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Tuftsin-AZT conjugate: potential macrophage targeting for AIDS therapy

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Abstract: The IgG-derived immunomodulating peptide tuftsin, Thr-Lys-Pro-Arg, is recognized by specific receptors on phagocytic cells, notably macrophages, and is capable of targeting proteins and peptides to these sites. Aiming to target 3′-azido-3′-deoxythymidine (AZT) to HIV-infected macrophages, a conjugate of AZT with tuftsin was synthesized. The AZT-tuftsin chimera possesses the characteristic capacities of its two components. Thus, like AZT, it inhibits reverse transcriptase activity and HIV-antigen expression, and similarly to tuftsin, it stimulates IL-1 release from mouse macrophages and augments the immunogenic function of the cells. Importantly, the conjugate is not cytotoxic to T-cells. The results suggest that the AZT-tuftsin conjugate might have potential use in AIDS therapy. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: AZT; tuftsin; HIV; macrophages

INTRODUCTION

The inhibitor of viral reverse transcriptase 3′-azido-3′-deoxythymidine (AZT) is one of the major drugs currently employed in AIDS therapy [1], affecting both enhanced survival and the quality of life in treated individuals. However, AZT application is accompanied by toxic side effects, particularly destruction of bone marrow cells that stems from its powerful cytotoxic nature [2]. Hence, administration of AZT, as well as other cytotoxic agents such as dideoxycytidine (ddC) or dideoxyinosine (ddI), directly to the human immunodeficiency virus (HIV)-infected cells is highly desirable. Decreasing the required drug doses and preventing or minimizing their action on non-infected cells would also reduce the harmful side effects. Potentially this objective may be achieved by covalent attachment of the therapeutically active agent of choice to a leading-targeting molecule, such as peptide or antibody, which is recognized by specific receptors of selected cells. Several attempts toward the realization of AZT-targeting were made, including its conjugation to the anti-transferin receptor antibody OX-26 for brain targeting [3], its attachment to a 14-amino acid residue peptide for targeting toward the chemokine receptor on the T cell-line [4], or to a neoglycoprotein-director toward CD4 lymphocytes [5].

The immunoglobulin G associated phagocytosis-stimulating-peptide tuftsin, Thr-Lys-Pro-Arg, augments a wide spectrum of biological activities expressed by phagocytic cells, primarily macrophages, monocytes and neutrophils [6]. Following rather avid binding to specific receptors on these cells, i.e. binding constants of $\sim 10^{-8}$ M [6–9], tuftsin notably enhances phagocytosis, cell motility, bactericidal and tumoricidal activities, immune response and the release of cytokines such as IL-1, IL-6 and TNF, indicating that the peptide may have significant and diverse clinical applications [6]. Initial studies on human cancer patients demonstrated that tuftsin is non-toxic and its administration leads to marked leukocytosis, i.e. elevation in white blood cells (WBC) and enhanced cytotoxicity of patients' mononuclear cells toward tumor target cells [6,10,11]. Leukopenia, i.e. a reduction in WBC count, occurs in a large proportion of AIDS patients. This may be due to HIV affecting the bone marrow stem cells, as well as due to the application of substances causing myelotoxic side effects [2]. With regard to clinical implications, the occurrence of leukopenia in AIDS patients means that certain therapies, e.g. chemotherapy of Kaposis sarcoma or lymphoma, or AZT therapy, should not be applied. Moreover, certain opportunistic infections are likely to be more frequent in patients with severe leukopenia than in those with normal or a mild reduction in WBC. The effect of tuftsin on leukocytosis leading

Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; DMEM, Dulbecco's modified Eagle's medium; HIV, human immunodeficiency virus; HPLC, high pressure liquid chromatography; PBS, phosphate buffer saline; PMC, 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl; TLC, thin layer chromatography; WBC, white blood cells.

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to an elevation in WBC indicates that co-administration of AZT, or other cytotoxic agents, with tuftsin may result in beneficial therapy.

A primary target cell of tuftsin is the macrophage [6], a major target cell and host of HIV. It is probable that HIV-infected macrophages directly participate in the pathogenesis of HIV-induced immunosuppression and in central nervous system dysfunction [12,13]. Because unique receptor sites for tuftsin are present on macrophages [6], the peptide can potentially direct drugs like AZT to cells. Although macrophage targeting of AZT was reported [14,15], its specific receptor-mediated delivery has never been realized. Tuftsin is capable of directing proteins and peptides to macrophages in a receptor-mediated fashion, with consequent cellular activation [16,17]. During activation of macrophages, there is a significant augmentation in the production of reactive oxygen compounds, such as $O_2^{-\bullet}$ and H_2O_2, as well as chloramines by these cells. These highly reactive compounds serve, in part, to kill bacteria, aberrant cancer cells and viruses [18]. Tuftsin augments most significantly the formation of $O_2^{-\bullet}$ and H_2O_2 in macrophages suggesting that it may serve as an anti-HIV drug [19]. Tuftsin is an endogenous peptide residing at position 289–292 of a specific leukocytes-associating γ-globulin fraction leukokinin. It is released from leukokinin following initial proteolysis by a splenic endocarboxypeptidase and a subsequent cleavage by leukokininase, a neutrophilic surface enzyme [20]. Consequently, in the absence of the spleen or in the event that its function is highly compromised, tuftsin remains attached to the parent protein carrier. It has been known that splenectomy results in lowered resistance to infection and bacterial invasion. This may be attributed, in part, to decreased tuftsin levels [21,22]. Reduced tuftsin activity, paralleled by decreased splenic functions, was found in patients suffering from AIDS or from AIDS-related complex (ARC) [23].

In view of the above, it seems that tuftsin may serve, concomitantly, both as a specific drug carrier toward macrophages and perhaps, in as yet unknown manner, by itself as an anti-AIDS measure.

The purpose of the present study was to prepare a conjugate between tuftsin and AZT and to evaluate its capacity to express the beneficial characteristics of its two components. This could lead to a possible novel anti-AIDS therapy.

EXPERIMENTAL SECTION

Fmoc-Arg-(Pmc)-O-AZT (I)

DCC (103 mg, 0.5 mmol) was added to an ice-cold solution of Fmoc-Arg(Pmc)-OH (663 mg, 1.0 mmol) in anhydrous dichloromethane (5 ml). After 20 min stirring, the mixture was filtered, washed with dichloromethane (3 ml) and the filtrate and washings were added to a suspension of AZT (134 mg, 0.5 mmol) (Sigma, USA) in anhydrous dichloromethane. DMAP (33 mg, 0.27 mmol) was added and the reaction mixture was kept for 30 min at 0 °C. After 4 h at room temperature, the precipitated DCU was removed by filtration and the filtrate was evaporated to dryness in vacuum. The crude product was purified by low pressure chromatography on a silica gel 60 4 × 20 cm column (dichloromethane–methanol 98 : 2 v/v as the eluant). Yield 365 mg (80%); m.p. 118°–119 °C; $[\alpha]D^{20°}$ + 14.3 (c 1.2 : chloroform) Anal. Calcd. for $C_{45}H_{53}N_9O_{10}S$ (912.02) : C 59.62, H, 5.85, N 13.82. Found: C, 58.71, H 5.93, N 13.62. ^{1}H NMR ($CDCl_3$, 200 MHz) data are shown in Table 1.

H-Arg-(Pmc)-O-AZT (II)

Fmoc-Arg-(Pmc)-O-AZT (1.03 g, 1.125 mmol) was dissolved in morpholine (20 ml). After 60 min at room temperature, ethyl ether (350 ml) was added and the resulting precipitate was collected by filtration, washed with ether and dried. Yield 581 mg (75%); m.p. 75°–78 °C (softening); [a] $D^{20°}$ + 15.3 (c 1.1, chloroform), single spot by TLC (on TLC silica gel, 60 F254, Merck A.G.) in *n*-butanol : acetic acid : water (3 : 1 : 1, v/v).

Anal. Calcd. for $C_{30}H_{43}N_9O_8S$ (689.79); C 52.2, H 6.28, N 18.27: Calcd. for $C_{30}H_{43}N_9O_8S{\cdot}H_2O$ (707.80): C 50.96, H 6.40, N 17.81. Found: C 50.96, H 6.31, N 17.83.

Boc-Thr-Lys(Boc)-Pro-Arg(Pmc)-O-AZT (III)

Boc-Thr-Lys(Boc)-Pro-OSu (580 mg, 0.9 mmol) and H-Arg(Pmc)-O-AZT (525 mg, 0.76 mmol) were dissolved in anhydrous dichloromethane (20 ml). After 24 h at room temperature, the reaction mixture was diluted with ethyl ether (300 ml) and the resulting precipitate was collected by filtration, washed with ether and purified by low pressure chromatography, as above [dichloromethane : methanol 98 : 2 v/v (2000 ml) and 95 : 5 v/v (2000 ml) as the eluant]. Fractions containing single spot material by TLC in 1-butanol : acetic acid : water (3 : 1 : 1, v/v) were combined and evaporated to dryness. Yield of product: 650 mg, 70%; m.p. 118°–120 °C $[a]D^{20°}$ – 24° (c 0.84, chloroform). Anal. Calcd. for $C_{55}H_{85}N_{13}O_{16}S$ (1216.43): C 54.30, H 7.04, N 14.97; Calcd. for $C_{55}H_{85}N_{13}O_{16}S{\cdot}2H_2O$ (1252.45) : C 52.74, H 7.16, N 14.53. Found: C 52.70, H 6.86, N 14.54. Amino acid ratios: Thr 1.00, Lys 1.00, Pro 1.02, Arg, 1.01. Analytical HPLC elution profile of the title compound (column 3.5 × 150 mm, Delta Pak C 18–100 A, spherical 5 µm. Eluant A, 0.1% trifluoroacetic acid in aqueous 90%) acetonitrile; B, aqueous 0.1% trifluoroacetic acid. Elution: isocratic 30% A over 2 min, then linear gradient 30%–70% A over 15 min, 70%–90% A over 5 min) showed a single peak. ^{1}H NMR ($CDCl_3$, 200MHz) data are shown in Table 1.

H-Thr-Lys-Pro-Arg-O-AZT (Tuftsin-AZT) (IV)

Boc-Thr-Lys(Boc)-Pro-Arg(Pmc)-O-AZT (470 mg, 0.38 mmol) was dissolved in aqueous 95% trifluoroacetic acid (10 ml). After 45 min at room temperature, the solvent was removed in vacuum and the residue was triturated with ethyl ether (300 ml), collected by filtration, washed with ether and dried (316 mg, 75%); $[\alpha]D^{20°}$ – 37.10 (c 0.72, water); single spot by TLC in 1-butanol : acetic acid : water (3 : 1 : 1, v/v). Amino acid ratios: Thr 0.98, Lys 1.00, Pro 1.00, Arg 0.99. The analytical HPLC elution profile using the same column and solvents

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Table 1 Proton NMR, 200 MHz

	Compound 1[a] ppm	Compound 2[b]	Compound 3[c] ppm
AZT protons			
H-3 thymine	9.71	10.54	
H-6 thymine	7.13	7.08	7.55
CH_3 thymine	2.00–1.65	1.88	1.96
H-1′ deoxyribose	5.84	5.55	6.26
H-2′a	2.70–2.45	2.90	2.62
H-2′b	2.45–2.30	2.45	2.62
H-3′	4.50–4.20	4.80–4.55	4.60–4.40
H-4′	3.95	3.87	4.38
H-5′a	4.50–4.20	4.80–4.55	4.60–4.40
H-5′b	4.15	4.10	4.60–4.40
Amino acid residues			
NH-Arg	6.05		
Hα-Arg	4.50–4.20	4.80–4.55	4.60–4.40
2Hδ-Arg	3.15	3.05	3.26
Hα-Pro		4.80–4.55	4.60–4.40
2Hβ-Pro		2.30–1.90	
Hα-Lys		4.80–4.55	4.72
2Hε-Lys		3.05	3.08
NH-Thr		5.66	
Hα-Thr		4.23	3.97
Hβ-Thr		4.23	4.18
CH_3-Thr		1.12	1.38

[a] Fmoc-Arg(Pmc)-OAZT in $CDCl_3$.
[b] Boc-Thr-Lys(Boc)-Pro-Arg(Pmc)-OAZT in $CDCl_3$.
[c] H-Thr-Lys-Pro-Arg-OAZT in D_2O.

as above (elution: isocratic 10% A over 2 min, then linear gradient 10%–60% A over 15 min, 60%–90% A over 5 min) showed a single peak. ^{1}H NMR (D_2O, 200 MHz) data are shown in Table 1. Mass spectra were determined using MALDI-TOF technique: found: 749.69 (Calcd. 749.82).

Biological Activity of the AZT-Tuftsin Conjugates

To study the immunomodulatory cytotoxic and the inhibitory effect of the tuftsin-AZT conjugates on HIV-infected cells, the following materials and methods were used.

Macrophages

Mice [female; BALC/C × (C3H.eb)Fl] were injected intraperitoneally (3 ml/mouse) with thioglycollate broth (Difco Laboratories, Detroit, MI, USA). Four days later, macrophages were aspirated, centrifuged and resuspended to a concentration of 10^6 cells/ml in Dulbecco's modified Eagle's medium (i.e. phosphate buffered saline (PBS), pH 7.4, supplemented with 5×10^{-4} M $MgCl_2$ and 10^{-3} M $CaCl_2$, DMEM, Gibco, Grand Island, NY, USA). The cell preparation consistently contained >90% macrophages (as determined by evaluating the cell population capable of phagocytosis and by staining to nonspecific esterase) of which more than 95% were viable, as assessed by Trypan blue exclusion. Cells (10^6/well) were cultured in Costar microtiter plates (24 wells, Cambridge, MA, USA) at 37 °C in an atmosphere of 5% CO_2.

In vitro 'education' of spleen cells

A macrophage suspension (10 ml) was plated on a 100 mm plastic tissue culture Petri dish (Falcon 3033, Falcon Plastics, Oxnard, CA, USA). The dishes were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 in air, to provide maximum adherence of macrophages as a monolayer. The supernatant was then discarded and the cultures were washed three times with PBS to remove most of the lymphocytes and to obtain as pure a population of macrophages as possible. To feed the macrophages with antigen, 4 ml of DMEM containing 50 µg of KLH per ml was added to each Petri dish for 4 h at 37 °C. In the experimental groups, the medium also contained tuftsin, its conjugate with AZT (AZT-tuftsin) or AZT at various concentrations. At the end of the incubation period, excess free antigen was washed out by three sequential washes with excess PBS. At this stage, 10 ml of spleen cells (10^7 cells/ml) with more than 90% viability (as assessed by the trypan blue dye exclusion test) was added on top of the antigen-fed monolayers and incubated at 37 °C in an atmosphere of 5% CO_2 in air overnight. The non-adherent cells were then gently collected, reseeded on another set of Petri dishes (10 ml/dish), and incubated for 90 min to absorb as many residual adherent cells as possible and thus to minimize contamination with macrophages. The cells were then irradiated with 1000 rads, using a Dermavolt x-ray machine (Siemens x-ray tube: 56 kV, 20 mA, 0.5 Al filter, at a dose rate of 750 rads/min). The suspension was collected, centrifuged and washed twice at

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250 × **g** for 10 min. The supernatants were discarded and the cells were resuspended in Eagle's medium and adjusted to a concentration of 10^8 nucleated cells/ml. Fifty microliters of this suspension was injected into the hind foot pads of syngeneic mice. On day 7, the popliteal lymph nodes were removed and a cell suspension was prepared in PBS. The cells were washed once and resuspended in a culture medium (RPMI 1640 supplemented with 0.5% syngeneic mouse serum and 50 μM 2-mercaptoethanol) to a concentration of 5×10^6 cells/mI. A 0.1 ml sample of these cells was cultured in tissue microtiter plates (Greiner, Nurtingen, Germany) in the presence of antigen or control reagents at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 72 h, 2 μCi of tritiated thymidine (Israel AEC, Negev, Israel) was added and the cells were allowed to incubate for 4 h. The cells were then collected by a 'Titerick' cell harvester (Skatronas, Lierbyen, Norway) on a glass filter, washed twice with saline, dried and placed in Bray's scintillation fluid. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Induction of interleukin-1 (IL-1).

Mouse macrophages were cultured for 20 h, then washed with Dulbecco's PBS and incubated for an additional period of 3 h in DMEM with different concentrations of tuftsin or of AZT-tuftsin conjugate, in the presence of antigen [keyhole limpet hemocyanin (KLH), 30 μg/ml, Calbiochem, Venice, CA, USA]. Assays of interleukin-1 supernatants of cultures were collected by centrifugation at 900 × **g** for 10 min, followed by a second centrifugation at 3000 × **g** for 15 min. Supernatants were assayed for IL-1 activity as described [24]. The assay is based on the ability of IL-1 to induce IL-2 production [as a co-stimulant with phytohemagglutinin (PHA)] by the T-cell lymphoma cell line LBRM-33-1A5. Briefly, 100 μl of LBRM-33- 1A5 cells (5×10^5/ml) were cultured in RPMI supplemented with 10% fetal calf serum (FCS) in 96-well flat bottom microtiter plates (Costar) in the presence of equal volumes of IL-1 supernatant (several dilutions) and 0.3% PHA (Wellcome, Dartford, England). Eighteen hours later, IL-2 activity was determined by removing 100 μl samples from the wells and incubating them with 100 μl of a IL-2-dependent continuous T-lymphocyte line (CTLL; 5×10^4 cells/ml) for an additional 20 h, followed by an 8 h pulse with 1 μCi of tritiated thymidine (36 Ci/mol; Israel AEC, Negev, Israel). An IL-2 standard mouse factor, prepared from concanavalin A stimulated mouse spleen cells, was used as a positive control. An IL-1 standard (contained in the supernatant obtained after overnight culturing of thioglycollate-induced macrophages) was arbitrarily assigned as 100% release. Results of the assay, expressed as % stimulation compared with the standard, represent mean values of three different experiments where each point is evaluated in triplicate.

Cells and Virus

HIV-1 was obtained from the culture supernatant of HUT 78 cells — chronically infected with HIV-1 and producing infective viral particles. The cells were a gift from Dr M. Essex (Harvard School of Hygiene, Boston, Mass., USA). Hut 78 is an OKT4 T-cell line that is permissive to HIV-1 replication, but partially resistant to the viral cytopathic effect.

Drug Treatment

T-cells (HUT 78 or CEM-SS), propagated in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS), L-glutamine and antibiotics, were pelleted at low speed centrifugation and then resuspended in the desired volume of cell-free supernatant containing HIV-1, yielding reverse transcriptase (RT) activity of stock virus of 100 000 cpm/ml. For the infection of T-cells with HIV-1, 0.25 ml stock virus per 1×10^6 cells was used. Following incubation for 2 h at 37 °C and removal of unbound virus by centrifugation, cells (1×10^5/ml) were resuspended in fresh growth medium containing various concentrations of the compounds to be tested. The infected cells were continuously exposed to the drugs. HIV-1 was measured by determination of particulate RT activity in culture fluids of treated and untreated cells, as described [25]. In short, at the time indicated, 6 ml of supernatant was collected and the cells were removed by centrifugation at 5000 × **g** for 10 min. Viral particles were concentrated from cell-free culture medium by ultracentrifugation (200 000 × **g** for 1 h in an SW40 rotor, Beckman), resuspended in 50 μl of 0.01 M Tris-HCI, pH 8.3, and assayed for RT activity using synthetic poly(rA). poly(dT) 12–18 (Pharmacia) as the template primer and 1 mM $MgCl_2$ as the divalent cation.

Cytotoxicity Studies

HUT 78 or CEM-SS T-cells (5×10^5 cells/ml) were exposed to multiple concentrations of the drug. Cell viability was determined by the trypan blue exclusion method and by the XTT assay as described [26]. This assay uses a newly developed tetrazolium reagent that is metabolically reduced by viable cells to yield a soluble colored formazan product measurable by conventional colorimetric techniques. At the indicated times, 200 μl of cell suspensions cultured in growth medium lacking phenol red were transferred to a 96-well microtiter plate (in triplicate) and a mixture (50 μl) of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H tetrazolium hydroxide and *N*-methylphenazonium methosulfate (PMS) was added. Following incubation for 4 h at 37 °C (to permit color development), the OD of the untreated as well as the treated cells was determined at 450 nm.

Determination of Antiviral Activity

The antiviral activity of the tested compounds was assayed by measuring the virion-associated RT activity in culture fluids of treated infected cells and by the determination of HIV-1 antigens in the same fluids by using a commercial ELISA kit (Abbot, E1A kit).

RESULTS AND DISCUSSION

The synthesis of tuftsinyl-AZT (H-Thr-Lys-Pro-Arg-O-AZT, III) was carried out by the solution methodology, as depicted in Scheme 1. Intermediate derivatives were purified by low pressure chromatography on silica gel F60. Compound II was obtained in 75% yield and was fully characterized by analytical HPLC, TLC, amino acid analysis and high resolution mass spectroscopy.

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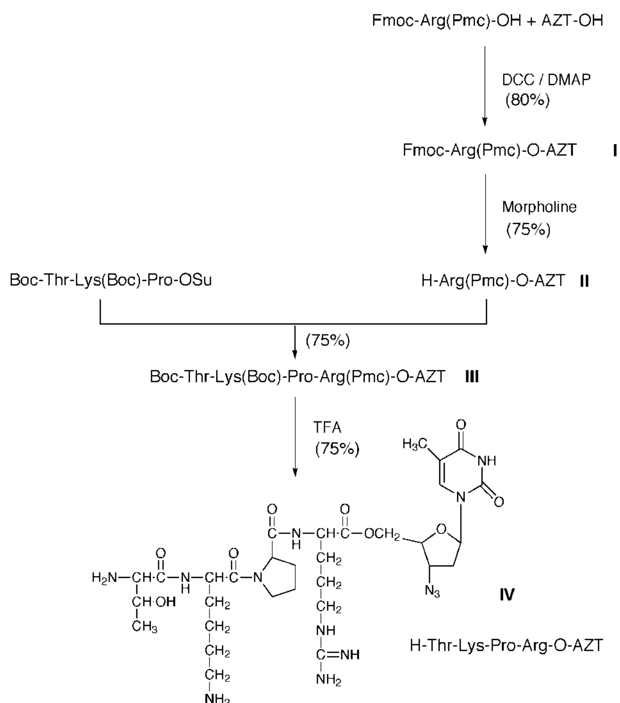

Scheme 1 Synthesis of tuftsinyl-AZT (numbers in parentheses refer to synthetic yields).

The ^{1}H-NMR data (200 MHz) of compounds I, II and of the AZT-tuftsinyl conjugate III are shown in Table 1.

The design of the conjugate, i.e. preferred attachment of AZT at the *C*-terminus of tuftsin, was based on previous structure–function studies in which the integrity of the free *N*-terminus of the Thr^1 residue and the ε-amino moiety of Lys^2 were found crucial for the manifestation of biological activity and binding [6,27,28].

To determine the cytotoxic effect of tuftsin, the AZT-tuftsin conjugate or AZT, uninfected HUT 78 cells (1×10^{-5} cells/ml) were cultivated in growth medium lacking phenol red and exposed to various drug concentrations. At the indicated times, the cell viability was determined by the XTT assay. The optical density at 450 nm, representing XTT formazan production in viable cells, was determined at 3 and 8 days after exposure of the cells to the drugs. The results illustrated in Table 2 represent the mean values for two separate sets of experiments. The results indicate that tuftsin, AZT-tuftsin conjugate or AZT were not toxic for HUT 78 cells at the tested concentrations. Co-treatment with AZT and tuftsin was found, as well, to be non-toxic (not shown). Moreover, at the higher concentrations (over 20 μg/ml), tuftsin and the AZT-tuftsin conjugate exhibited a stimulatory effect on cell proliferation, which was most evident on day 3 but not on day 8 post-treatment. These findings are in agreement with the potent capacity of tuftsin to augment circulatory levels of WBC counts [6,10].

Table 2 Effect of Tuftsin, AZT-Tuftsin Conjugate, and AZT on HUT 78 Cells (XTT assay)

Concentration of drug (μg/ml)	Viability (% of untreated cells)	
	3 days post-treatment	8 days post-treatment
Tuftsin		
0.001	104	134
0.01	102	124
0.1	101	104
1.0	100	102
10.0	102	78
20.0	142	101
40.0	168	101
50.0	163	99
AZT-tuftsin		
0.001	104	100
0.01	101	109
0.1	107	118
1.0	94	120
10.0	136	93
20.0	160	104
40.0	151	107
50.0	120	76
AZT		
0.001	83	118
0.01	101	104
10.0	83	73
20.0	86	89
40.0	83	81
50.0	88	81
Untreated cells	100	100

Table 3 Effect of 40 μg/ml Tuftsin or AZT-Tuftsin on CEM-SS Cells, 5 and 8 days Post-treatment (XTT assay)

Drug	Days post-treatment (% of control cells)	
	5	8
Control, untreated CEM-SS cells	100 ± 5	100 ± 9
Tuftsin	90 ± 7	90 ± 7
AZT-tuftsin	80 ± 6	101 ± 9
AZT	n.d.	n.d.

Such effects could not be demonstrated in cells treated with AZT alone. The XTT assay revealed that none of the drugs tested was cytotoxic to HUT 78 cells at concentrations below 50 μg/ml. Tuftsin or AZT-tuftsin conjugate at 40 μg/ml showed no cytotoxic effect on CEM-SS cells on days 5 and 8 post-treatment (Table 3).

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The Effect of the AZT-Tuftsin Conjugate on Virus Production by CEM-SS Cells

To evaluate the inhibitory effect of the AZT-tuftsin conjugate (compound IV, scheme 1) on HIV-infected CEM-SS cells, 2×10^6 cells were infected with HIV-1 (prepared from cell-free supernatants of HUT 78 chronically infected with HIV-1) and then incubated for 2 h at 37 °C. Infected as well as uninfected control cells were resuspended in fresh medium containing multiple concentrations of the drug and cultured at 37 °C. At the indicated time points, reverse transcriptase (RT) was determined on particles concentrated from the cell-free culture medium. The effect of the AZT-tuftsin conjugate on RT inhibition was compared with AZT as a positive control.

As shown in Figure 1, AZT-tuftsin suppressed the production of HIV-1 by CEM-SS cells under continuous inhibition conditions. Treatment of infected cells with increasing concentrations of AZT-tuftsin, i.e. 0.001, 0.01, 0.1, 1.0, 10.0 and 20.0 µg/ml for 8 days, yielded 68, 76, 86, 86, 89 and 90% inhibition of virus production, respectively, as evident from the RT activity. However, at the lowest concentrations tested, i.e. 0.0001 µg/ml, AZT-tuftsin exhibited a rather substantial stimulatory effect on virus production, as evident from the RT hyperactivity. We do not have, as yet, an explanation for this finding. Incubation of HIV-1-infected CEM cells in the presence of AZT alone resulted in a substantial decrease in the level of RT activity in the culture supernatant, similar to the decreased activity shown by AZT- tuftsin. At a concentration of 0.001 µg/ml, the inhibition was 77% (68% for AZT-tuftsin). Higher concentrations of AZT (0.1 mg/ml and more) showed up to 91% reduction in RT activity. Co-treatment of cells with AZT and tuftsin revealed inhibition values very close to those manifested by AZT alone (not shown). These results indicate that AZT-tuftsin is a potent inhibitor (like AZT) of HIV-1 replication in CEM-SS cells. The antiviral effect of AZT-tuftsin and AZT was confirmed in a different system using the expression of HIV-1 antigens in CEM cells. The results shown in Figure 2 confirm previous results indicating that AZT-tuftsin, like AZT, is a potent inhibitor of viral replication in this system. Notably, the molecular weight of AZT is 267, whereas that of the AZT-tuftsin conjugate is 995. Thus, in terms of content, the drug AZT within the conjugate complex amounts to about 27% of the total weight. Thus for example 86% RT-inhibition is observed with ~0.1 µM and ~0.37 µM of AZT-tuftsin and AZT, respectively.

Guided by the fact that tuftsin augments the immunogenic activity of macrophages [29] it was decided to evaluate whether this capacity was not lost upon its conjugation to AZT, i.e. a measure of functional integrity. Thus macrophages were incubated in the presence of various concentrations of AZT-tuftsin, tuftsin and AZT with a constant amount of antigen. Spleen cells were seeded for 'education' on the macrophage monolayer and were then injected into the hind foot pads syngeneic mice. The popliteal lymph-node cells were then tested for response to the antigen. Tuftsin and AZT-tuftsinyl were found (Figure 3) to augment antigen processing and presentation by mouse peritoneal macrophages as the maximal activity of the conjugate was twice that of tuftsin alone (~5 fold of basal level compared with ~2 fold, respectively). AZT had no effect while co-treatment with AZT and tuftsin yielded similar values to those obtained with tuftsin alone (not shown). Further, and along with the above line, tuftsin and tuftsinyl-AZT augmented the release of IL-1 from mouse macrophages in a nearly identical manner (Figure 4). For example, at 5×10^{-8} M, both compounds induced ~175% stimulation of cytokine

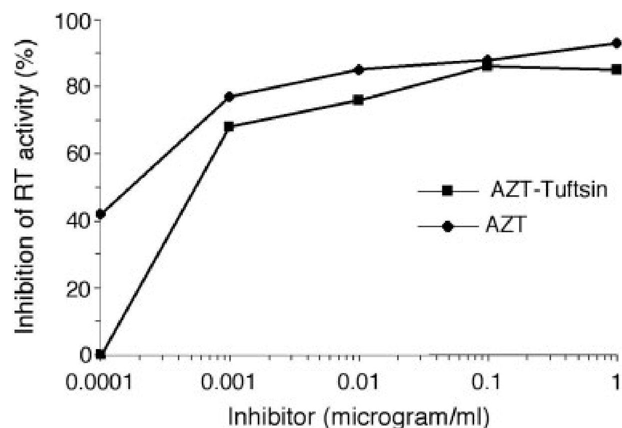

Figure 1 Inhibition of reverse transcriptase (RT) activity by AZT and AZT-tuftsin conjugate, 8 days post-infection and treatment. The results are mean values obtained from two separate experiments that varied by no more than 5%–10%.

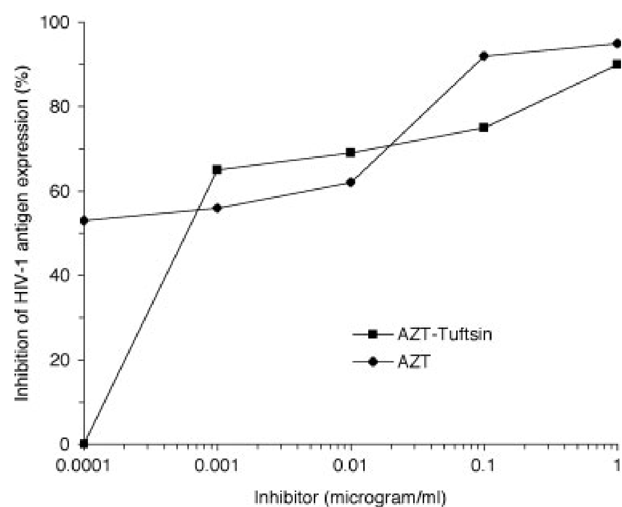

Figure 2 Inhibition of HIV-1 antigen expression by AZT and AZT-tuftsin conjugate, 8 days post-injection and treatment. The results are mean values obtained from three separate experiments that varied by no more than 5%–10%.

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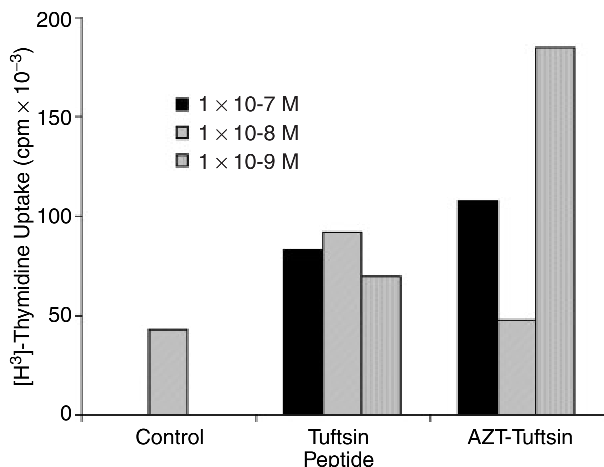

Figure 3 Augmentation of the immunogenic activity of antigen-fed macrophages by tuftsin and AZT-tuftsin conjugate. The results are mean values of three separate experiments that varied by no more than 5%–10%.

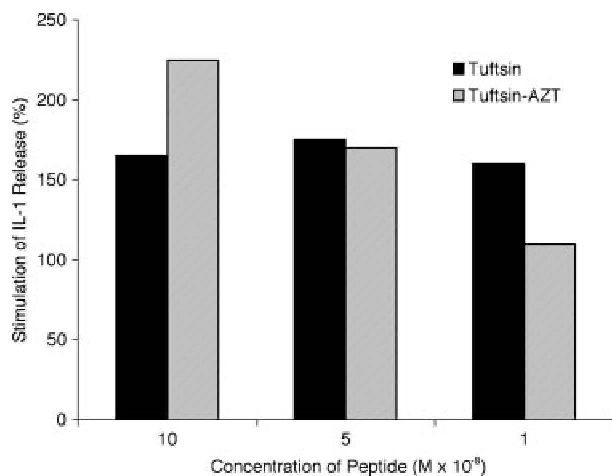

Figure 4 Augmentation of IL-1 secretion from macrophages induced by tuftsin and AZT-tuftsin conjugate. The results are mean values obtained from two separate experiments that varied by no more than 5%–10%.

release. AZT alone had no effect compared with the control while co-treatment with tuftsin was as effective as tuftsin alone (not shown).

Thus, considering the above two parameters for immunomodulation, it appears that the conjugate AZT-tuftsin augments cellular function to the same extent as tuftsin and, perhaps, even somewhat better.

CONCLUSION

In summary, it has been demonstrated that a conjugate of AZT and the macrophage-associating peptide AZT-tuftsin is endowed with the unique capacities of its components and thus may be of potential use in the treatment of HIV-infected macrophages.

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