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Peptide T-araC Conjugates: Solid-Phase Synthesis and Biological Activity of N^4-(Acylpeptidyl)-araC

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Abstract—Due to the capability of peptidyl derivatives of araC to behave as prodrugs of this antimetabolite, and because of the well known biological properties of peptide T and its analogues (in particular that of targeting $CD4^+$ cells), new peptide T-araC conjugates were prepared and tested in vitro for antiproliferative activity. The aim was that of specifically delivering the antitumor drug to $CD4^+$ cells. N^4-(Acylpeptidyl)-derivatives of araC were synthesized by a new general approach involving solid-phase synthesis, which allows mild conditions, avoids the usually required protection of the glycoside portion of nucleosides and affords high yields of the final products. After the demonstration that peptide T-araC conjugates were able to activate chemotaxis by binding CD4 receptor on monocyte membranes, the antiproliferative activity was evaluated against a panel of leukemia lymphoma and carcinoma cell lines derived from human tumors, three $CD4^+$ cell lines included. Title compounds resulted effective as antiproliferative agents at concentrations 4- to 10-fold higher than those of araC alone, did not preferentially inhibit $CD4^+$ cells and proved stable not only in cell culture medium containing 20% FCS, but also in human plasma. All this suggests their potential utility in vivo.

Introduction

1-(β-D-Arabinofuranosyl)cytosine (araC) is one of the most effective drugs currently used in the treatment of acute leukemia and other hematopoietic malignancies. In combination with other antitumor agents it is also used against solid tumors. The activity of araC depends upon conversion to triphosphate and, following incorporation by cellular DNA polymerases into growing DNA strands, it causes chain termination and DNA fragmentation. Very recently, araC has been reported to also induce apoptosis of neoplastic cells.[1]

Major obstacles to the clinical use of araC are its relevant toxicity, low plasma levels and high sensitivity to enzymatic degradation. The last two drawbacks have been partially overcome by masking the amino group of the drug with lipophilic substituents,[2] while the problem of toxicity has been addressed by adopting the strategy of targeted drug delivery.

Peptide prodrugs of araC have been designed with the goal of obtaining selective targeting and, therefore, selective cytotoxicity.[3] Moreover, different conjugates of araC with short peptides containing α,α-disubstituted amino acids have been synthesized as prodrugs which proved fairly active in regulating the apoptosis of human leukemia cells.[4]

Among the synthetic peptides, peptide T (Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr) is an hydrophilic CD4-binding octapeptide derived from the second variable domain of gp120,[5–7] which has been shown to elicit different biological effects. Both peptide T and its analogue [D-Ala[1]]T[1-8]-NH_2 inhibit in vitro the HIV-1 multiplication, likely by competitive inhibition of the gp120-CD4 interaction.[5] Moreover, they prevent the neurotoxic effects of gp120[8] and improve the neuropsychiatric and clinical status of AIDS patients.[9,10] Recently, Phipps and MacFadden[11] have suggested that the capability of peptide T to inhibit in vitro and in vivo the

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effects of tumor necrosis factor-α (TNF-α) could account for its antiretroviral effects. Finally, [D-Ala^1] T[1-8]-NH_2 has been successfully employed as an antipsoriasis agent,[12–15] whereas peptide T has been shown to modulate protein tyrosine kinases (PTK) in activated T lymphocytes,[16] whose activity regulates cell growth.[17]

Our current interest in drug targeting strategies centered on arabinofuranosyl-nucleosides,[18–20] has now led us to investigate the feasibility of a dual targeting approach based on the combination of araC and peptide-T. The approach is based on the following considerations: (1) several lines of evidence indicate that certain tumor cells seem to lose their capability to undergo apoptosis because of the TNF-α induced overproduction of proteins that protect cells from TNF-α cytotoxicity.[21,22] Peptide-T and its analogues could restore, via TNF-α modulation, the balance between resistance and susceptibility of tumor cells to apoptosis;[23] (2) peptidyl conjugates are suitable candidates for the preparation of araC prodrugs, particularly those possessing good water solubility; (3) the free α-amino function of peptide T is not necessary for the interaction with the CD4 receptor[24–26] and, thus, it can be used to link araC; (4) finally, the chemical conjugation, rather than merely the association, of two molecules having different biodistribution, may allow the concomitant delivery to the site were the activity will take place.

Based on these premises, the conjugates composed of [D-Ala^1]T[1-8]NH_2 and the pentapeptide T[4-8] carrying an N-terminal araC, were synthesized and preliminarily evaluated for their in vitro capability to activate monocyte chemotaxis by binding CD4 receptors. The conjugates were then tested for their ability to inhibit the proliferation of a panel of leukemia, lymphoma and carcinoma cell lines derived from human tumors, $CD4^+$ cell lines included. Moreover, considering that araC is characterized by low distribution and high sensitivity to enzymatic degradation in plasma, both of which can be prevented by masking the amino group with appropriate functions (i.e. peptides), we also evaluated the half lives of conjugates in human plasma.

Results and Discussion

Chemistry

Title compounds were synthesized through a new synthetic approach involving solid-phase synthesis methodologies. This allowed us to take advantage of a simple, smooth and automated procedure that makes the protection of the glycoside portion unecessary. It should be noted that, although the synthesis of other N^4-(acylpeptidyl)-araC derivatives has been reported,[3,4,27] solid-phase methodologies have never been used. When compared to previous methods, our approach gives the following advantages: (1) it avoids harsh procedures, such as those involving strong bases (LiOH and BuLi),[4] which are unsuitable for labile peptides; (2) it avoids protection/deprotection steps of the glycoside moiety;[27] and (3) it can be extended to the synthesis of other peptide-nucleoside derivatives.

Briefly, the N^4-(α- or β-aspartyl)-araC derivatives (**2**–**4**) were prepared through an original and fast synthetic route starting from protected aspartic acid (commercially available), which then reacted with araC using 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (WSC) as coupling agent. *t*-Butyl-protecting groups (*t*Bu) were next removed by trifluoroacetic acid (TFA) and the final conjugates were protected at the α-amino function by introducing *t*-butoxycarbonyl groups (BOC) (Scheme 1). Peptide-nucleoside conjugates **5a,b** and **6a,b** were synthesized by solid-phase methods (the synthesis of N^4-(β-acylpeptidyl)-araC derivative **6a** is shown in Scheme 2 as a representative example).

Stepwise syntheses were carried out by fluorenylmethyloxycarbonyl (Fmoc) chemistry using a fully automated continuous-flow peptide synthesizer, and no special efforts were made to optimize repetitive steps. The N^{α}-Fmoc amino acids carrying standard side-chain protective groups and the N-terminal building blocks Boc-Asp(araC)-OH or Boc-Asp-araC were converted to active esters with *N*-hydroxybenzo-triazole (HOB*t*) and diisopropylcarbodiimide (DIPCDI) in the synthesizer. After completion of the synthesis, the protected peptide-nucleosides were cleaved from the resin and the amino acid side-chains were simultaneously deprotected by treatment with a TFA:H_2O:Et_3SiH (88:5:7) mixture. The resulting crude products were purified by preparative HPLC. Homogeneity of products was assessed by analytical HPLC and amino acid analysis, NMR and/or mass spectrometry performed structure assessment. Using this synthetic strategy, aspartyl-nucleosides were used directly as N-terminal residues in solid-phase peptide synthesis and moreover aspartyl-araC adducts were never subjected to basic or reductive treatments which could compromise their structural integrity.

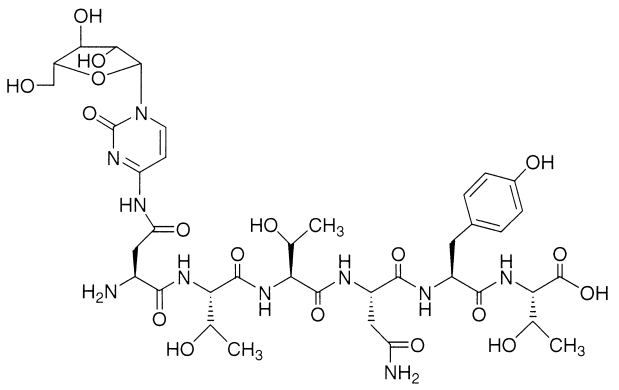
Biological Studies

Chemotactic assay

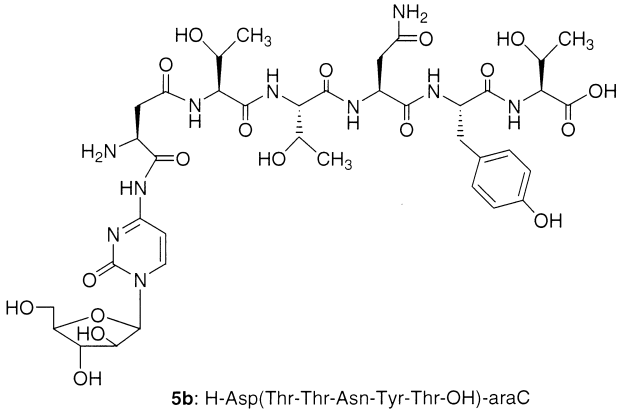
Dose–response and potency of title compounds as agonists were evaluated in an in vitro monocyte chemotaxis assay. Compounds **5a,b** and **6a,b** and peptide T were used at concentrations ranging from 10^{-12} to 10^{-5} M (Fig. 1). Compounds **5b** and **6b** showed potent chemotactic agonist capacity, being maximally effective at 10^{-11} M, a concentration which is easily reached in plasma.[28] The behavior of the above compounds was very similar to that of the peptide T. Compound **6a** was also active, but it showed a potency lower than that of peptide T; in fact, its maximum chemotactic activity was observed at 10^{-9} M. Finally, compound **5a** was the less active of the series.

Antiproliferative activity

The antiproliferative activity of araC and peptide T, alone or in combination, and that of the araC prodrug derivatives **5a,b** and **6a,b** was evaluated against a panel



5a: H-Asp(araC)-Thr-Thr-Asn-Tyr-Thr-OH



5b: H-Asp(Thr-Thr-Asn-Tyr-Thr-OH)-araC

of leukemia, lymphoma and carcinoma cell lines derived from human tumors (Table 1). Among them were three $CD4^+$ cell lines (i.e. the lymphoblastoid CEM and MT-4 and the monocytic U937 cells).

When used alone, peptide T (Table 1) was found to be devoid of antiproliferative activity at concentrations as high as 100 μM, whereas araC showed an antiproliferative activity whose potency differed according to the cell line tested. CEM cells were the most susceptible ($IC_{50} = 0.03\,\mu M$) followed, in decreasing order of potency, by U937 and MT-4 ($IC_{50} = 0.12$ and $0.36\,\mu M$, respectively) and by B-lymphoblastoid and carcinoma cell lines ($IC_{50} = 2.0$–$7.2\,\mu M$). When araC was used in combination with peptide T up to 50 μM, no change was observed in the IC_{50} of the nucleoside analogue. As far as araC prodrugs were concerned, compounds **5a,b** and **6a,b** were effective as antiproliferative agents only at concentrations 4- to 10-fold higher than those of araC alone, no matter whether the cell lines carried or not the CD4 receptor on their plasma membrane.

Stability to enzymatic degradation

In order to evaluate the susceptibility of title compounds to enzyme-mediated hydrolysis, conjugates **5a,b** and **6a,b** were incubated at 37 °C for up to 4 days in cell culture medium (RPMI), in the absence or presence of 20% fetal calf serum (FCS). At the end of incubation, whose duration corresponded to the time necessary to detect the effects of title compounds on cell proliferation in vitro, samples were evaluated for the presence of intact peptide T–araC conjugates. The latter were recovered unmodified at concentrations close to those present at the beginning of incubation, thus suggesting that the various conjugates were not cleaved to release free araC. Notably, test compounds proved fully stable also after a 4 h incubation in human plasma (data not shown).

Conclusions

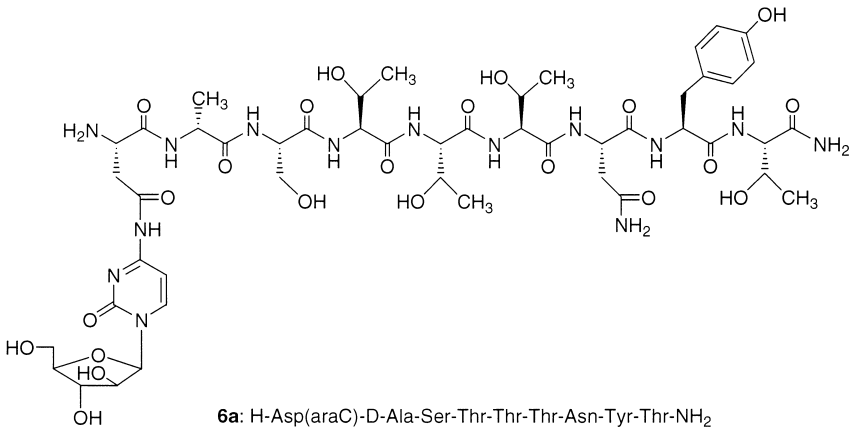
This study was undertaken to synthesize new prodrugs, namely peptidyl conjugates of the potent antiproliferative agent araC. Our objectives were: (i) to selectively deliver the antitumor drug to cells targeted by peptide T (in our case $CD4^+$ cells); (ii) to slow down, if not prevent, araC degradation by serum catabolic enzymes. To this end, four representative conjugates were obtained through a facile solid-phase synthesis starting from the appropriate araC–aminoacyl derivative. Although with different potency, all compounds showed potent agonist capacity in an in vitro monocyte chemotaxis assay, which is a consequence of their binding to CD4 receptors. Nevertheless, when tested as antiproliferative agents in vitro, these compounds showed no preference for inhibition of $CD4^+$ cells. Moreover, the IC_{50} was 4- to 10-fold higher than those of araC alone. Since the conjugates were quite stable in 20% FCS, the antiproliferative activity could not be ascribed to free araC. Therefore, release of free araC following the action of intracellular hydrolytic enzymes may be the rate limiting step, responsible for the lower potency of title araC prodrugs.

In conclusion, solid-phase synthesis of peptidyl-araC derivatives is a mild and versatile procedure that does not require protection of araC as described in previously reported procedures. In our opinion the method can be easily extended to other nucleosides containing amino functions. Further studies are currently under way to generalize this procedure and its potential application in combinatorial chemistry (i.e. SAR and development of new nucleoside prodrugs). Finally, the conjugation with peptide T failed to improve the antimetabolite activity of araC. However, the consideration that the chemical combination allows the concomitant delivery to the cells of the two active principles, supports the evaluation of the antiproliferative potential in vivo of this kind of araC prodrugs. This observation is further supported by the stability of the conjugates in extracellular fluids.

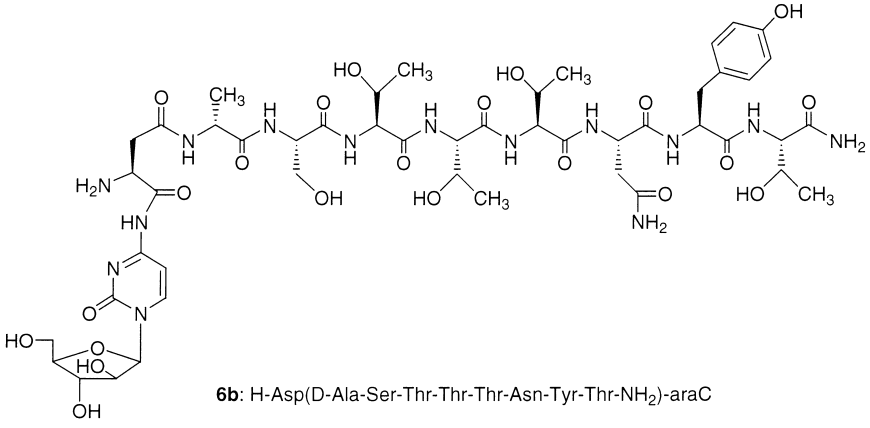
Experimental

General

Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined



6a: H-Asp(araC)-D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2

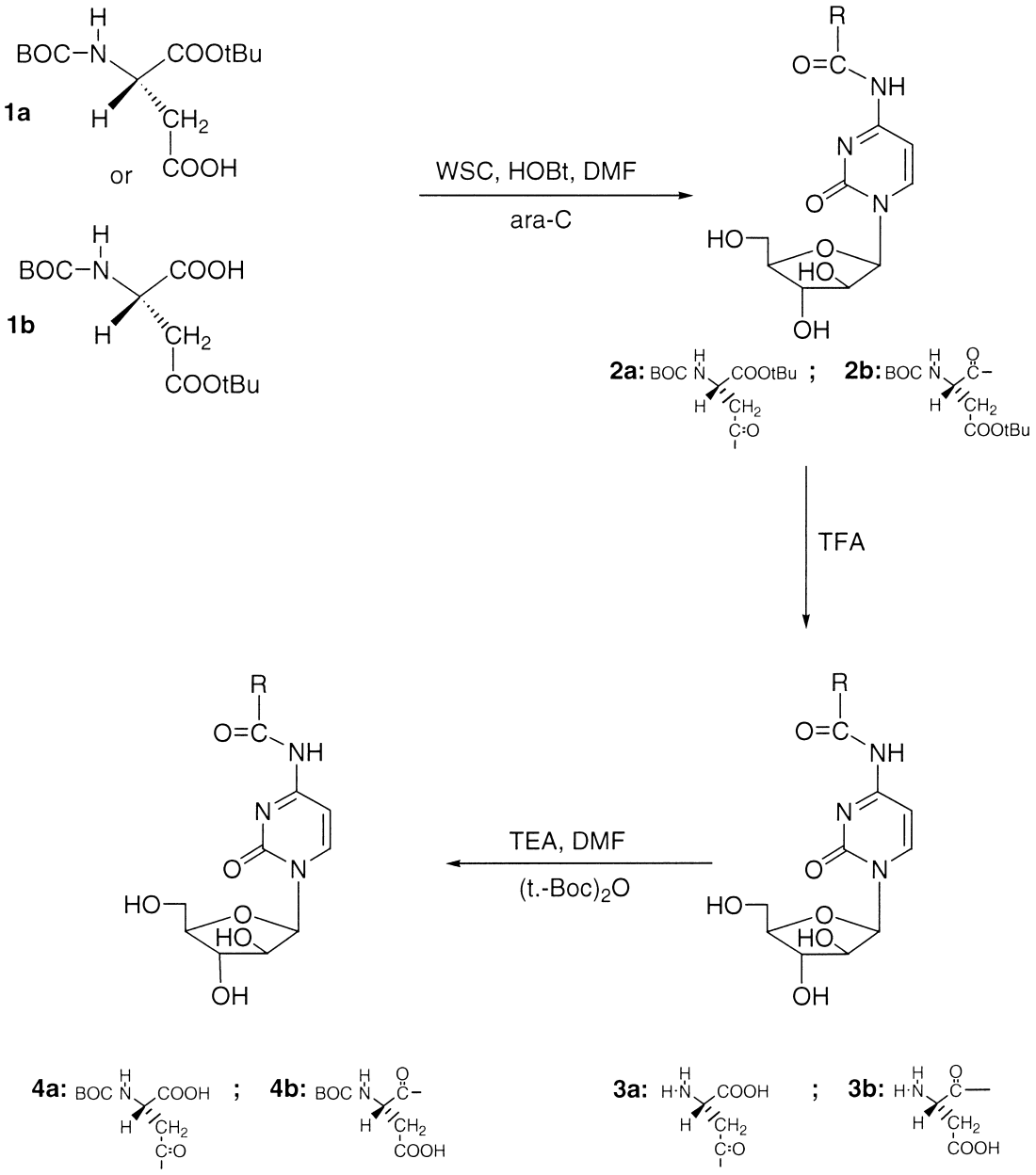


6b: H-Asp(D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2)-araC

with a Perkin–Elmer 141 polarimeter with a 10-cm water-jacketed cell. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) were obtained using the HPG2025A mass spectrometer. HPLC analysis was performed on Beckman System Gold with a Beckham ultrasphere ODS column (5 μM; 4.6×250 mM). Analytical determinations were carried out by two solvent systems: A = water, B = acetonitrile, both containing 0.1% TFA, a linear gradient was initiated after injection of a sample and run from 100% A to 100% B in 25 min. All analogues showed a purity higher than 99% following analytical HPLC monitored at 220 and 254 nM. Preparative reversed-phase HPLC was carried out with a Water Delta Prep 3000 using a Delta Pack C 18-300 A column (30 mm×30 cm, 15 μm, spherical). The mobile phase and the gradient used were identical to that of analytical determinations. Chromatography was performed at a flow rate of 30 mL/min. Column chromatography was performed with Merck 60–200 mesh silica gel. ^{1}H NMR spectra were obtained on a Bruker AC 200 spectrometer using DMSO-d_6 as the reference solvent. Amino acid analyses were carried out using PITC methodology as the amino acid derivatization reagent (Pico-Tag, Waters-Millipore, Waltham, MA, USA). Lyophilized samples of compounds (50–100 pmol) were placed in heat-treated borosilicate tubes (50×4 mm), sealed and hydrolyzed using 200 μL 6 N HCl containing 1% phenol in the Pico-Tag work station for 1 h at 150 °C. A Hypersil ODS column (250×4.6 mM, 5 μM particle size) was employed to separate the PITC-amino acid derivatives. For TLC precoated plates of silica gel F254 were used (E. Merck, Darmstadt, FRG) in the following solvent system: (A) 1-butanol:acetic acid:H_2O (3:1:1), (B) EtOAc:pyridine:acetic acid:H_2O (12:4:1.2:2.2), (C) CH_2Cl_2:MeOH:toluene (8.5:1:0.5), (D) $CHCl_3$:MeOH:benzene:H_2O (8:8:8:1). Ninhydrin 1%, fluorescamine and chlorine spray reagents were employed to detect the peptides. Purity of target compounds was also assessed by high resolution mass and combustion analyses. Where not differently stated, microanalyses were in agreement with calculated values (SD ± 0.4%).

General procedures

Method A. Coupling reaction with WSC/HOBt. To a solution of protected aspartyl-derivative (Boc-Asp(O-But)OH or Boc-AspOtBu 318 mg, 1.1 mmol) in dry DMF (2 mL) were added WSC (210 mg, 1.1 mmol), HOBt (0.168 g, 1.1 mmol) and araC (0.24 g, 1 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then 18 h at room temperature. After evaporation the oily residue was dissolved in ethyl acetate (30 mL) and washed successively with 5% $NaHCO_3$, 0.1 M HCl, and brine, dried ($MgSO_4$), filtered and evaporated. The crude product was purified by silica gel column chromatography and recrystallization.

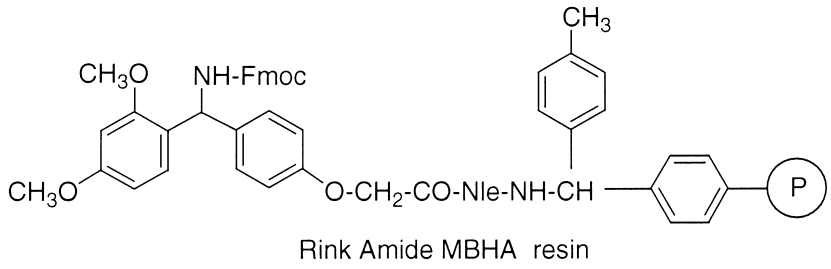


Scheme 1.

Method B. Deprotection. The protected peptidyl-nucleosides, above obtained, were treated with aqueous 90% TFA (2 mL) at room temperature for 1 h. After evaporation in vacuum at 20 °C the residue was triturated with ethyl ether and the resulting solid was collected and dried to give a white solid. The crude residues were used in the next step without any further purification.

Method C. Introduction of Boc-protecting group. 177 μL (0.168 g, 0.77 mmol) of di-*tert*-butyl dicarbonate were added dropwise within an hour to a stirred solution of 0.25 g (0.7 mmol) of aspartyl-nucleoside in 2 mL DMF containing 100 μL (0.7 mmol) of triethylamine (TEA). The reaction mixture was stirred overnight at room temperature. After evaporation the oily residue was treated with 10 mL of 0.1 M HCl and extracted with 2×20 mL portions of ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and evaporated. The solid residue was purified by preparative HPLC and structural verification was achieved by ^{1}H NMR and mass spectrometry.

Boc-Asp(araC)-O*t*Bu (2a). According to coupling method A, *N*-α-*t*-butoxycarbonyl-aspartic acid α-*t*-butyl ester was reacted with araC to give 0.41 g (79% yield) of purified product: R_f 0.87 (A), 0.44 (C); HPLC 2.45 (1); mp 121–123 °C; $[\alpha]_D^{21}$ −22.8° (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.37 (br, 18H, 2×*t*But), 2.60 (ddd, 2H, *J* = 15.9, 7.2 and 6.04 Hz,-CH_2-Asp), 3.45–3.60 (m, 2H, H5′,5″), 3.79–3.82 (m, 1H, H4′), 3.84–3.88 (m, 1H, H3′), 3.90–3.96 (m, 1H, H2′), 4.21–4.28 (m, 1H,-CH-Asp), 5.07 (br, 1H, OH5′), 5.48–5.54 (m, 2H, OH3′ and OH2′), 6.05 (d, 1H, *J* = 3.82, H1′), 7.15 (d, 1H, *J* = 7.5 Hz, H-5), 7.24 (d, 1H, *J* = 8.5 Hz, NH), 8.08 (d, 1H,



1. 20% piperidine/DMF
2. Fmoc-Xaa-OH/DIPCDI/HOBt

Fmoc-D-Ala-Ser(tBu)-Thr(tBu)-Thr(tBu)-Thr(tBu)-Asn(Trt)-Tyr(tBu)-Thr(tBu)-**resin**

1. 20% piperidine/DMF
2. Boc-Asp(ara-C)-OH (**4a**)

Boc-Asp(ara-C)-D-Ala-Ser(tBu)-Thr(tBu)-Thr(tBu)-Thr(tBu)-Asn(Trt)-Tyr(tBu)-Thr(tBu)-**resin**

1. 20% piperidine/DMF
2. TFA/H_2O/Et_3SiH (88:5:7)

6a: H-Asp(araC)-D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2

Scheme 2.

H-6), 10.87 (sbr, 1H, NH). MS (M + H) + 515.2 (calcd 515.2), anal ($C_{22}H_{34}N_4O_{10}$) C, H, N.

Boc-Asp(O*t*Bu)-araC (2b). This compound was obtained from *N*-α-*t*-butoxycarbonyl-aspartic acid β-*t*-butyl ester and 1-β-D-arabinofuranosylcytosine according to coupling procedure A to give 0.36 g (70% yield) of a white product: R_f 0.88 (A), 0.47 (C), HPLC 2.93 (1); mp 133–135 °C; $[\alpha]_D^{21}$ −18.9° (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.37 (br, 18H, 2×*t*But), 2.54 (ddd, 2H, *J* = 15.9, 7.2 and 6.2 Hz,-CH_2-Asp), 3.25–3.36 (m, 2H, H5′,5″), 3.82–3.86 (m, 1H, H4′), 3.90–3.94 (m, 1H, H3′), 3.98–4.06 (m, 1H, H2′), 4.44–4.48 (m, 1H,-CH-Asp), 5.08 (br, 1H, OH5′), 5.49–5.51 (m, 2H, OH3′, OH2′), 6.05 (d, 1H, *J* = 3.82, H1′), 7.15 (d, 1H, *J* = 7.5 Hz, H-5), 7.24 (d, 1H, *J* = 8.5 Hz, NH), 8.08 (d, 1H, H-6), 10.77 (sbr, 1H, NH). MS $(M + H)^+$ 515.2 (calcd 515.2), Anal ($C_{22}H_{34}N_4O_{10}$) C, H, N.

Boc-Asp(araC)-OH (4a). The *N*-protected derivative was obtained by deprotection of Boc-Asp(araC)-O*t*Bu (procedure B) and successive introduction of *t*-butoxycarbonyl protecting group (method C). The crude product was purified by preparative HPLC and finally lyophilized to give 0.267 g (83% yield) of a white solid: R_f 0.72 (A), 0.17 (C), HPLC 1.98 (1); mp 155–157 °C; $[\alpha]_D^{21}$ −35.6° (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.36 (br, 9H, *t*But), 2.82 (ddd, 2H, *J* = 15.9, 7.2 and 6.2 Hz,-CH_2-Asp), 3.40–3.62 (m, 2H, H5′,5″), 3.74–3.80 (m, 1H, H4′), 3.85–3.89 (m, 1H, H3′), 3.92–4.06 (m, 1H, H2′), 4.16–4.29 (m, 1H,-CH-Asp), 5.00 (br, 1H, OH5′), 5.55–5.62 (br, 1H, OH3′), 5.73 (d, 1H, *J* = 7.5, OH2′), 6.06 (d, 1H, *J* = 3.53, H1′), 7.06 (d, 1H, *J* = 7.5 Hz, H-5), 7.14 (d, 1H, *J* = 8.5 Hz, NH), 7.53 (d, 1H, H-6), 11.08 (sbr, 1H, NH), 12.50 (br, 1H,-COOH). MS (M + H) + 459.1 (calcd 459.1), anal ($C_{18}H_{26}N_4O_{10}$) C, H, N.

Boc-Asp-araC (4b). The *N*-protected aspartyl α-nucleoside was obtained as for its β-isomer 0.247 g (77% yield): R_f 0.78 (A), (C) 0.19 (C); HPLC 2.11 (1); mp 160–162 °C; $[\alpha]_D^{21}$ −30.9° (*c* 1.0, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.36 (br, 9H, *t*But), 2.70 (ddd, 2H, *J* = 15.9, 7.2 and 6.04 Hz,-CH_2-Asp), 3.48–3.59 (m, 2H, H5′,5″), 3.80–3.84 (m, 1H, H4′), 3.90–3.92 (m, 1H, H3′),

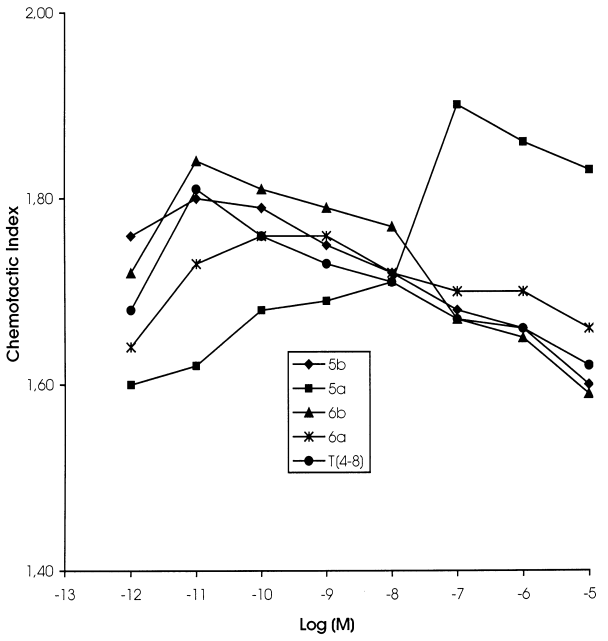


Figure 1. Chemotactic activity of human monocytes toward peptidyl-nucleosides **5a,b** and **6a,b** and peptide T [4–8] at varying concentrations. The points are mean of eight separate experiments. SE, is in the 0.02–0.08 chemotactic index range.

3.95–3.98 (m, 1H, H2′), 4.19–4.35 (m, 1H,-CH-Asp), 5.10 (br, 1H, OH5′), 5.58–5.62 (br, 1H, OH3′), 5.76 (d, 1H, $J=6.5$, OH2′), 6.05 (d, 1H, $J=3.57$, H1′), 7.05 (d, 1H, $J=7.5$ Hz, H-5), 7.30 (d, 1H, $J=8.5$ Hz, NH), 7.53 (d, 1H, H-6), 11.02 (sbr, 1H, NH), 12.80 (br, 1H,-COOH). MS (M+H)+ 459.1 (calcd 459.1), Anal ($C_{18}H_{26}N_4O_{10}$) C, H, N.

General procedure for solid-phase synthesis (SPPS)

Peptidyl-araC derivatives were prepared by solid-phase synthesis (Scheme 2) with a continuous-flow instrument with on-line UV monitoring (Milligen/Biosearch 9050). The stepwise syntheses were carried out by Fmoc chemistry and no special efforts were made to optimize the repetitive steps. For the aspartyl α- or β-araC pentapeptides 0.5 g (0.09 meq) Fmoc-Thr(*t*Bu)-Wang resin (Novabiochem, Läufelfingen, Switzerland) was used. For the aspartic acid α- or β-nucleoside-[D-Ala[1]]-Peptide T amides 0.5 g (0.06 meq) MBHA resin (Novabiochem) functionalized with the linker 4-(2′,4′-dimetylphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucine[29] (Novabiochem) was used. The functionalized resin was swelled in DMF for 1 h and packed in the reaction column. *tert*-Butyl (*t*Bu) was used as side-chain protecting group for serine, tyrosine and threonine, the trityl-protecting group was used for asparagine. N^{α}-Fmoc amino acids as well as Boc-Asp(araC)-OH and Boc-Asp-araC in a 4-fold excess using DIPCDI in the presence of HOBt, always in 4-fold excess for 1 h. The Fmoc group was cleaved with a flow of 20% piperidine in DMF for 25 min. After completion of the synthesis, each protected nucleoside peptide was cleaved from the resin, and the amino acid side chains were simultaneously deprotected by treatment with modified reagent B (88% TFA, 5% H_2O, 7% Et_3SiH). 7 mL for 1 h at room temperature. The resin was removed by filtration and washed with TFA (2×1 mL), the filtrate and washing were combined, evaporated under vacuum at 25 °C and the oily residue triturated with ethyl ether (10 mL). The resulting solid was collected by centrifugation and purified by preparative HPLC (purification yield 79–88%). The homogeneity of the purified products was accessed by analytical HPLC. Structural verification was achieved by amino acid analysis and mass spectrometry.

H-Asp(araC)-Thr-Thr-Asn-Tyr-Thr-OH (5a). Synthesis, cleavage of the resin-bound peptidyl-nucleoside and purification by HPLC, according to the general procedure (SPPS), gave compound **5a** 0.067 g (yield 79%): HPLC 3.43 (1), 4.71 (2); mp 190–192 °C; $[\alpha]_D^{21}$ −13.5° (*c* 1.0, MeOH). MS (M+H)+ 938.9 (calcd 938.9). Amino

Table 1. Antiproliferative activity of peptide T, araC and their conjugates

Compound	IC_{50} (μM)[a]						
	Wil2-NS[b]	CCRF-SB[c]	CCRF-CEM[d]	MT-4	U937[e]	HeLa[f]	KB[g]
5a	23.2	25.1	0.15	3.93	0.80	18.5	60.0
5b	24.5	32.3	0.23	2.87	0.62	9.1	39.0
6a	22.6	26.2	0.16	3.78	0.77	17.3	64.0
6b	18.9	43.0	0.24	2.8	0.66	10.0	42.0
FUDR	21.6	0.3	0.04	0.06	0.2	5.7	6.4
Peptide T	> 100	> 100	> 100	> 100	> 100	> 100	> 100
araC		4.2	0.03	0.36	0.12	2.0	7.8
PeptideT + araC	5.3	6.8	0.03	0.42	0.14	2.1	7.4

[a]Inhibitory concentration 50: compound concentration required to reduce cell multiplication by 50% under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Values represent the mean of three independent experiments. Variation among duplicate samples is less than 22%.
[b]WIL2-NS, human spenic B-lymphoblastoid cells.
[c]CCRF-SB, human acute B-lymphoblastic leukemia.
[d]CCRF-CEM, human acute T-lymphoblastic leukemia; MT-4, human $CD4^+$ T-cells containing an integrated HTLV-1 genome.
[e]U937, human histiocytic lymphoma.
[f]HeLa, human cervix carcinoma.
[g]KB, human oral epidermis carcinoma.

acid analysis: Asp 2.06 (2), Thr 2.91 (3), and Tyr 1.00 (1). Anal. ($C_{38}H_{54}N_{10}O_{18}$) C, H, N.

H-Asp(Thr-Thr-Asn-Tyr-Thr-OH)-araC (5b). The peptidyl-nucleoside was obtained according to the general procedure (SPPS): 0.073 g (yield 87%): HPLC 3.29 (1), 4.12 (2); mp 187–191 °C; $[\alpha]_D^{21}$ −11.6° (*c* 1.0, MeOH). MS $(M+H)^+$ 938.9 (calcd 938.9). Amino acid analysis: Asp 2.03 (2), Thr 2.86 (1), and Tyr 1.00 (1). Anal. ($C_{38}H_{54}N_{10}O_{18}$) C, H, N.

H-Asp(araC)-D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2 (6a). Preparation and purification were conducted according to general procedure (SPPS): 0.062 g (yield 86%): HPLC 4.19 (1), 5.21 (2); mp 166–170 °C; $[\alpha]_D^{22}$ −8.5° (*c* 1.0, MeOH). MS $(M+H)^+$ 1199.1 (calcd 1199.1). Amino acid analysis: Asp 1.97 (2), Ser 0.91 (1), Thr 3.87 (4), Ala 1.00 (1), Tyr 1.04 (1). Anal. ($C_{48}H_{72}N_{14}O_{22}$) C, H, N.

H-Asp(D-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-NH_2)-araC (6b). Preparation and purification were conducted according to general procedure (SPPS): 0.067 g (yield 93%): HPLC 4.27 (1), 5.33 (2); mp 170–173 °C; $[\alpha]_D^{22}$ −6.6° (*c* 1.0, MeOH). MS $(M+H)^+$ 1199.1 (calcd 1199.1). Amino acid analysis: Asp 2.05 (2), Ser 0.93 (1), Thr 3.79 (4), Ala 1.00 (1), Tyr 0.94 (1). Anal. ($C_{48}H_{72}N_{14}O_{22}$) C, H, N.

Metabolic stability assays. The kinetics of peptidyl-araC derivatives degradation were studied in phosphate buffer, culture medium (RPMI) and human plasma. Fifty microlitres of a solution of each compound (30 mg/mL in DMSO or acetonitrile:H_2O, 1:1) were added to 500 μL of phosphate buffer (0.2 M, pH 7.4), or RPMI containing 20% fetal calf serum. Alternatively, test compounds were incubated with plasma (300 μL) in a total volume of 750 μL of 10 mM Tris–HCl buffer, pH 7.5. Incubations were performed at 37 °C for different times: up to 240 min in the case of human plasma and up to 4 days in the case of RPMI containing 20% FCS. The incubation was terminated by addition of ethanol (80 μL), the mixture poured at −21 °C and after centrifugation (5000 rpm for 10 min), aliquots (20 μL) of the clear supernatant were injected into the RP-HPLC column. HPLC was performed on a Spherisorb 5-OD52 column (4.6×250 mM). Elution was carried out by a linear gradient as described above (see Experimental procedures, general).

Biology

Monocyte chemotaxis. Peptidyl-araC derivatives **5a,b** and **6a,b** were evaluated for their ability to stimulate the monocyte-directed migration (chemotaxis). Mononuclear cells were isolated from heparinized blood of normal human volunteers. Chemotaxis was evaluated, as previously described,[30,31] using a 48 multiwell chemotaxis chamber (Neuroprobe, Inc., Milano, Italy) and estimating the distance (μM) migrated by the leading-front of the cells. Each compound was dissolved in DMSO at an intial concentration of 10^{-2} M, diluted before use with Krebs–Ringer phosphate buffer containing 0.5 mg/ml bovine serum albumin (Sigma–Aldrich, Milano, Italy), and tested in the range 10^{-5}–10^{-12} M. To obtain accurate comparisons, the results for the individual compounds were expressed in terms of chemotactic index, which is the ratio: migration towards test attractant versus migration towards buffer. Migration in the presence of buffer alone was 35μM ± 2 SE. Peak response migration for CHO-Met-Leu-Phe-OH (fMLP) occurred at 10^{-8} M and was 68 μM ± 3 SE (chemotactic index 1.94 ± 0.03 SE). In order to confirm that synthetic peptides bound the CD4 receptor, chemotactic effects induced by test conjugates were blocked by low doses (0.1–0.2μg/mL) of OKT4, a specific monoclonal antibody for the CD4 molecule.

Antiproliferative activity

Compounds. Test compounds were dissolved in DMSO at an initial concentration of 200 mM and then were serially diluted in culture medium.

Cells. Cell lines were from American Type Culture Collection (ATCC). The following cell lines were used for the evaluation of antiproliferative activity: Wil2-NS, human splenic B lymphoblastoid cells; CCRF-CEM, human acute T-lymphoblastic leukemia; C8166 and MT-4, human $CD4^+$ T-cells containing an integrated HTLV-1 genome; U937, human histiocytic lympohoma; HeLa, human cervix carcinoma; KB, human nasofaryngeal carcinoma. Leukemia- and lymphoma-derived cells were grown in RPMI-1640 medium supplemented with 10% FCS, 100 units/mL penicillin and 100 μg/mL streptomycin. Solid tumor-derived cells were grown in their specific media supplemented with 10% FCS and antibiotics. Cell cultures were incubated at 37 °C in a humidified, 5% CO_2 atmosphere. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

Antiproliferative assays. Exponentially growing leukemia and lymphoma cells were resuspended at a density of 1×10^5 cells/mL in growth medium containing serial dilutions of test drugs. Cell viability was determined after 96 h at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method, as previously described by Denizot and Lang[32] and by Pauwels et al.[33] Activity against solid tumor-derived cells was evaluated in exponentially growing cultures seeded at 5×10^4 cells/mL and allowed to adhere for 16 h to culture plates before addition of the drugs. Cell viability was determined by the MTT method 4 days later. Cell growth at each drug concentration was expressed as percentage of untreated controls and the concentration resulting in 50% growth inhibition (IC_{50}) was determined by linear regression analysis.

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