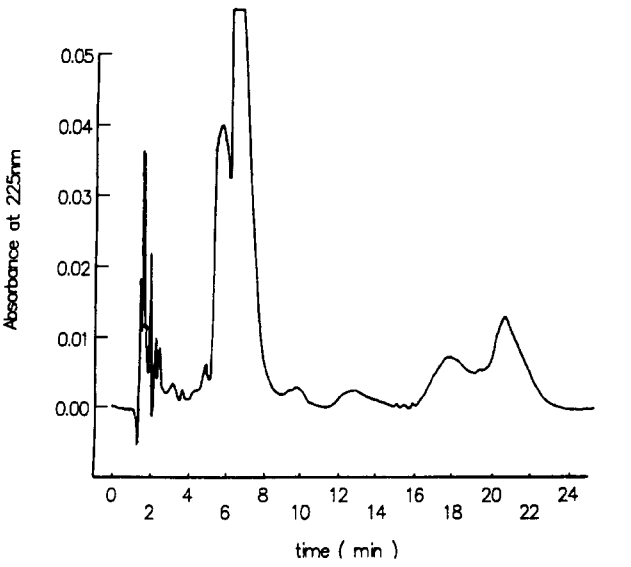

Figure 2. RP-HPLC of crude peptide IV. Column: Licrospher RP-18, 250 × 4.0 mm, 10 μm (Merck). Eluent: 10 mM ammonium acetate, 16.4% CH_3CN. Flow rate: 1.5 mL/min. Sample: 20 μg in 20 μL of eluent.

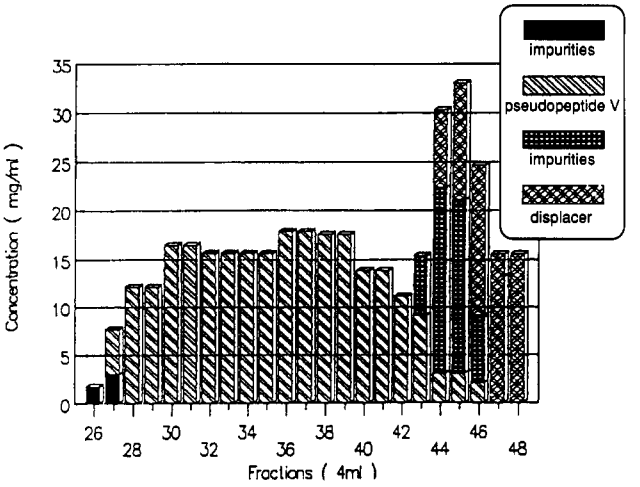

Figure 3. RP-HPDC of crude peptide IV. Column: Lichroprep RP-18, 250 × 20 mm, 25–40 μm. Carrier: 0.1% TFA. Displacer: 50 mM benzyltributylammonium chloride. Flow rate: 2 mL/min. Sample: 1.1 g in 5 mL of 0.1% TFA.

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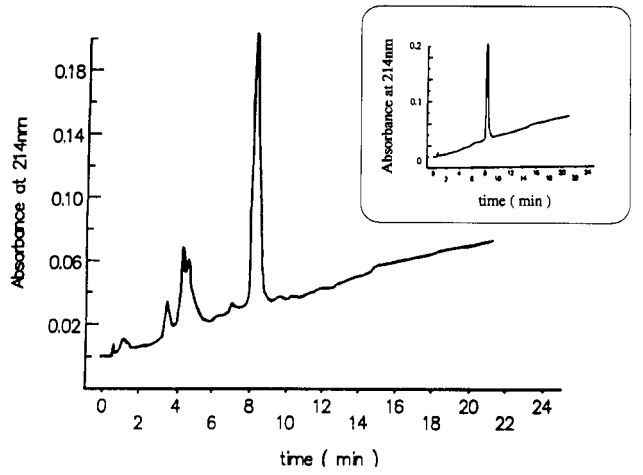

Figure 4. FPLC of crude peptide V. Column: Mono S, 50 × 5 mm, 10 μm (Pharmacia). Eluent: A, 0.1 M NH_4Cl; B, 1 M NH_4Cl; linear gradient of B from 0% to 100% in 20 min; pH 3. Flow rate: 1 mL/min. Sample: 30 μg in 20 μL of 0.1 M NH_4Cl. Inset: FPLC analysis of pure peptide V.

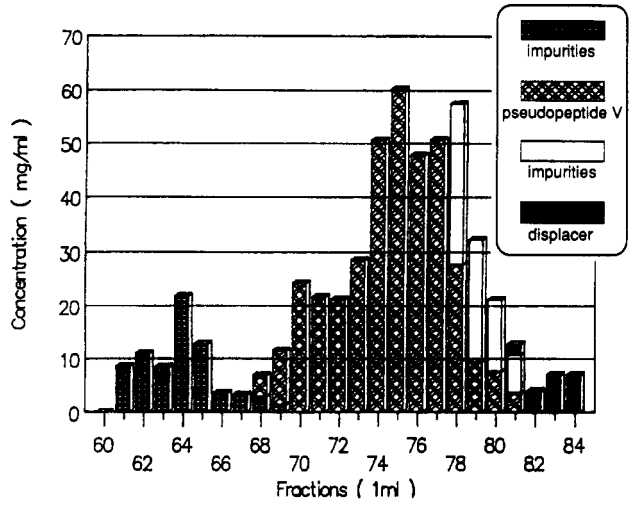

Figure 5. Ion-exchange displacement chromatography of crude peptide V. Column: S Sepharose FF, 450 × 10 mm, 45–164 μm (Pharmacia). Carrier: 1 mM HCl. Displacer: 50 mM triethylenetetraamine, pH 3. Flow rate: 0.5 mL/min.

The purification of the intermediate IV and of the final product V was carried out by HPDC according to Horvath.[36]

When compared to conventional linear elution chromatography, HPDC has the following advantages: (i) higher load capacity per gram of stationary phase (10-fold more), (ii) lower volumes of eluants (100-fold less), and (iii) high concentration of products in the eluate. Most notable is the possibility to resolve closely related peptide impurities which are not easily separated via linear elution.[37]

After a careful choice of the chromatographic critical parameters, we have been able to separate both crude peptide intermediate IV and tuftsin analogue V preparations in the range of hundreds of milligrams to grams. In Figures 2–5 the analytical chromatograms and relative HPDC's of compounds IV and V are reported, respectively. Most important, fractions in the eluate consisted of pure products in aqueous solutions devoid of salts and with a very low acid content (0.1% trifluoroacetic acid or 1 mM HCl).

The elution of both compounds IV and V from the column with low acidity aqueous carriers and in very high concentration (from 20 to 60 mg/mL) markedly facilitated their high-yield final recovery by lyophilization. In fact, as observed in the previous synthesis of retro–inverso peptide analogues containing *gem*-diamine residues at the chain ends, repeated lyophilizations from acidic aqueous solutions were harmful to the integrity of the retro–inverso molecules.

Attempts to purify compounds IV and V by reversed-phase and ion-exchange linear-elution chromatography, respectively, gave disappointing results due to poor load capacity and extensive tailing.

It has been recently shown by HPDC that few as well as several grams of crude peptide mixture can be purified in the same chromatographic conditions just by enlarging the column diameter.[38]

Displacement chromatography should then be of great help in the purification of the multigram quantities of *retro-inverso*-tuftsin needed for preclinical and clinical studies.

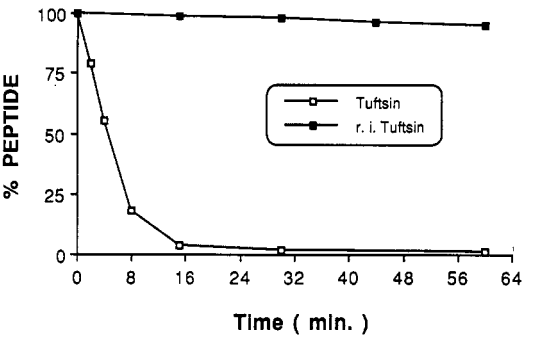

Figure 6. Time course of tuftsin and *retro-inverso*-tuftsin breakdown by human plasma peptidases. The retro-inverso analogue is also stable either to leucine aminopeptidase (EC 3.4.11.1) or amino acid arylamidase (EC 3.4.11.2) from hog kidney in vitro.

Biological Results

We first tested the in vitro stability of normal tuftsin and its retro-inverso analogue against human plasma peptidase(s). As reported in Figure 6, after 50 min only a slight hydrolysis (less than 2%) was observed upon incubation of the *retro-inverso*-tuftsin with a pool of 50 different human plasma samples. The difference after 360 min of incubation was not statistically significant (data not shown). In contrast, normal tuftsin was almost completely hydrolysed in less than 8 min.

On the basis of this evidence, we compared the immunostimulating activity of the two molecules in terms of their ability to enhance the primary antibody response of mice to the T-dependent antigen sheep red blood cells (SRBC).

The specific antibody response of murine splenic lymphocytes to SRBC was significantly augmented by the retro-inverso analogue of tuftsin inoculated iv together with the antigen. In addition, the increase observed was

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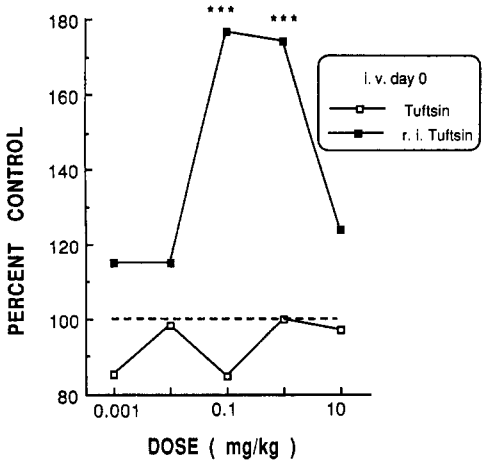

Figure 7. Effect of different doses of tuftsin and *retro-inverso*-tuftsin on the in vivo primary PFC response to SRBC. Drugs were injected iv together with SRBC on day 0. The SRBC-specific PFC response of spleen cells was evaluated 4 days later and expressed as percent of the control response of animals receiving the antigen alone. Statistical significance: $***p < 0.01$ versus control group.

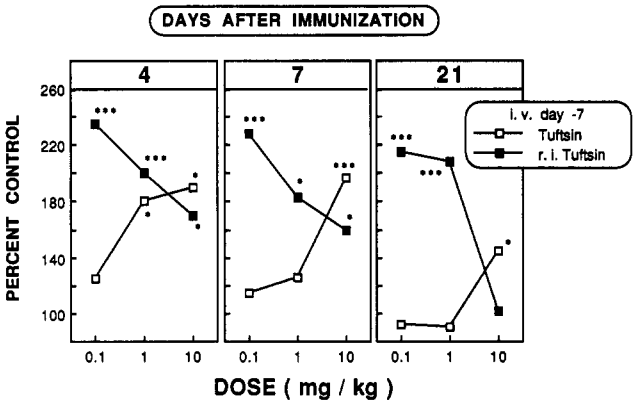

Figure 8. Kinetics of the effect of tuftsin and *retro-inverso*-tuftsin on the in vivo primary PFC response to SRBC. Mice received iv different doses of tuftsin and *retro-inverso*-tuftsin 7 days before the iv inoculum of SRBC. The SRBC-specific PFC response of spleen cells was evaluated at different times after immunization with SRBC and expressed as percent of the control response of animals receiving the antigen alone. Statistical significance: $*p < 0.05$, $***p < 0.01$ versus control group.

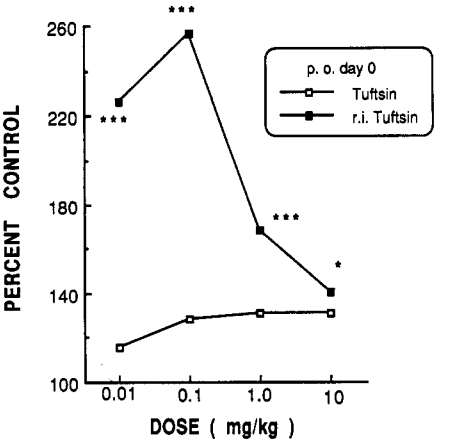

Figure 9. Effect of tuftsin and *retro-inverso*-tuftsin on the primary PFC response to SRBC. Mice received drugs orally (po) on the same day as the iv inoculation of antigen. Direct anti-SRBC splenic PFC were estimated 4 days later and expressed as percent of the control response of animals receiving the antigen alone. Statistical significance: $*p < 0.05$, $***p < 0.01$ versus control group.

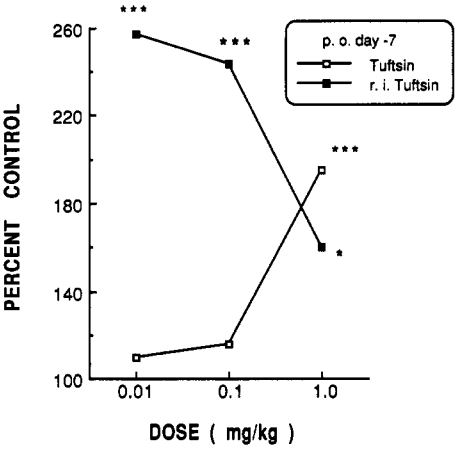

Figure 10. Effect of tuftsin and *retro-inverso*-tuftsin on the primary PFC response to SRBC. Drugs were administered po to mice on day −7. On day 0 mice were injected iv with SRBC. Direct anti-SRBC splenic PFC were estimated 4 days after immunization and expressed as percent of the control response of animals receiving the antigen alone. Statistical significance: $*p < 0.05$, $***p < 0.01$ versus control group.

dose-dependent with a maximal activity at 0.1 mg/kg (Figure 7). Conversely, normal tuftsin never showed a significant immunostimulating effect at all the doses tested.

The kinetics of the primary antibody response to SRBC was investigated by treating mice iv with different doses of tuftsin or its retro-inverso analogue 7 days before the antigen administration (day 0), according to a previous report by Florentin et al.[8] Both peptides were found to significantly increase the splenic PFC response to SRBC, at all times tested after immunization (Figure 8).

Once again the PFC enhancement was dose-dependent, being maximal at 10 mg/kg for normal tuftsin and at 0.1 mg/kg for the retro-inverso analogue.

To test whether the activity of tuftsin and its retro-inverso analogue on the primary PFC response to SRBC was specific in its dependence upon the schedule of treatment, or more likely upon the chemical modification of the molecule, we performed a set of experiments injecting mice orally (po) by intragastric intubation with various doses of drugs on day 0 or −7. Results reported in Figure 9 show that the *retro-inverso*-tuftsin given on day 0 was able to enhance the immune response to SRBC, whereas the native molecule proved to be completely inactive at all doses tested.

As observed with the iv treatment, the oral dose of *retro-inverso*-tuftsin needed to obtain the maximal immunopotentiating effect was 0.1 mg/kg.

Again, both normal and *retro-inverso*-tuftsin molecules administered po on day −7 were capable of enhancing the anti-SRBC PFC response of spleen cells. However, as shown in Figure 10, the PFC enhancement induced by normal tuftsin, as compared with the control, was statistically significant at a dose of 1 mg/kg, whereas *retro-inverso*-tuftsin proved to be more active in terms of number of PFC anti-SRBC even at the dose of 0.01 mg/kg. These were also the highest and lowest doses used of normal and *retro-inverso*-tuftsin, respectively.

The in vivo effect of tuftsin and its retro-inverso analogue on NK activity was also investigated. Twenty-four hours after ip administration of the tetrapeptides, spleen cells from both treated and control mice were tested for their ability to lyse YAC-1 tumor cells. As shown in Figure 11, only mice treated with 250 μg of normal tuftsin ex-

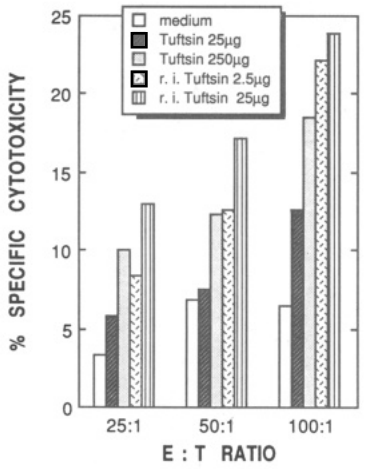

Figure 11. NK activity against YAC-1 tumor cells of murine splenocytes obtained 1 day after the ip administration of medium alone or containing different doses of normal or *retro-inverso* tuftsin. Statistical significance: *retro-inverso*-tuftsin (25 μg) versus corresponding dose of tuftsin: $p < 0.01$.

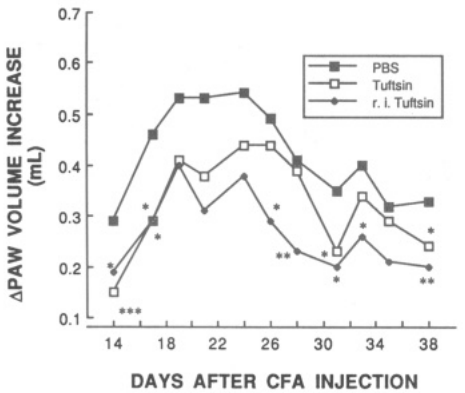

Figure 12. Development of adjuvant arthritis in Lewis male rats administered orally with 1 mL/kg of PBS (control group), 1 mg/kg of tuftsin, or 0.1 mg/kg of *retro-inverso* tuftsin. Results are the mean of 10 animals in each experimental group. For reasons of clarity, standard error mean values have been omitted, but were less than 10% in all cases. The experiment has been repeated twice with identical results. Statistical significance: $*p < 0.05$, $**p < 0.025$, $***p < 0.01$ versus control group.

hibited a significant increase of splenic NK activity. However, the same level of cytotoxicity was observed with the in vivo administration of 2.5 μg of *retro-inverso*-tuftsin. A further increase of the splenic NK activity was observed with 25 μg of the same retro–inverso peptide.

Finally, the effect of the two peptides on a model of chronic inflammation, the rat adjuvant arthritis, was investigated. The data reported in Figure 12 show that the control rats injected with complete Freund adjuvant (CFA) developed the first signs of the secondary lesion, i.e. swelling of the contralateral paw 14 days after the injection, with the arthritis reaching its peak between 18 and 26 days. In the animals treated with tuftsin (1 mg/kg) and its retro-inverso analogue (0.1 mg/kg), the extent of the inflammatory response was clearly reduced. The reduction was statistically significant on days 14, 17, 31, 38 for normal tuftsin and on days 14, 17, 26, 31, 38 for *retro-inverso*-tuftsin.

Discussion

Relevant effects on tuftsin activities, ranging from partial to complete inhibition, have been observed with its analogues carrying even minute structural modifications.

However, it has to be still elucidated whether the loss or change of activity of these analogues was due to their rapid enzymatic degradation or to a major perturbation of the "biologically active" conformation, i.e. the one seen by tuftsin receptor(s). Various approaches were carried out to develop tuftsin analogues stable to leucine aminopeptidase which is localized in neutrophils and is considered to be mainly responsible for the inactivation of the molecule and the appearance of the metabolite inhibitor Lys-Pro-Arg.[39] Apart from the classical substitution of L-threonine with the D isomer,[40] to increase resistance to enzymatic degradation, several investigations have adopted other strategies such as, (i) the elongation of the tetrapeptide chain by addition of further amino acid residues to the N-terminus;[41] (ii) the cyclization,[42] substitution, or derivatization of the threonyl residue;[43] and (iii) the introduction of an isopeptide bond into the molecule by replacing Thr-Lys with Thr-ε-Lys.[44] In spite of such various modifications in position 1 of the molecule, many activities of tuftsin were preserved, suggesting that the threonyllysyl terminus is not so critical in influencing the conformational properties of the peptide.

Thus, from structure–function studies of the tuftsin analogues prepared so far, it seems reasonable to infer that the entire tetrapeptide sequence is necessary for the expression of the full spectrum of tuftsin activities.

In order to clarify this issue we have synthesized the partially modified retro-inverso analogue of tuftsin H-ThrΨ[NHCO]Lys-Pro-Arg-OH and tested it for its biological activity.

Such an analogue, which contains a single backbone modification at the extremity of the chain but largely retains the spatial, electronic, and hydrogen bond typical of the original peptide bond, can be topologically considered very similar to tuftsin in the extended conformation as suggested by Goodman and colleagues in the discussion of the general conformational properties of short, linear retro-inverso peptide chains.[45,46]

Probably, the lower energy conformation (β-turn or hairpin with a two-split-ends structure), suggested by theoretical calculations as well as spectroscopical studies, should also be minimally perturbed.[47–49]

Indeed, we have demonstrated that the tuftsin analogue with the reversed peptide bond between Thr and Lys possesses an enhanced immunostimulatory activity in different experimental systems when compared with normal tuftsin. A precise assessment of the possible mechanism responsible for the potentiated stimulation by *retro-inverso*-tuftsin is quite complex, mainly because the

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effects of the modified compound on the immune system have been so far marginally investigated.

It has been repeatedly reported that retro–inverso analogues are less susceptible than natural peptides to hydrolysis by proteolytic enzymes.[35,45,46] The more potent activity of the *retro-inverso*-tuftsin administered orally or intravenously, as compared with the native peptide, may be related to its higher stability in gastric and plasma environments. In fact, *retro-inverso*-tuftsin is completely resistant to human plasma peptidase(s) in vitro, while under the same conditions normal tuftsin undergoes an early enzymatic degradation (Figure 6). Similar results were obtained when the stability of the retro-inverso analogue was tested against isolated aminopeptidases and tissue homogenates (hog brain, liver, kidney, and human lung) in vitro. High concentrations of the enzymatically split metabolite H-Lys-Pro-Arg-OH may exert a negative feedback control of tuftsin level. This tripeptide and particularly the dipeptide H-Pro-Arg-OH are probably recognized by specific receptors on cell surface.[16]

On the other hand, the presence of an intact Thr side chain could be crucial for expression of maximal activity since its replacement drastically reduces the stimulatory effect of tuftsin.[50]

Retro-inverso peptide N-termini undergo chemical degradation due to some instability of their monoacyl–*gem*-diamine moieties. In regard to the synthesis of the tuftsin retro-inverso analogue of this study, we have isolated the major degradation product and demonstrated its identity by FAB-MS analysis. It is the pseudotripeptide $H_2NCOCH[(CH_2)_4NH_2]CO$-Pro-Arg-OH, according to Loudon's mechanism of hydrolysis of the monoacyl–*gem*-diamines.[51] Since the extent of degradation of the tuftsin analogue at pH = 7.0 and 37.0 °C is less than 10% in 3 h, the concentration of the degradation products and hence their effect on the activity tests was judged negligible. We believe that the absence of the COOH terminal tripeptide metabolite could explain the difference in potency between tuftsin and its retro-inverso analogue observed in the in vivo tests.

When injected iv in mice 7 days before the antigen administration, both molecules enhanced the primary immune response to SRBC, measured as the number of antibody secreting cells in the spleen. However, the retro–inverso analogue proved to be more active than tuftsin, being able to induce the same level of immunostimulation at 100 times lower doses. The difference in potency was even more evident when molecules were injected iv together with the antigen. In fact, while the retro-inverso analogue was able to enhance the number of PFC specific for SRBC, the natural peptide proved to be completely inactive.

As far as normal tuftsin is concerned, our results confirm those obtained by Florentin and co-workers,[8] who have reported that in the same experimental system a single iv injection of tuftsin could enhance the antibody response to the T-dependent antigen TNP-KLH, only when given 7 days before immunization.

Interestingly, the adjuvant-like effect of the retro-inverso analogue, as compared with normal tuftsin, was also pronounced while administering orally either 7 days before or together with the antigen inoculum. These findings further indicate that time and route of administration are not critical parameters for the immunostimulating activity of the *retro-inverso*-tuftsin, as occurs for the native molecule and other biological response modifiers.

The more potent stimulation induced by *retro-inverso*-tuftsin on the immune response of mice to SRBC, may be related to a higher stability toward either plasma or gastric enzymes conferred to the molecule by retro-inversion.

A more pronounced effect of *retro-inverso*-tuftsin compared to the native tetrapeptide was also observed on NK activity. In fact, while in agreement with previous results,[8] the natural tetrapeptide had a slight effect on the cytotoxic activity of spleen cells against YAC-1 tumor cells, while the in vivo administration of 100-fold lower doses of the *retro-inverso*-tuftsin resulted in a marked increase of the NK activity. With regard to this, it has been recently found that, as compared with the native tetrapeptide, *retro-inverso*-tuftsin is able to induce the synthesis of higher levels of interferon-γ (IFN-γ) by human peripheral blood mononuclear cells in vitro (Paulesu et al., personal communication). Since IFNs have been shown to strongly increase the cytotoxic activity of NK cells,[52] this finding suggests that the increase of NK activity observed in our experiments may be the result of IFN-γ induction in vivo.

A different range of potency between the two tetrapeptides was again observed in rat adjuvant arthritis, an immunologically mediated chronic inflammatory reaction. The *retro-inverso*-tuftsin was about 10-fold more potent than the native tetrapeptide in reducing the development of the inflammation. This antiinflammatory action, like NK activity, may be ascribed, at least in part, to an increased IFN-γ production. In fact, it has been shown that antibodies to IFN-γ are able to modulate the development of the rat adjuvant arthritis.[53] Moreover, although the mechanism of action of IFN-γ in the inflammation is quite complex, IFN-γ inducers are antiinflammatory agents[54] and the IFN-γ treatment has been reported to be beneficial in both rheumatoid and psoriatic arthritis.[55]

Taking these findings as a whole, it is suggested that the in vivo exposure to *retro-inverso*-tuftsin results in an early activation of macrophages either as accessory or regulatory or effector cells.

The results discussed above also indicate that the reversal of the first peptide bond in the tuftsin sequence does not significantly perturb the topology of the molecule, since only the preservation of the right orientation of all side chains can guarantee a correct interaction with putative tuftsin receptor(s).[56] Moreover, they indicate that the backbone N-terminus can tolerate structural modifications with retention of activity. A tuftsin analogue with the Thr-Lys bond replaced by the CH_2NH amide surrogate shows an activity equivalent or superior to that of tuftsin.[57]

As far as the synthesis is concerned, this report describes for the first time a practical procedure for the use of 5-substituted Meldrum's acid derivatives in the preparation of retro-inverso analogues of biologically active peptides. Meldrum's acid derivatives were suggested some years ago as a logical and general simplification of the synthesis of retro–inverso peptides.[58] As they are protected and ac-

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tivated malonic acid residues, many synthetic manipulations involving the malonyl residue can be eliminated.[59]

However, Meldrum's acid derivatives have never been employed in practice in the synthesis of peptidomimetics due to their sluggish acylation of the free amino groups of amino acid and peptide derivatives. As described in the Experimental Section, Meldrum's acid and its 5-substituted derivatives rapidly and efficiently react only after α-NH_2 trimethylsilylation of amino acid esters and protected peptides to give the corresponding malonylic acid derivatives in good to excellent yield (one-pot procedure). The malonylic acid derivatives carry a free COOH and can be used directly as "carboxylic components" in the synthesis of retro-inverso peptides, thus avoiding the saponification step of the previous procedure.[60]

The facile preparation of Meldrum's acid derivatives and their efficient coupling to D-amino acid amides and to suitably protected chiral geminal alkyl diamines, will certainly provide further impetus to the synthesis of retro-inverso analogues of biologically active peptides.[31]

Experimental Section

Materials. 2,2-Dimethyl-1,3-dioxane-4,6-dione was purchased from Janssen Chimica (Beerse, Belgium) and recrystallized from acetone/water. All the other materials were of reagent grade and used without further purification. Acetonitrile of HPLC grade, Milli-Q-purified water, and distilled trifluoroacetic acid were used for preparation of chromatography solvents. Melting points were measured with a Büchi capillary melting point apparatus and are uncorrected. A Merck-Hitachi gradient HPLC system, equipped with a Model 655A-61 processor, a Model 655A-11 pump, and a Model 655A-71 proportioning valve, and a PYE UNICAM PU 4025 UV detector, operating at 230 nm, were used. A 4 mm × 25 cm Hibar reverse-phase (Lichrosorb RP-18, 10 um) column was employed. Buffer A was 10% acetonitrile in 0.1% aqueous trifluoroacetic acid, while buffer B was 0.1% trifluoroacetic acid in acetonitrile. Unless otherwise stated, analytical runs were conducted using a two-step linear gradient from 0% to 40% B in 20 min and from 40% to 80% B in 10 min.

^1H NMR spectra were obtained with a Varian XL-400 instrument.

2,2-Dimethyl-5-[4-(trifluoroacetamido)butyl]-1,3-dioxane-4,6-dione [Lys(CF_3CO) Meldrum's Acid Derivative]. Trifluoroacetic anhydride (29.4 mL, 210 mmol) was added dropwise throughout 2 h to a solution of 4-aminobutyraldehyde diethyl acetal (32.2 g, 200 mmol) and 4-(dimethylamino)pyridine (25.6 g, 210 mmol) in dichloromethane (600 mL) stirred under N_2 at 0 °C. The reaction mixture was allowed to reach room temperature and stirred for 1 h; the solid was filtered off and the filtrate was washed with water, dried over Na_2SO_4, and evaporated under reduced pressure to obtain a colorless oil (47.8 g, 186 mmol) which was suspended in HCl (1 N, 500 mL) and stirred at room temperature until a homogeneous solution was obtained (about 15 min). The pH was brought to 6 by addition of solid $NaHCO_3$ and the solution was heated on a steam bath for 15 min; after cooling at room temperature, the solvent was partially evaporated under reduced pressure to obtain a volume of about 200 mL.

The aqueous solution was extracted several times with dichloromethane and the combined organic extracts were dried over Na_2SO_4 and evaporated to yield an oily mixture (27 g) containing about 60% (by HPLC) of aldehyde. The oil was added to a solution of Meldrum's acid (12.1 g, 84 mmol) and $NaCNBH_3$ (3.72 g, 59 mmol) in *N,N*-dimethylformamide (60 mL). After stirring at room temperature for 1 h, water (200 mL) was added and the pH adjusted to 4.5 with HCl (1 N). The resulting precipitate was collected by filtration, washed with diethyl ether, and dried in vacuo. Yield: 17.5 g (28%).[62] Mp: 131–132 °C. HPLC: 18.74 min (100% pure). ^1H NMR (DMSO-d_6) 9.4 (t, NH), 4.45 (t, $C_\alpha H$), 3.18 (q, $C_\epsilon H_2$), 1.9 (q, $C_\beta H_2$), 1.8 (s, CH_3), 1.65 (s, CH_3), 1.50–1.35 ppm (m, $C_\gamma H_2$–$C_\delta H_2$). Anal. Calcd for $C_{12}H_{16}O_5NF_3$ (311.258): C, 46.30; H, 5.15; N, 4.50. Found: C, 46.48; H, 5.31; N, 4.61.

H-Pro-Arg(NO_2)-OBzl (I). Intermediate I was prepared as reported by Fridkin et al.[61]

HO-(*R,S*)mLys(CF_3CO)-D-Thr(*t*-Bu)NH_2 (II). *O-tert*-Butyl-D-threonine amide (3.5 g, 20 mmol) and TMSAc (8 mL, 40 mmol) were dissolved in 90 mL of dichloromethane and refluxed for 6 h. To the solution cooled at room temperature was added the Meldrum's acid derivative $(CH_3)_2C(COO)_2CH(C-H_2)_4NHCOF_3$ (5.6 g, 18 mmol) prepared as previously reported.[62] After stirring for 18 h, the reaction mixture was extracted with a 5% citric acid solution and then with water and finally dried over sodium sulfate. The solution was evaporated under vacuum to give an oil which was triturated in acetone/water. The yield was 5.2 g, corresponding to 66%. HPLC: 14.5 min, 16.2 min (corresponding to the two diastereomers). Both peaks showed identical EI-MS spectra. HPLC: 14.9 min, 16.2 min (corresponding to the two diastereomers). Anal. Calcd for $C_{17}H_{28}O_6N_3F_3$ (427.424): C, 47.77; H, 6.6; N, 9.83. Found: C, 47.90; H, 7.0; N, 9.92.

H_2N-D-Thr(*t*-Bu)-(*R,S*)mLys(CF_3CO)-Pro-Arg(NO_2)-OBzl (III). DCC (1.0 g, 5 mmol) was added to a solution of the pseudopeptide amide II (2.1 g, 5 mmol) and HOBt (0.7 g, 5 mmol) in 30 mL of dichloromethane and 4 mL of DMF. The mixture was stirred for 30 min at 0 °C and for 30 min further at room temperature. The reaction mixture was then filtered and the filtrate added to a solution of prolyl-N^ω-nitroarginine benzyl ester hydrochloride (2.1 g, 5 mmol) in 20 mL of dichloromethane and triethylamine (0.7 mL, 5 mmol). After stirring for 18 h at room temperature and evaporation under vacuum, the oily residue was dissolved in saturated sodium bicarbonate aqueous solution and ethyl acetate. The organic layer was separated, and washed with water, dried over sodium sulfate, and evaporated under vacuum to yield a solid product. The yield was 3.8 g (92%). HPLC: 23.3 min, 23.9 min. Anal. Calcd for $C_{35}H_{52}O_{10}N_9F_3$ (815.854): C, 51.53; H, 6.42; N, 15.45. Found: C, 51.33; H, 6.89; N, 15.87.

H_2N-D-Thr(*t*-Bu)-(*R,S*)mLys-Pro-Arg-OH (IV). One gram of palladium "sponge" and ammonium formate (0.3 g, 4.5 mmol) were added to a solution of the protected pseudotetrapeptide III (1.25 g, 1.5 mmol) in 20 mL of methanol. After stirring for 2 h at room temperature, the palladium was filtered off and the filtrate was evaporated under vacuum. The oily residue was dissolved in 10 mL of methanol and 20 mL of water, and the pH adjusted to 12 using a 1 N aqueous solution of NaOH in a "pH-stat" apparatus. After stirring for 1 h at room temperature, the reaction mixture was acidified to pH 5 with 1 N aqueous solution of HCl and evaporated under vacuum. The crude material (1.1 g), whose analytical chromatogram is reported in Figure 2, was purified by HPDC. The chromatographic conditions are described in the caption of Figure 3. The concentration and purity of the fractions were measured by isocratic RP-HPLC analysis carried out at the same conditions of Figure 2, using 10 mM ammonium acetate and 16.4% acetonitrile as eluent. The pure compound was recovered from the pool of fractions 29–43 with a yield of 0.92 g (87%). Anal. Calcd for $C_{28}H_{49}O_9N_8F_3$ $^1/_2H_2O$ (707.758): C, 47.51; H, 7.10; N, 15.83. Found: C, 47.70; H, 6.80; N, 16.20.

2HCl·H-gThr-(*R,S*)mLys-Pro-Arg-OH (V). The trifluoroacetate salt of the partially protected pseudotetrapeptide (IV) (0.55 g, 0.79 mmol) dissolved in 30 mL of a 1:1 mixture of acetonitrile and water was reacted with TIB (0.52 g, 1.2 mmol). After stirring for 6 h at room temperature, the reaction mixture was evaporated under vacuum and the residue dissolved in 15 mL of concentrated HCl and stirred for 8 min at 0 °C. After evaporation under vacuum the residue was redissolved in water and evaporated to dryness several times. Finally, the solution was lyophilized and the residue, after FPLC analysis (Figure 4), was purified by ion-exchange displacement chromatography on a S Sepharose FF column (450 × 10 mm, 45–164 μm; Pharmacia) with aqueous solutions of 1 mM HCl (carrier) and 50 mM triethylenetetraamine (displacer). The collected fractions were

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analyzed on a Supelco LC-18 DB column (133 × 4.6 mm, 3 μm; Supelchem, Milan, Italy) with a linear gradient of B (0.1% TFA, 60% CH_3CN) from 2% to 5% in A (0.1% TFA) in 5 min; the relative histogram is reported in Figure 5. The pure pseudopeptide V was recovered as the hydrochloride salt from fractions 70–77 in a yield of 0.305 g (72%). The corresponding chromatographic profile is shown in the insert of Figure 4. One- and two-dimensional H NMR spectra in DMSO-d_6 confirmed the identity of the product. The characteristic resonances of *gem*-diamino CH (4.9 ppm) and malonyl CH (3.55 ppm) were observed in the spectra. All other resonances were assigned upon examination of the dimensional maps. The two diastereomers were named A and B for the peak assignments (ppm from TMS): 8.65 ($N_\alpha H$ gThr (B), d), 8.4 ($N_\alpha H$ gThr (A), d), 8.3 ($N_\alpha H$ Arg (A), d), 8.2 ($N_\alpha H$ gArg (B), d), 7.8 ($N_\epsilon H^+$ mLys, br), 7.7 ($N_\epsilon H$ Arg, t), 6.9–7.5 ($N_{\omega,\omega'}H_2^+$ Arg, br), 5.75 (OH gThr, br), 4.9 ($C_\alpha H$ gThr, d), 4.4 ($C_\alpha H$ Pro (A), m), 4.35 ($C_\alpha H$ Pro (B), m), 4.15 ($C_\alpha H$ Arg, m), 3.9 ($C_\beta H$ gThr, br s), 3.55 ($C_\alpha H$ mLys + $C_{\delta'}H_2$ Pro, m), 3.45 ($C_{\delta''}H_2$ Pro, m), 3.10 ($C_\delta H_2$ Arg, br d), 2.75 ($C_\epsilon H_2$ mLys, br s), 2.05 ($C_{\beta'}H_2$ Pro, m), 1.6–1.9 ($C_{\beta''}H_2$ Pro, $C_\gamma H_2$ Pro, $C_\beta H_2$ mLys, $C_\beta H_2$ Arg, m), 1.55 ($C_\gamma H_2$ Arg, $C_\delta H_2$ mLys, m), 1.3 ($C_\gamma H_2$ mLys, m), 1.1 ppm ($C_\gamma H_3$ gThr, d). Anal. Calcd for $C_{21}H_{42}O_6N_8Cl_2$ (573.524): C, 43.98; H, 7.38; N, 19.54. Found: C, 44.50; H, 6.98; N, 19.95. FAB mass spectra, run on a Kratos MS 80 as previously described for partially modified retro-inverso peptide analogues, gave for [M + H] m/z 501 as expected.[63] A detailed analysis of the fragmentation pattern by tandem mass spectrometry has been carried out as reported for other partially modified retro-inverso homologues and will be published elsewhere.

Stability Studies. The stability of tuftsin and *retro-inverso*-tuftsin chains against human plasma proteases (Figure 6) and isolated enzymes was studied following a previously described method.[64]

Plaque Forming Cell (PFC) Assay. Antibody producing cells in spleen of mice were determined according to the Cunningham and Szenberg[65] slide technique. Briefly, C3H/HeNCr 1BR male mice (Charles River Italia, Calco, Italy), aged 10–12 weeks and weighing 25 g, received iv on day 0 a single inoculum of 2×10^8/0.2 mL of sheep red blood cells (SRBC, Sclavo, Siena, Italy), previously washed and resuspended in pyrogen-free saline. The same mice were injected iv and po (intragastric intubation) on day 0 or −7 with saline alone or containing different doses of normal (Sigma Chemical Co., St. Louis, MO) or *retro-inverso*-tuftsin. For use as indicator cells in the PFC assay, pellets of washed SRBC were resuspended to 10% in Eagle's minimum essential medium (M.A. Bioproducts, Walkersville, MD). Direct anti-SRBC PFC were evaluated 4 days after immunization in the presence of guinea pig serum (Sclavo, Siena, Italy) as a source of complement at a final dilution of $^1/_{64}$. Results evaluated as the geometric mean of PFC/spleen from three mice assayed individually were expressed in Figures 7–10 as percent of the control response of animals receiving the antigen alone.

Cytotoxicity Assay. BALB/c female mice, aged 6–8 weeks, originally obtained from Charles River Italia (Calco, Italy) and bred in our animal facilities, were injected ip on day −1 with pyrogen-free saline alone or containing various amounts of normal and *retro-inverso*-tuftsin. On day 0, pooled spleen cells from three mice of control and experimental groups were obtained by gentle teasing. After filtration through nylon gauze, the single cell suspensions were washed twice and adjusted to the desidered concentration with RPMI-1640 medium (GIBCO-Europe, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Sterile Systems, Logan, UT), 2 mM L-glutamine, 0.1 mM nonessential amino acids (GIBCO, Grand Island, NY), 25 mM HEPES buffer, 1.25×10^{-5} M 2-mercaptoethanol, and 50 μg/mL gentamycin sulfate (Sigma Chemical Co., St. Louis, MO), hereafter referred to as complete medium. The FBS used throughout the experiments was free of LPS contamination. The YAC-1 tissue culture line, a Moloney virus-induced lymphoma of A/Sn origin,[66] was maintained in vitro in complete medium and used as target cells in the cytotoxicity assay. Tumor cells, suspended in 1 mL of complete medium, were incubated for 90 min at 37 °C with 150 μCi (7.4 MBq) of $Na_2{}^{51}CrO_4$ (Amersham International, Amersham, Great Britain). After washing, 1×10^4 labeled target cells were incubated for 4 h at 37 °C with effector spleen cells at various effector to target (E:T) ratios in 0.6-cm round-bottomed wells (Costar, Cambridge, MA), and the assay was performed as described.[67] The results, expressed as percentage (%) of specific cytotoxicity (Figure 11), were calculated from the average counts per minute (cpm) of triplicate samples as follows:

$$100 \times \frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm incorporated} \times 0.8 - \text{cpm spontaneous release}}$$

Adjuvant Arthritis. The inflammatory response was induced in Lewis male rats (200–220 g) as previously described.[68] Rats were randomized in three groups of 10 animals each and injected on day 0 with 0.2 mL of CFA (10 mg/mL *Mycobacterium tuberculosis*, Difco, Detroit, MI) into the right hind paw. The development of the arthritis was assessed by measuring the volume of the controlateral hind paw (secondary lesion) with a water plethysmometer (U. Basile, Comerio, Italy). Tuftsin (1 mg/kg) and *retro-inverso*-tuftsin (0.1 mg/kg) were administered orally three times a week throughout 4 weeks starting from day 0. This dosing regimen was established on the basis of preliminary experiments. The control group received the vehicle (PBS, 1 mL/kg).

Statistical Analysis. Statistical significance was calculated by Student's t test.

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