Peptidomimetic HIV Protease Inhibitors: Phosphate Prodrugs with Improved Biological Activities

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The rapid spread of the acquired immunodeficiency syndrome (AIDS) epidemic and the difficulties encountered in the development of an efficacious vaccine have stimulated a world-wide quest for therapeutic agents to arrest the replication of the causative virus in AIDS, human immunodeficiency virus (HIV). One promising possibility to interrupt the viral life cycle is the use of inhibitors of the virally encoded protease responsible for viral maturation.[1,2] Among the most potent inhibitors reported thus far are peptidomimetic compounds containing transition-state inserts in place of the dipeptidic cleavage sites of the substrates.[3,4] A large number of reported peptidomimetic inhibitors, however, possess very low aqueous solubility at physiological pH due to the high lipophilicity inherent in these active compounds. These compounds also appeared to be rapidly cleared by the liver. Using the previously reported active peptidomimetic inhibitor U-75875 (Noa-His-CVD-Ile-Amp)[5-7] as a starting template (K_i < 1 nM), a series of compounds were prepared to identify a potential site for the incorporation of a prodrug moiety to address the issues of aqueous solubility and maintenance of blood levels after intravenous administration.

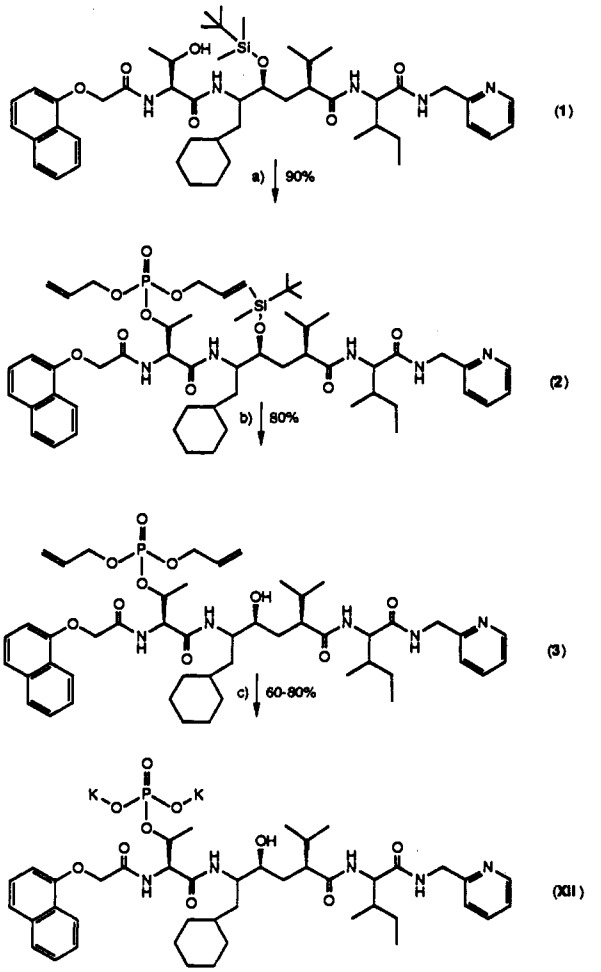
Peptidomimetic compounds were prepared using standard solution-phase methods as described previously.[5] The preparation of a representative phosphate-containing compound **XII** is illustrated in Scheme I. Reaction of compound 1 with diallyl *N,N*-diethyl phosphoramidite[8] and tetrazole in tetrahydrofuran, followed by *in situ* oxidation with *m*-chloroperbenzoic acid, afforded compound **2** in 90% yield. The silyl ether protecting group was removed with trifluoroacetic acid in dichloromethane to give compound **3** in 80% yield. Removal of the diallyl protecting group was accomplished by treatment with formic acid, *n*-butylamine, triphenylphosphine, and tetrakis(triphenylphosphine)palladium(0)[9] in tetrahydrofuran to give the desired phosphate-containing compound, which was isolated as the corresponding potassium salt **XII** (60–80% yield).

In the series of compounds which contain the hydroxyethylene isostere (compounds **I–VII** in Table I), the reference compound **I** is a potent inhibitor in the enzyme assay[10] and also showed potent p24 inhibitory activity in the CV-1 cell assay[11] (IC_{50} value of 0.3 μM). Substituting the isoleucine residue at the P_2' site with a serine or a threonine residue resulted in compound **II** or **III**, respectively; they maintained high enzyme inhibitory activity. However, there was a significant loss of activity in the CV-1 cell assay. With a hypothesis that the loss in antiviral activity might have been a result of decreased lipophilicity, the histidine residue at the P_2 site of compounds **II** and **III** was substituted with a valine residue (compounds **IV** and **V**, respectively). There was no improvement in the activity in the CV-1 cell assay. However, substitution of the histidine residue at the P_2 site of compound **I** with a serine or a threonine residue resulted in compounds **VI** and **VII**, which showed potent activity in the CV-1 cell assay. These two compounds became targets for the introduction of the phosphate moiety (*via infra*).

In the series of inhibitors which contain the dihydroxyethylene isostere (compounds **VIII–X** in Table I), we studied compounds with the C-terminal 1(*S*)-amino-2(*R*)-hydroxyindane[12] in order to study compounds with reduction in size. Substitution of a serine or a threonine residue at the P_2 site of the reference compound **VIII** resulted in compounds **IX** and **X**, respectively. The last compound maintained high antiviral activity in the CV-1 cell assay, and also became a target for the introduction of the phosphate moiety (*via infra*).

The three parent/phosphate pairs are shown in Table II. As anticipated, the aqueous solubility of these phos-

Scheme I.[a] Preparation of the Phosphate-Containing Compound XII



[a] (a) $Et_2NP(OCH_2CH{=}CH_2)_2$, tetrazole, THF; MCPBA, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) $[(C_6H_5)_3P]_4Pd$, $(C_6H_5)_3P$, HCO_2H, n-$C_4H_9NH_2$, THF.

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Table I. HIV-1 Protease Inhibitory Activity and Antiviral Activity as Assessed in the CV-1 Cell Assay[a]

		K_i (nM)	CV-1/vvK-1
I	Noa-His-CVA-Ile-Amp	<5	90% at 1 μM
II	Noa-His-CVA-Ser-Amp	<5	54% at 10 μM
III	Noa-His-CVA-Thr-Amp	<5	39% at 10 μM
IV	Noa-Val-CVA-Ser-Amp	8	48% at 10 μM
V	Noa-Val-CVA-Thr-Amp	10	42% at 10 μM
VI	Noa-Ser-CVA-Ile-Amp	<10	72% at 1 μM
VII	Noa-Thr-CVA-Ile-Amp	<10	74% at 1 μM
VIII	Noa-His-CVD-Ahi	<5	59% at 1 μM
IX	Noa-Ser-CVD-Ahi	30	39% at 1 μM
X	Noa-Thr-CVD-Ahi	<10	78% at 1 μM

[a] Noa = 1-naphthoxyacetyl; CVA = 5(S)-amino-6-cyclohexyl-4(S)-hydroxy-2(S)-isopropylhexanoyl; CVD = 5(S)-amino-6-cyclohexyl-3(R),4(R)-dihydroxy-2(R)-isopropylhexanoyl; Amp = 2-(aminomethyl)pyridine; Ahi = 1(S)-amino-2(R)-hydroxyindane; *For the enzyme inhibitory assay*, hydrolysis of 2.5 mM synthetic substrate H-Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-OH by recombinant HIV-1 protease, in the presence or absence of inhibitor, was measured at 30 °C in 100 mM sodium acetate, pH 5.5, containing 10% glycerol, 5% ethylene glycol, and 0.1% Nonidet P-40 in a total volume of 50 μL; the reaction was stopped after 30 min by the addition of 75 μL of 1% trifluoroacetic acid, and samples were subjected to HPLC analysis. *For the antiviral activity assy*, CV-1 cells were seeded at 2×10^5 cells per well in 24-well Costar dishes and infected 4–6 h later with vVK-1 at 5 plaque-forming units per cell; compound were added at the indicated final concentration 2 h after virus addition; after 24 h, the cell lysates were subjected to electrophoresis on 12% SDS–polyacrylamide gels and proteins were electroblotted onto nitrocellular and analyzed by protein immunoblotting; the levels of immunoreactive proteins were quantified by densitometry; inhibition refers to the mean percent decrease in p24 levels determined from the duplicate drug-treated samples compared to the nondrug-treated controls.

Table II. Inhibition of p24 Production in HIV-1_{IIIB}-Infected Human Peripheral Blood Mononuclear Cells[a]

			IC_{50} (nM)	IC_{90} (nM)	correlation coefficient
VI	Noa-Ser	-CVA-Ile-Amp	2.4	41.4	0.9611
XI	Noa-Ser(OPO_3K_2)	-CVA-Ile-Amp	9.6	46.7	0.9507
VII	Noa-Thr	-CVA-Ile-Amp	4.4	17.6	0.9821
XII	Noa-Thr(OPO_3K_2)	-CVA-Ile-Amp	8.3	21.7	0.9646
X	Noa-Thr	-CVD-Ahi	8.3	33.9	0.9619
XIII	Noa-Thr(OPO_3K_2)	-CVD-Ahi	70.4	510.2	0.9704
U-75875	Noa-His	-CVD-Ile-Amp	50.2	222.7	0.9992

[a] IC_{50} and IC_{90} values were calculated by subjecting the linear section of the dose–response curve to linear regression and estimating the 50% and 90% p24 inhibitory concentrations.

phate-containing compounds at pH 7 was found to be high (>10 mg/mL), while that of the parent compounds was very low (<0.003 mg/mL). Not unexpectedly, the phosphate-containing compounds showed poor enzyme inhibitory activity (K_i > 100 nM). The antiviral activity of these three pairs of compounds were studied in the HIV-1_{IIIB}-infected human peripheral blood mononuclear cells. The dose effects are shown in Figure 1, and the results summarized in Table II. The PBMC assay was selected because these cells represent the primary target cells for HIV-1 infection in patients and thus is suggested to be the most clinically relevant. The antiviral activity result of U-75875 is included as a reference standard. Compounds **VI**, **VII**, and **X** showed much more potent antiviral activity (with IC_{50} values of 5-10 nM) as compared to the previous reference standard U-75875 (with an IC_{50} value of 50 nM). It was, therefore, a pleasant surprise to note that the identification of serine or threonine substitution for the purpose of phosphate prodrug incorporation led to compounds with significant enhancement of the antiviral activity. Of very great significance was the observation that the phosphate prodrugs **XI** and **XII** could be demonstrated in this cell culture assay to elicit essentially the same potent antiviral activity as their corresponding parent compounds.

Due to the potent antiviral activity of the two pairs of peptides, **VI**, **XI** and **VII**, **XII**, they were then selected for pharmacokinetic evaluation. Figure 2 shows the serum concentrations found after intravenous administration of 2 mg/kg of these compounds to rats, and suggests significantly higher drug levels of the phosphate-containing compounds. It should be noted that the rate of metabolic dephosphorylation of compounds **XI** and **XII** to the parent compounds in systemic circulation was slow. Compound **VII** exhibited very low serum values at the early time points, and this was speculated to be due to precipitation after injection of this very poorly soluble compound. Consequently, pharmacokinetic analysis[13] (summarized in Table III) was only calculated for the three other compounds.[14] Clearance and volume of distribution were reduced and the area under the curve was substantially increased for the phosphate-containing compounds.

In summary, replacement of histidine at the P_2 site of a reference HIV protease inhibitory peptide with serine or threonine resulted in compounds with marked improvement in their antiviral potency. The hydroxyl functionality served as a handle for the incorporation of the phosphate group. The resulting highly water soluble phosphate-containing compounds maintained high antiviral activity in cell culture and significantly higher blood

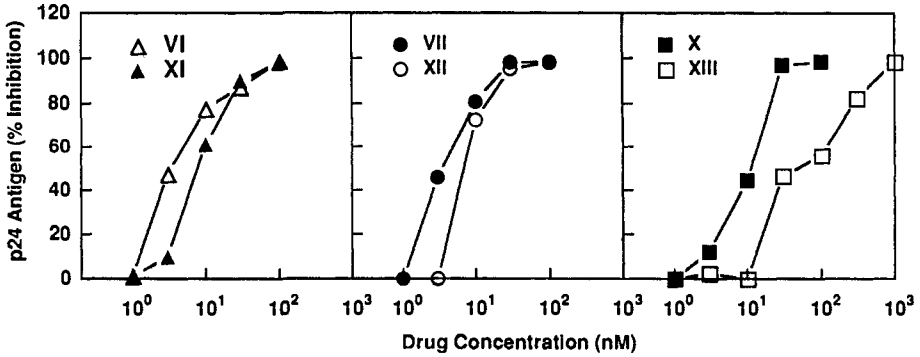

Figure 1. Dose effect on p24 inhibition in HIV-1_{IIIB}-infected human peripheral blood mononuclear cells. Human PBMC (5×10^4 cells) were seeded into 96-well culture dishes and infected by incubating for 90 min with stock virus at 0.003 multiplicity of infection. Stock solutions at 10 mM of test compounds in DMSO were diluted into culture medium. Infected cells in triplicate wells were maintained in the absence (controls) or continued presence of test compound for 7 days in a humidified CO_2 incubator. Culture supernatants were harvested and the extent of p24 core antigen production was measured by an ELISA specific for HIV-1 p24 antigen (Coulter).

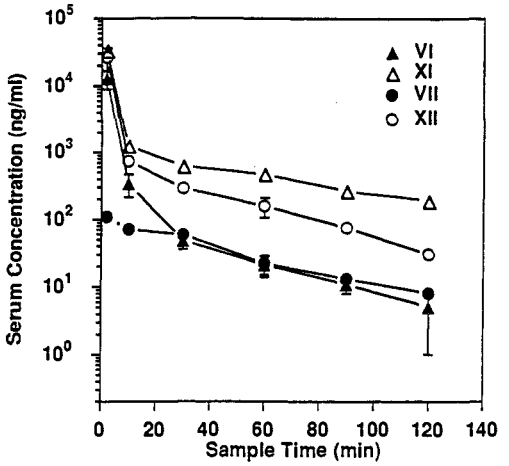

Figure 2. Time-course serum concentrations after intravenous administration to rats. Compounds **XI** and **XII** were formulated in 0.5% Tween 80, 4% ethanol, and 5% dextrose (1.20 mg/mL); compound **VI** in a similar vehicle acidified with HCl to 20 mM; and compound **VII** in 10% DMSO, 10% ethanol, 0.5% Tween 80 (v/v), and 5% dextrose, and acidified with HCl to 20 mM. Fasted male Upjohn Sprague–Dawley rats (420–500 g) were anesthetized with dial-urethane (0.6 mg/kg ip), and compounds were administered as bolus intravenous injections through the tail veins. Blood samples were collected through a capillary tube from the orbital sinus at 2, 10, 30, 60, 90, and 120 min after dosing. Sera was harvested by centrifugation of whole blood at 12000*g*. After solid-phase extraction, 100-μL samples were injected onto a Phenomonex IB-Sil C_{18} column and detected with a fluorescence detector (at 220 nm emission cutoff filter of 295 nm). A mobile phase of acetonitrile/water/triethylamine adjusted to pH 4.5 with phosphoric acid was used for compounds **VI** and **VII** (48:52:0.3) and compounds **XI** and **XII** (38:62:0.3).

Table III. Selected Pharmacokinetic Parameters Calculated from Time-Course Serum Concentrations[a]

treatment	$t_{1/2\beta}$ (min)	Cl_T (mL/min/kg)	$V\beta$ (mL/kg)	$AUC_{0-\infty}$ (ng/mL·min)
VI (n = 4)	33.2 ± 5.4	29.1 ± 4.3	1387 ± 286	73,460 ± 11,127
XI (n = 4)	50.3 ± 2.7	9.9 ± 0.9	727 ± 79	247,800 ± 20,174
XII (n = 3)	28.2 ± 2.1	14.7 ± 2.5	608 ± 141	173,870 ± 30,453

[a] The serum concentration–time data were fitted to mathematical equations using nonlinear regression analysis (NONLIN84), and the distribution and disposition rate constants, α and β, respectively, their half-lives, $t_{1/2\alpha}$ and $t_{1/2\beta}$, respectively, and the area under the serum concentration versus time curve ($AUC_{0-\infty}$) were estimated. The total body clearance (Cl_T = dose/AUC) and volume of distribution (V_β = dose × β/AUC) were also calculated.

levels *in vivo*. The availability of this series of prodrugs offers the opportunity to consider potential parenteral formulation of these compounds as therapeutic agents for the treatment of HIV infection.

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