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A Picomolar Inhibitor of Resistant Strains of Human Immunodeficiency Virus Protease Identified by a Combinatorial Approach[1]

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In order to identify inhibitors of various drug-resistant forms of the human immunodeficiency virus protease (HIV PR), we have designed and synthesized pseudopeptide libraries with a general structure Z-mimetic-Aa_1-Aa_2-NH_2. Five different chemistries for peptide bond replacement have been employed and the resulting five individual sublibraries tested with the HIV PR and its drug-resistant mutants. Each mutant contains amino acid substitutions that have previously been shown to be associated with resistance to protease inhibitors, including Ritonavir, Indinavir, and Saquinavir. We have mapped the subsite preferences of resistant HIV PR species with the aim of selecting a pluripotent pharmaceutical lead. All of the enzyme species in this study manifest clear preference for an L-Glu residue in the P2′ position. Slight, but significant, differences in P3′ subsite specificity among individual resistant PR species have been documented. We have identified three compounds, combining the most favorable features of the inhibitor array, that exhibit low-nanomolar or picomolar K_i values for all three mutant PR species tested.

Key Words: **HIV protease; combinatorial library; inhibitor testing; viral resistance.**

HIV-1 protease (PR)[3] has been recognized as a target for rational drug design of anti-AIDS compounds (for a review, see 1). Hundreds of active inhibitory compounds have been published and, to date, five HIV PR inhibitors have been approved for clinical use by the Food and Drug Administration, including Saquinavir (2), Ritonavir (3), Indinavir (4), Nelfinavir, and Amprenavir (for a review, see 5). The main reason for the continuing interest in anti-PR drug discovery is that under the selection pressure of such inhibitors, mutations evolve within PR which allow the virus to replicate in the presence of drug (for a review, see Refs. 5 and 6 and references therein). Such drug-resistant variants have been found both in patients and in tissue culture. Each protease inhibitor elicits unique mutations but there is a considerable overlap and some mutations therefore exhibit cross-resistance. Most of the primary mutations conferring resistance to PR inhibitors are located in the inhibitor binding cleft; some of them, however, are located in relatively distant

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[3] Abbreviations used: Abu, L-α-aminobutyric acid; Aib, α-aminoisobutyric acid; Apb, (3*S*)-3-amino-4-phenylbutanoic acid; Cha, L-cyclohexylalanine; DIC, 1,3-diisopropylcarbodiimide; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FAB MS, fast atom bombardment mass spectrometry; Fmoc, fluoren-9-ylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; Hph, L-homophenylalanine; Hse, L-homoserine; MBHA, 4-methylbenzhydrylamine; Nle, L-norleucine; Nph, L-4-nitrophenylalanine; Nva, L-norvaline; Orn, L-ornithine; Phg, L-phenylglycine; Pns, phenylnorstatine, (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid; PR, protease; Pst, phenylstatine, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid; RB, "reduced bond," (2*S*,5*S*)-5-amino-3-aza-2-benzyl-6-phenylhexanoic acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Thi, L-thiazolidine-3-carboxylic acid; Tic, L-1,2,3,4-tetrahydroisoquinoline-4-carboxylic acid; Tic(OH), L-1,2,3,4-tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid; Z, benzyloxycarbonyl.

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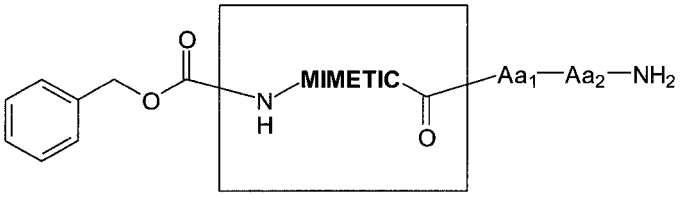

FIG. 1. General scheme of the library.

parts of the enzyme molecule, thus affecting the binding of the inhibitor by indirect conformational changes. Typical mutations associated with Saquinavir resistance occur at G48V and L90M. Those associated with Ritonavir are found at V82A and/or I84V and mutations A71V, V82T, and I84V are associated with Indinavir resistance (7). In the later stages of treatment, secondary mutations appear in the more distant parts of the enzyme that may compensate for the partial loss of proteolytic function caused by the primary mutations.

It is therefore of increasing importance to search for novel pharmaceutical lead compounds, based on different chemistries, that might complement the available range of inhibitors and help to overcome the problem of viral resistance. One of the possible approaches is the use of combinatorial chemistry.

The concept of synthetic combinatorial libraries represents a powerful tool for synthesis and evaluation of vast numbers of compounds in order to obtain potent drugs or drug leads (8, 9; for a review, see 10). There are many examples of the successful application of this approach to the identification of possible inhibitors of medicinally relevant enzymes (11, 12; for a review, see 13), including also identification of potent inhibitors of HIV protease (13–16). These results encouraged us to use a similar approach for the identification of compounds that inhibit a number of inhibitor-resistant mutants of HIV protease. We have therefore designed and synthesized five series of pseudopeptide libraries, based on the structures of potent inhibitors designed recently in our laboratory (17), and tested them with recombinant HIV PR and three mutant forms thereof, resistant against most common anti-HIV PR drugs. We have found slight, but significant, differences in substrate specificity of individual resistant species and identified three compounds capable of inhibiting all tested PR mutants with subnanomolar activity.

METHODS

Synthesis of peptide libraries. All peptide sublibraries were synthesized on Rink amide linker/MBHA-polystyrene resin (18), using Fmoc/*tert*-butyl-HOBt/DIC strategy. The libraries were assembled by the split/mix method with 48 amino acid building blocks and five N-terminal peptidomimetic building blocks (see Fig. 1). The amino acid building blocks contain most of the naturally occurring L-amino acids and their D enantiomers (except for D-Thr, D-Ile, L-Cys, and D-Cys) as well as 11 noncoded L-α-amino acid derivatives: cyclohexylalanine (Cha), homophenylalanine (Hph), norleucine (Nle), norvaline (Nva), ornithine (Orn), phenylglycine (Phg), 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), 1,2,3,4-tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid (Tic(OH)), aminobutyric acid (Abu), homoserine (Hse), and thiazolidine-4-carboxylic acid (Thi). Moreover, two additional building blocks were used: aminoisobutyric acid (Aib) and D-Tic. In this way, 11,520 pseudopeptides in 240 mixtures were prepared. Cleavage of all the peptides from the resin was performed with TFA/DMS/water (95:2.5:2.5) mixture. TFA and scavengers were evaporated in a desiccator over KOH *in vacuo.* Peptide libraries were precipitated with dry ether and filtered, and the precipitates were dissolved in 20% aqueous acetic acid and lyophilized. The composition of libraries was verified by amino acid analysis. All libraries were screened for HIV-1 protease-inhibitory activity.

Individual compounds from the sublibraries with apparent inhibition activities, as identified by library screening, were resynthesized as single peptides by the solid-phase approach (vide supra). Synthesized pseudopeptides were characterized by amino acid analysis and FAB MS and screened for HIV protease inhibition activity.

HIV PR and resistant mutants. Expression and purification of HIV PR have been described previously (17, 30). The cloning and expression of HIV PR mutants will be published elsewhere (Weber *et al.,* manuscript in preparation). Briefly, DNA coding regions of individual HIV PR mutants (HIV PR^{SAQ}, HIV PR^{RIT}, and HIV PR^{IND}) were amplified from the corresponding proviral DNA clones containing G48V and L90M mutations (HIV PR^{SAQ}), V82A mutation (HIV PR^{RIT}), or A71V, V82T, and I84V mutations (HIV PR^{IND}). The PR coding region and the adjacent sequences encoding for the first 21 amino acids of the *pol* polyprotein were cloned into the pET24a expression vector. The host strain *Escherichia coli* BL21(DE3) (Novagen) was used to overexpress the PR precursors. The enzymes were isolated from inclusion bodies by solubilization in 6 M urea followed by batch chromatography on QAE Sephadex and FPLC using a Mono S column (Pharmacia). The purity of enzyme preparations was judged by SDS–polyacrylamide gel electrophoresis and the content of active, correctly folded species determined by active-site titration using a tight-binding HIV PR inhibitor, IIId (17).

Screening of the peptide libraries. An internally quenched fluorescent substrate of HIV PR (2-aminobenzoyl-Thr-Ile-Nle-Nph-Gln-Arg-NH_2, (19)) was used for activity screening. In a typical experiment, a 96-well plate was incubated for 30 min with blocking buffer (0.25% bovine serum albumin and 0.05% Tween 20 in 0.17 M borate buffer, pH 8.5). Subsequently, 50 μL of a 0.5–12 nM preparation of HIV PR or a mutant thereof was added and preincubated for 30 min at room temperature with 10–20 μL of tested library in 30% DMSO/water (final concentration 20–260 nM per compound, depending on the enzyme tested). The reaction was initiated by the addition of 50μL of millimolar substrate solution in DMSO/water. The final concentration of DMSO in the reaction mixture did not exceed 2%. Substrate cleavage could be viewed using a Perkin–Elmer LS50B fluorimeter (excitation wavelength 337 nm, emission wavelength 420 nm), with readouts determined in 10-min intervals. In all cases, several wells containing either only substrate, only enzyme, reaction mixture with active HIV PR inhibitor IIId (17), or no inhibitor were used in duplicates as negative or positive controls, respectively. After initial screenings, putative candidate inhibitors were resynthesized and tested.

Determination of K_i values. The inhibition constants of the selected inhibitors were determined at pH 4.7 and 6.5 as described previously (20). Selected inhibitors (compounds I–III, Table II) were HPLC purified and characterized by amino acid analysis and FAB MS. A spectrophotometric assay with the chromogenic substrate Lys-Ala-Arg-Val-Nle*Nph-Glu-Ala-Nle-NH_2 based on the decrease of absorbance at 305 nm upon substrate cleavage was employed. In a typical experiment, 6–10 pmol of the enzyme was added to 1 mL of 0.1 M acetate buffer, pH 4.7, or 0.1 M phosphate buffer, pH 6.5,

containing 0.3 M NaCl, 4 mM EDTA, 20 nmol of substrate, and various concentrations of the inhibitors, dissolved in DMSO. The final concentration of DMSO was always less than 2.5%.

The K_i values were estimated using a competitive inhibition equation according to Williams and Morrison (20) and Dixon (21) as described (22).

Molecular modeling. Molecular modeling was performed using the INSIGHT II package (MSI). We used the experimental structure of complex HIV-1 PR–acetylpepstatin (5HVP in PDB) as a backbone template on which the inhibitor III has been fitted. The position of water molecule 301, bridging flaps with two carboxyl oxygens of the inhibitor backbone, was maintained. Molecular mechanics optimization based on the AMBER force field (Amber 4.0 program) was used for prediction of the inhibitor binding mode in the HIV PR binding cleft. All atoms of the whole complex were regarded as flexible throughout the minimization process except the α-carbons outside the active-site region.

RESULTS AND DISCUSSION

The use of various protease inhibitors in the treatment of HIV has resulted in the emergence of HIV variants with mutations in the protease (PR) gene. Such variants may result in treatment failure and it is therefore important to identify drugs that may specifically inhibit these viruses. In an attempt to select a novel structure that inhibits drug resistance associated mutants of HIV, we have designed and synthesized a mixture-based synthetic combinatorial library. This library is composed of five sublibraries based on the general structure shown in Fig. 1. This leading structure was derived from subnanomolar inhibitors of HIV PR designed in our laboratory previously (17, 23, 24). The sublibraries have a common structural feature, an N-terminal benzyloxycarbonyl group (reported to be beneficial in many successful inhibitors of proteolytic enzymes (25)) followed by a building block mimicking the cleavable bond found in peptide substrates and two amino acid building blocks (Aa_1 and Aa_2). We have introduced 48 individual building blocks into positions Aa_1 and Aa_2, involving most of the naturally occurring L-amino acids and their D enantiomers (except for D-Thr, D-Ile, L-Cys, and D-Cys) and 13 noncoded amino acid derivatives. The resulting 48 mixtures, each composed of 48 individual compounds, were linked to one of five individual peptide bond mimetics, protected at the N-terminus by a benzyloxycarbonyl group. The mimetics, depicted in Fig. 2, have been chosen to represent successful examples of a peptide bond replacement in HIV protease. They involve the Phe-Phe reduced bond (RB, mimetic A (24)), phenylstatine (Pst, mimetic B (26)), 3-amino-4-phenylbutanoic acid (Apb, mimetic C), phenylnorstatine (Pns, mimetic D (27)), and phenylnorstatinylphenylalanine (Pns-Phe, mimetic E).

Only mimetic A is a typical isosteric moiety for a peptide bond replacement since all others have different numbers of atoms in their backbones compared to the peptide bond. As depicted in Fig. 2, mimetic B contains only five atoms in its pseudopeptide backbone, and mimetics C and D contain one carbon atom less. We therefore designed mimetic E, a pseudodipeptide Pns-Phe (spanning as much as seven atoms in its backbone), in order to investigate the potential of a larger P1–P1′ replacement and to mimic the Pns-Pro-based inhibitors that have been successfully employed for the inhibition of HIV-1 protease (28 and the references therein). To our knowledge, the β-amino acid structure of construct C has not previously been used as a peptide bond mimetic in HIV PR inhibitor design.

In order to simplify our terminology, we define our mimetics as groups mimicking P1–P1′ positions despite their different structural characteristics (e.g., various numbers of backbone atoms). Thus, our mixture-based combinatorial library is composed of five sublibraries, with each one composed of 48 mixtures consisting of 48 individual compounds. The library is thus composed of 240 mixtures with a total diversity of

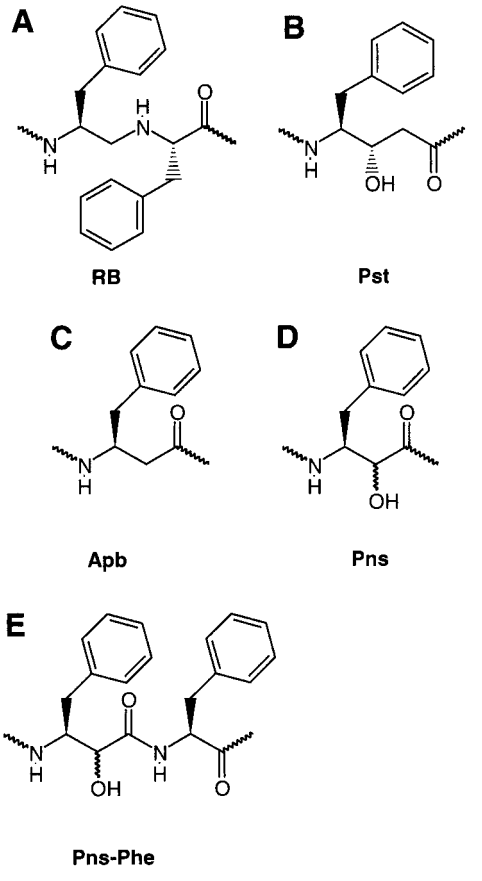

FIG. 2. Mimetic groups used in this study. Only the peptide bond replacements are shown. A, Reduced peptide bond (RB, (2*S*,5*S*)-5-amino-3-aza-2-benzyl-6-phenylhexanoic acid); B, phenylstatine (Pst, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid); C, Apb ((3*S*)-3-amino-4-phenylbutanoic acid); D, phenylnorstatine (Pns, (2*RS*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid); E, Pns-Phe ((2*RS*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoylphenylalanine).

11,520 individual compounds. The composition of individual sublibraries was checked by amino acid analysis.

The libraries were used to select a compound potently inhibiting a panel of HIV PR variants known to be resistant to clinically used HIV PR inhibitors. In search of a multipotent inhibitor, we have used three variants—PR^{RIT} (V82A), PR^{SAQ} (G48V, L90M), and PR^{IND} (A71V, V82T, I84V)—in the primary screening. Each variant contains mutations frequently found in the HIV genome of patients undergoing highly active antiretroviral therapy (HAART). These mutations influence substrate binding especially in the S2–S3 and S2′–S3′ pockets. The enzymes were cloned from infectious full-length plasmids, which carry the respective point mutations in an otherwise isogenic DNA background (Klimkait, unpublished) of the HIV-1 clone pNL4-3 (29). DNA segments containing the protease gene were inserted into the expression vector pET24a, expressed in *E. coli* as described previously (30) and purified to homogeneity as judged from SDS–gel electrophoresis. The enzymes differ slightly in their specific activities as measured by k_{cat}/K_m value (Weber *et al.*, manuscript in preparation) and thus the reaction conditions had to be adjusted for individual enzymes to achieve similar response in the assay.

In order to rapidly screen compound mixtures and select inhibitors, we developed an assay based on an internally quenched fluorescence substrate of HIV PR (19). Relative inhibition of protease activity was expressed as a ratio between an uninhibited reaction control and the tested one. Numerous negative and positive controls were performed to ensure reproducibility of the results, and all measurements were done in duplicate.

Enzyme Preferences in P2′ Position

For all the mimetic groups and all four HIV PR species, all five sublibraries show strong and almost exclusive preference for L-Glu in the P2′ position (according to Schechter and Berger notation (31)). Other building blocks that reveal inhibitory activity in the P2′ are L-Gln, L-Val, and to some extent also L-Tyr (see Fig. 3). It should be noted that due to the experimental setup of our screening procedure, the detection limit for an individual compound to be scored as positive was in the range of nanomolar IC_{50} values. Less active inhibitors or their mixtures were thus intentionally beyond the detection limit of our screening method.

Interestingly, for the sublibrary B, we detect significant and reproducible inhibitory activity of mixtures containing D-Pro in P2′ (compare Fig. 3, line 26). In order to elucidate this observation, we have synthesized all individual 48 compounds that form the mixture tested in the experiment shown in Fig. 3. None of these single compounds showed an inhibitory activity (data not shown). It could be speculated, therefore, that the inhibitory activity of the compound mixture containing peptidomimetic B and D-Pro in the P2′ position is caused by a superposition of relatively weak inhibitory activities of individual compounds that, as individuals, were beyond the detection limit of our measurement.

A major feature of the substrate specificity of HIV PR is the preference for the L-Glu residue in the P2′ position (32–34). We observed this preference for all sublibraries tested, irrespective of the peptide bond mimetic occupying the P1–P1′ position. This observation is in contrast with substrate specificity data obtained on a large set of substrates (35, 36) that suggest a cross dependence between the nature of the amino acid residues occupying the P1–P1′ subsites and the preferential amino acid residue in the P2′ position of the substrate. It should be noted, however, that the subsite preferences determined by a large series of peptide substrates should not be directly applied for inhibitor design. The requirements for a peptide to be cleaved by an enzyme are more complex than the binding of an inhibitor to the protease binding cleft. Moreover, the K_i values of useful HIV PR inhibitors are at least three orders of magnitude lower than the binding constants of the best proteolytic substrates. Substrate specificity data derived from peptide substrate cleavage should thus be applied with caution to inhibitor design.

Structural Requirements for P3′ Position

In order to identify compounds responsible for the inhibitory effect, we synthesized an additional five subsets of single compounds with the general formula Z-mimetic-L-Glu-Aa_2-NH_2, employing again the five different peptide bond mimetics depicted in Fig. 2. All five subsets (consisting of 48 separately prepared inhibitors each) were synthesized by parallel solid-phase peptide synthesis and screened using the four HIV PR species described above. We have thus mapped the requirements of HIV PR and its resistant mutants for the residue occupying position P3′ of the inhibitor.

An example of the outcome of an analysis of an individual library with HIV-1 PR^{SAQ} mutant is shown in Fig. 4. The graph depicts an activity pattern obtained for mimetic E (Pns-Phe; see Fig. 2). All the relative activities are normalized to the value of uninhibited reaction (0 inhibition) and to the positive control, 1 μM inhibitor of HIV PR IIId, identified in previous studies ((17); 100% inhibition).

This particular library shows a marked preference for L-Glu and bulky, aromatic residues in the position Aa_2, corresponding to the P3′. The results are again in good agreement with generally accepted rules for subsite preferences of HIV PR (32–34): the inhibitor-based mapping

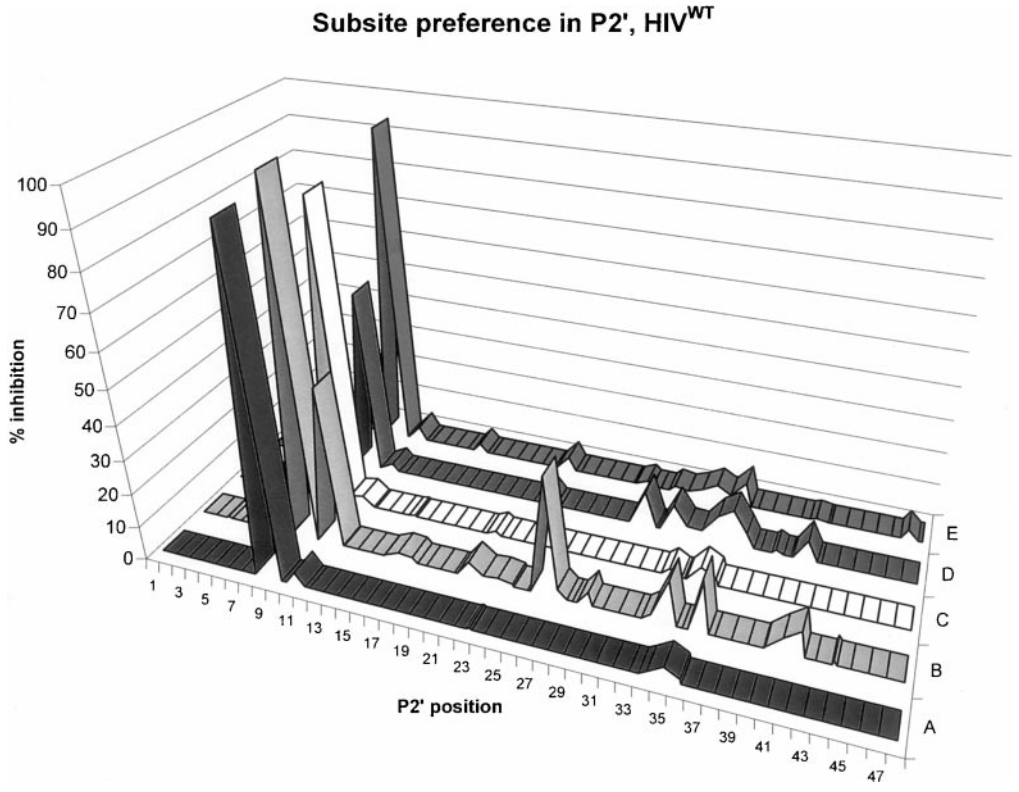

FIG. 3. Schematic summarizing representation of P2′ specificity for five tested pseudopeptide libraries. Relative inhibition (*y* axis) is shown against Aa_2 residue (P2′ position, *x* axis) for individual libraries. The relative inhibition is normalized against a positive and negative control (see Methods for details). *x* axis: 1, L-Ala; 2, D-Ala; 3, L-Arg; 4, D-Arg; 5, L-Asn; 6, D-Asn; 7, L-Asp; 8, D-Asp; 9, L-Glu; 10, D-Glu; 11, L-Gln; 12, D-Gln; 13, Gly; 14, L-His; 15, D-His; 16, L-Ile; 17, L-Leu; 18, D-Leu; 19, L-Lys; 20, D-Lys; 21, L-Met; 22, D-Met; 23, L-Phe; 24, D-Phe; 25, L-Pro; 26, D-Pro; 27, L-Ser; 28, D-Ser; 29, L-Thr; 30, L-Trp; 31, D-Trp; 32, L-Tyr; 33, D-Tyr; 34, L-Val; 35, D-Val; 36, L-Abu; 37, L-Aib; 38, L-Cha; 39, L-Hph; 40, L-Hse; 41, L-Nle; 42, L-Nva; 43, L-Orn; 44, L-Phg; 45, L-Thi; 46, L-Tic; 47, D-Tic; 48, L-Tic(OH).

of the P2′–P3′ preferences yields bulky or aromatic side chains of amino acids in P3/P3′ such as Tyr, Phe, Trp, or Val and a hydrophobic or hydrogen-bonding residue in positions P2/P2′ such as Val, Ile, or Glu (23, 37).

When mimetic E was chosen as the P1–P1′ replacement, a slight difference in P3′ preferences could be observed for individual resistant mutants: PR^{SAQ} and PR^{IND} tolerate smaller (L-Ala) and hydrophilic (L-Glu) residues as opposed to PR^{RIT} or the PR^{WT}.

A summary of the P3′ preference mapping of individual PR species for three representative sublibraries (mimetics B, C, and E) is shown in Table I. For the sake of clarity, only the three most favorable structures in position Aa_2 (i.e., P3′) are depicted for each sublibrary. From this summary, information could be derived about (1) the interplay between the mimetic type and the P3′ preference for an individual inhibitor structure and (2) differences in P3′ preferences for individual resistant mutants.

The Chemistry of the P1–P1′ Mimetics Influences Preferences of the HIV PRs in Distant Subsites

Table I suggests that the preferences for residues in P3′ are influenced by the nature of the peptidomimetic in the P1–P1′ positions. Most notably, introduction of Pns-Phe into P1–P1′ changes the P3′ preference of the enzymes toward L-Glu. Not surprisingly, bulky hydrophobic residues are the most preferred of all tested enzymes in the P3′ position, again in good agreement with experiments using synthetic substrates as well as statistical analyses of relative occurrences of individual residues in this position (33, 36). Specifically, L-homophenylalanine (Hph) has been identified as the most favorable structure from all 48 residues tested. To our knowledge, this structure has not been used for an HIV PR inhibitor design before.

Substrate Specificity Differences between Individual Resistant Mutants

For most peptidomimetic groups in P1–P1′, all four enzymes follow a similar pattern of P3′ subsite preference (bulky, hydrophobic residues L-Hph, L-Trp, L-Phe, L-Tyr, and L-Phg or hydrophilic L-Glu; compare Table II). When Pns-Phe was used as a P1–P1′ mimetic (Table I, mimetic E), slight variations in the substrate specificity could be observed; for example, HIV^{SAQ} and HIV^{IND} toler-

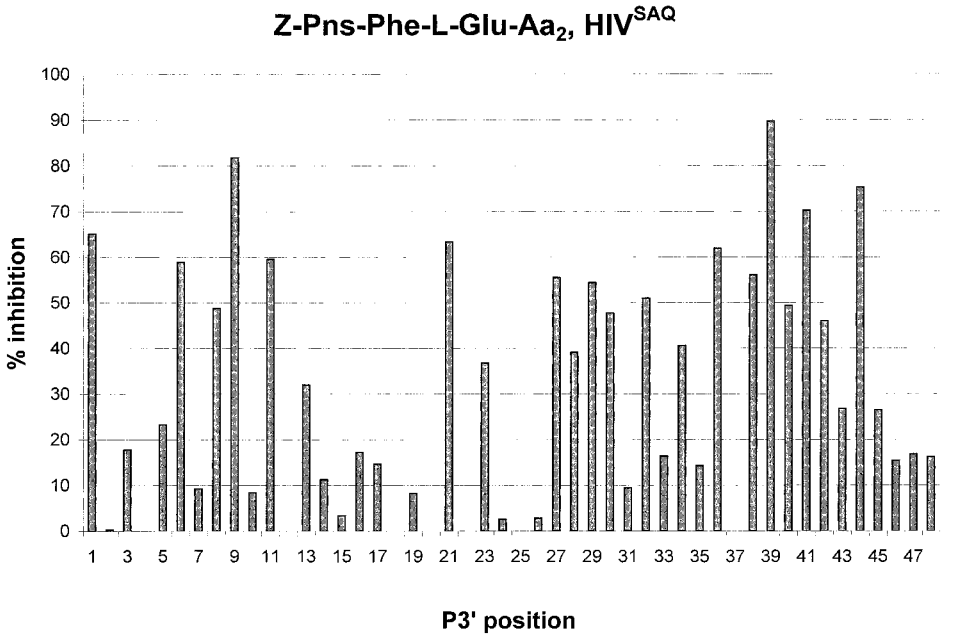

FIG. 4. An example of the outcome of an analysis of individual compounds. The relative inhibitory activity of 48 compounds with the general formula Z-Pns-Phe-L-Glu-Aa_2-NH_2 as measured with HIV-1 PR^{SAQ} are depicted on the *y* axis. For the *x* axis notation, see legend of Fig. 3.

ate small (L-Ala) or hydrophilic (L-Glu) residues in P3′. Interestingly, in this library, all protease species show significant preference for D-Asp and D-Asn over their corresponding L counterparts (Fig. 4, columns 5–8). The subsite preference shift might be partly explained by the fact that mimetic E is two backbone residues "longer" than the corresponding peptide bond, and thus the Aa_2 residues might in fact interact with the S2′ pocket rather than S3′. A definitive answer would be provided by X-ray crystallographic analysis.

TABLE I

Z-Mimetic-L-Glu-Aa_2: Interplay between the Chemistry of Peptide Bond Replacement and the Preference in the P3′ Pocket

Mimetic group	Aa_2 of selected inhibitors of protease species							
	HIV-1 PR^{wt}		HIV-1 PR^{RIT}		HIV-1 PR^{IND}		HIV-1 PR^{SAQ}	
	P3′	Inhibition (%)	P3′	Inhibition (%)	P3′	Inhibition (%)	P3′	Inhibition (%)
B	L-Hph	98	L-Hph	98	L-Hph	100	L-Hph	97
	L-Trp	97	L-Trp	95	L-Trp	100	L-Phe	96
	L-Phe	95	L-Phe	91	L-Phe	95	L-Trp	94
C	L-Hph	73	L-Hph	57	L-Hph	76	L-Hph	84
	L-Trp	40	L-Trp	31	L-Trp	50	L-Phe	32
	L-Phe	23	L-Thr	20	L-Phe	23	L-Trp	16
E	L-Glu	67	L-Glu	86	L-Glu	89	L-Hph	89
	L-Hph	57	L-Hph	68	L-Tyr	78	L-Glu	81
	L-Tyr	47	L-Phg	58	L-Phe	69	L-Phg	75

Note. The three most advantageous substitutions in the P3′ positions as determined by screening of individual compounds are depicted for pseudopeptide mimetic B (PST, phenylstatine), C (Apb, (3*S*)-3-amino-4-phenylbutanoic acid), and E (Pns-Phe). Relative inhibition values for individual compounds normalized to the uninhibited reaction (0%) were determined in duplicate. Experimental details are given under Methods. For the three-letter abbreviations, see footnote 3.

TABLE II

K_i Values (nM) of Selected Compounds (Compounds I, II, and III) and Commercially Available Inhibitors of HIV PR Tested with All PR Species at pH 4.7[a]

Compound	HIV-1 PR^{SAQ}	HIV-1 PR^{RIT}	HIV-1 PR^{IND}	HIV-1 PR^{WT}
I, Z-Apb-Glu-Hph-NH_2	1.85 ± 0.06	0.12 ± 0.01	6.1 ± 0.3	0.1 ± 0.02
II, Z-Pns-Phe-Glu-Glu-NH_2	9.3 ± 0.2	0.11 ± 0.01	5.8 ± 0.2	0.18 ± 0.02
III, Z-Pst-Glu-Hph-NH_2	0.18 ± 0.03	0.024 ± 0.003	0.72 ± 0.12	0.032 ± 0.005
IV, Saquinavir	70.7 ± 4.1	2.00 ± 0.14	11.7 ± 0.8	0.04 ± 0.01
V, Ritonavir	0.26 ± 0.04	0.12 ± 0.02	0.95 ± 0.14	0.015 ± 0.003
VI, Indinavir	7.1 ± 0.6	3.3 ± 0.04	12.7 ± 0.09	0.12 ± 0.02

[a] Experimental details are given under Methods.

Novel HIV PR Inhibitors Selected from the Libraries

We have chosen three compounds exhibiting high inhibition potency in our screening: Z-Apb-L-Glu-Hph-NH_2 (compound I, Table II), Z-Pns-Phe-L-Glu-L-Glu-NH_2 (compound II), and Z-Pst-L-Glu-Hph-NH_2 (compound III) based on mimetics C, E, and B, respectively. As seen in Fig. 3 and Table I, these structures combine the most favorable features of our array of inhibitors. The compounds have been HPLC purified and their inhibitory activity on recombinant, purified resistant HIV PR mutants has been evaluated. For comparison, the inhibitory activity of three commercially available inhibitors of HIV PR (Saquinavir, Ritonavir, and Indinavir) on the same mutants has been determined. The results are summarized in Table II. All three compounds are subnanomolar inhibitors of HIV-1 PR^{WT} at pH 4.7 and exhibit also a strong inhibitory effect on resistant HIV PR mutants. The most potent compound, Z-Pst-L-Glu-Hph-NH_2 (compound III), reaches a picomolar K_i value for the inhibition of HIV PR and a subnanomolar K_i value for all the resistant mutants tested. Figure 5 presents a model of compound III bound into the binding cleft of HIV PR that may explain inhibitor efficiency. The inhibitor may extend over the flaps and the N-terminal benzyloxycarbonyl group therefore fits into a hydrophobic pocket composed of Ile84 and Phe53 residues. The other benzyl residue of the inhibitor (Hph) is relatively far from the S3′ ideal binding position, directed outside the HIV-1 PR cleft. Due to its flexibility, this residue makes a T-contact with the Phe153 of the protease. The T-contact has been both theoretically and experimentally proven to be a stable benzene–benzene conformation (38). Generally, it could be speculated from the model as well as from many structures of HIV PR-inhibitor complexes that an efficient binding requires proper side-chain fitting into the respective pockets only in S2–S2′. The other residues probably only tune the affinity of the inhibitor in a less specific manner. The additional flexibility of the Hph side chain might thus represent an advantage for binding to a resistant mutant PR molecule. The mutations conferring the resistant phenotype cause almost always a significant rearrangement of binding site geometry (1). All the inhibitors that are specifically designed to fill all individual binding subsites in the PR binding cleft might thus have less “elbow room” to bind the resistant mutants.

CONCLUSIONS

In this study, we utilized the methodology of pseudopeptide libraries for mapping of the subsite preferences of several resistant HIV PR species, with the aim of selecting a potent pharmaceutical lead capable of inhibiting various HIV viral strains. We have confirmed a mutual dependence of individual binding sites of the enzymes: specifically, cooperativity between the residues occupying P1–P1′ positions and the preferences for the P3′ position has been documented. Potential use of a β-amino acid (3-amino-4-phenylbutanoic acid) as a scissile bond mimetic and noncoded α-amino acids (homophenylalanine) as an amino acid building block should be further investigated. We have identified several potent inhibitors of all four HIV PR species, including an inhibitor with a picomolar K_i value for the wild-type HIV PR and a low subnanomolar value for the resistant HIV PRs. We built a model of the PR-inhibitor structure suggesting that added flexibility of the Hph moiety in the PR′ position might improve the inhibitor binding toward resistant PR mutants.

Resistant PR species, tested in this study, exhibit slight, but significant, variations in subsite specificity. This would be compatible, if not expected, for the design of inhibitors, which function as a substrate mimetic and bind in the respective place within the enzyme dimer. The identification of highly potent next-generation HIV PR inhibitors that may overcome the notorious problem of clinical inhibitor resistance should therefore involve a search for such minute differences, which could consequently be utilized in inhibitor design.

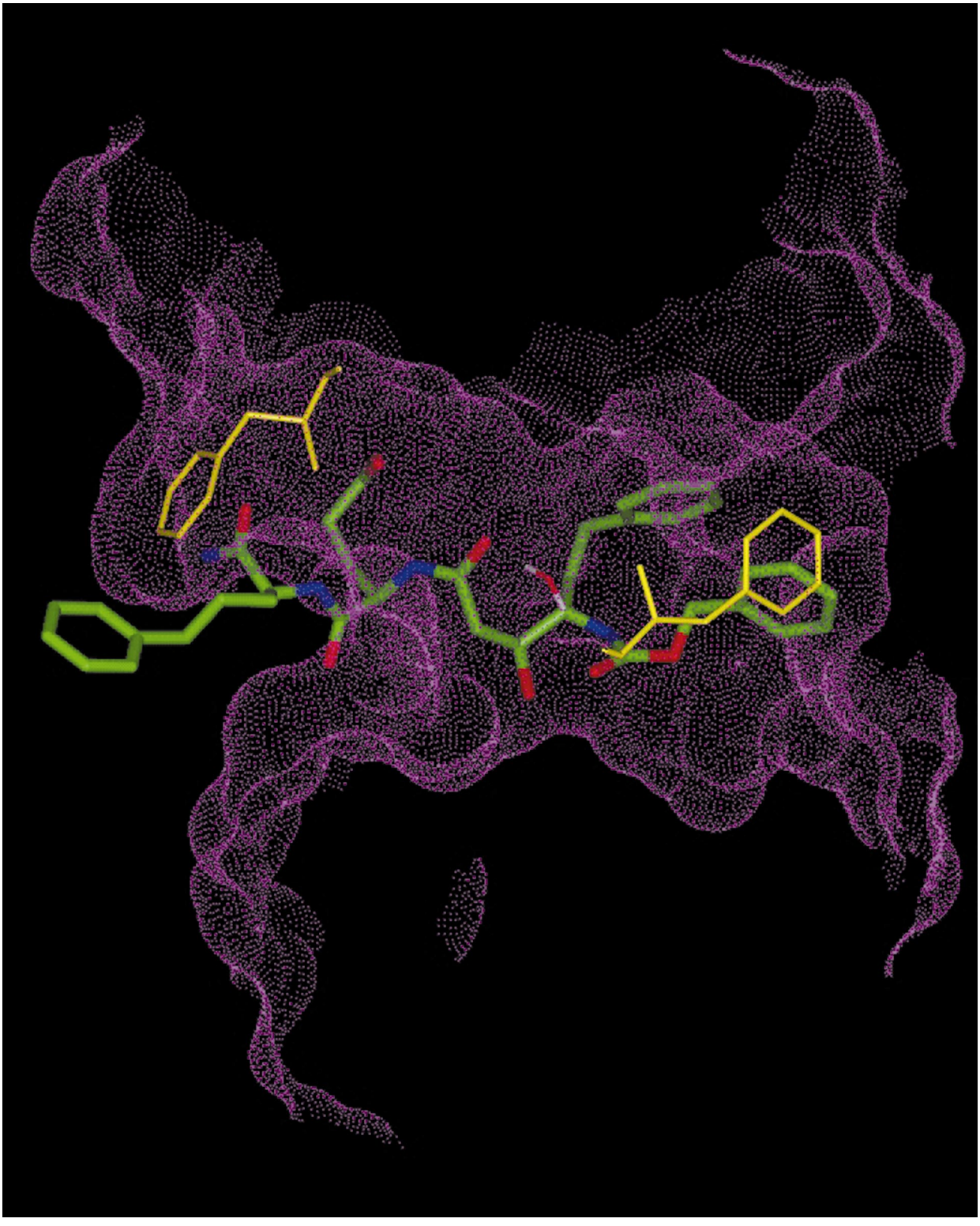

FIG. 5. A model of Z-Pst-Glu-Hph-NH_2 (compound III) inside the HIV-1 PR^{WT} binding cavity shown as a Connolly surface. A water molecule bridging flaps with inhibitor and Phe53 and Phe153 residues (yellow) in contact with the P3′ residue of the inhibitor (green) are visualized. For details, see Methods.

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