HIV Protease Inhibition: Limited Recent Progress and Advances in Understanding Current Pitfalls

Fátima Rodríguez-Barrios and Federico Gago

Department of Pharmacology, University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain

Abstract: The identification of HIV-1 protease (HIVp) as a target for therapeutic intervention against AIDS was soon followed by major efforts to understand its substrate specificity, reaction kinetics and three-dimensional structure, both in the free state and in complex with a number of ligands including substrate mimics, products, and inhibitors. On the whole these studies have been extremely successful and have had a major impact on our understanding of ligand-receptor interactions and enzyme inhibition mechanisms. HIVp has also become a paradigm for the development and testing of new drug-design methodologies both *in vitro* and in silico. Even though thousands of potential HIVp inhibitors exhibiting amazing chemical diversity have been synthesized or identified from natural sources, only a few have turned out to be useful for human therapy. Although the alternative goal of preventing enzyme dimerization has been achieved as a proof of concept, this approach has not yet yielded a clinical candidate. The review covers the general strategies that led to some of the most useful inhibitors, the reasons for our limited success in effectively inhibiting this retroviral target in a clinical setting, current progress with second-generation inhibitors, and new avenues for research.

1. INTRODUCTION

The genome of human immunodeficiency virus type 1 (HIV-1) encodes 15 distinct proteins [1], three of which provide essential enzymatic functions: an integrase, a reverse transcriptase (RT), and a protease (HIVp). Once the viral DNA integrates into the host genome, the proviral genome is transcribed to messenger RNA which is then translated into viral proteins by cellular enzymes. The viral *gag* and *pol* gene products are expressed as large peptide chains that need to be cleaved by HIVp at ten asymmetric and nonhomologous sequences to yield the functional proteins. The fact that this step is essential for maturation of the virus and production of infectious particles accounts for the early [2,3] and current interest [4-6] in HIVp as a prime target for antiretroviral drug design.

HIVp presents the Asp-Thr-Gly sequence characteristic of aspartyl proteases but, in contrast to its eukaryotic counterparts, it is a homodimeric enzyme. Preliminary molecular models of HIVp based on known structures of eukaryotic aspartic proteases and the Rous sarcoma virus protein were soon replaced by the actual three-dimensional structure determined by X-ray crystallography [7,8]. In a relatively short time HIVp became one of the most intensively studied proteins ever and nowadays the number of structures of native and mutant HIV proteases, both liganded and unliganded, that have been determined number in the hundreds [9]. Most of these are publicly available from the standard repository of macromolecular structures (Protein Data Bank: http://www.rcsb.org/pdb/) as well as from a specific database established at the National Cancer Institute in the United States (http://mcl1.ncifcrf.gov/hivdb/index.html) [10]. The wealth of structural information that has been distilled from these HIVp-ligand complexes, together with complementary information about the thermodynamics involved in ligand-receptor interactions [11], can be used to advantage to derive general drug design rules that are applicable to other pharmacologically important targets [12]. In fact, a large number of these complexes have been used by computational scientists to develop and test novel theoretical approaches to both ligand docking [13] and the calculation of ligand binding affinities [14-19].

2. DESCRIPTION OF HIVP AND ITS HYDROLYTIC MECHANISM

HIVp is a homodimer with each monomer having 99 residues. Despite the fact that the dimer is crystallographically symmetric in the absence of any bound ligand other than water, the protease in the crystal is inherently asymmetric, as is the natural substrate. In fact, although the recognition subsites that confer sequence selectivity are structurally equivalent in the apoenzyme (S1 and S1', S2 and S2', and so on), the amino acids that preferentially occupy equivalent locations on both sides of the scissile bond are not identical, e.g. Phe-Pro or Tyr-Pro.

The active site is located at the bottom of a cavity in the dimer interface and is covered by two β−hairpins, called 'flaps', one from each monomer. The flaps have been shown to be very flexible [20], most likely to facilitate substrate binding and product release, and they also participate in the binding of inhibitors, as shown in Fig. (**1**). The catalytic site intersects the diad axis so that each monomer contributes one of the two catalytic aspartic acid residues. Each active site triad Asp25-Thr26-Gly27 is located in a loop whose structure is stabilized by a "firearm's grip" network of hydrogen bonds [3,9]. The result is that the carboxylic groups of Asp25 from both subunits, which may have different ionization states depending on whether or not an

*Address correspondence to this author at Department of Pharmacology, University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain; E-mail: federico.gago@uah.es

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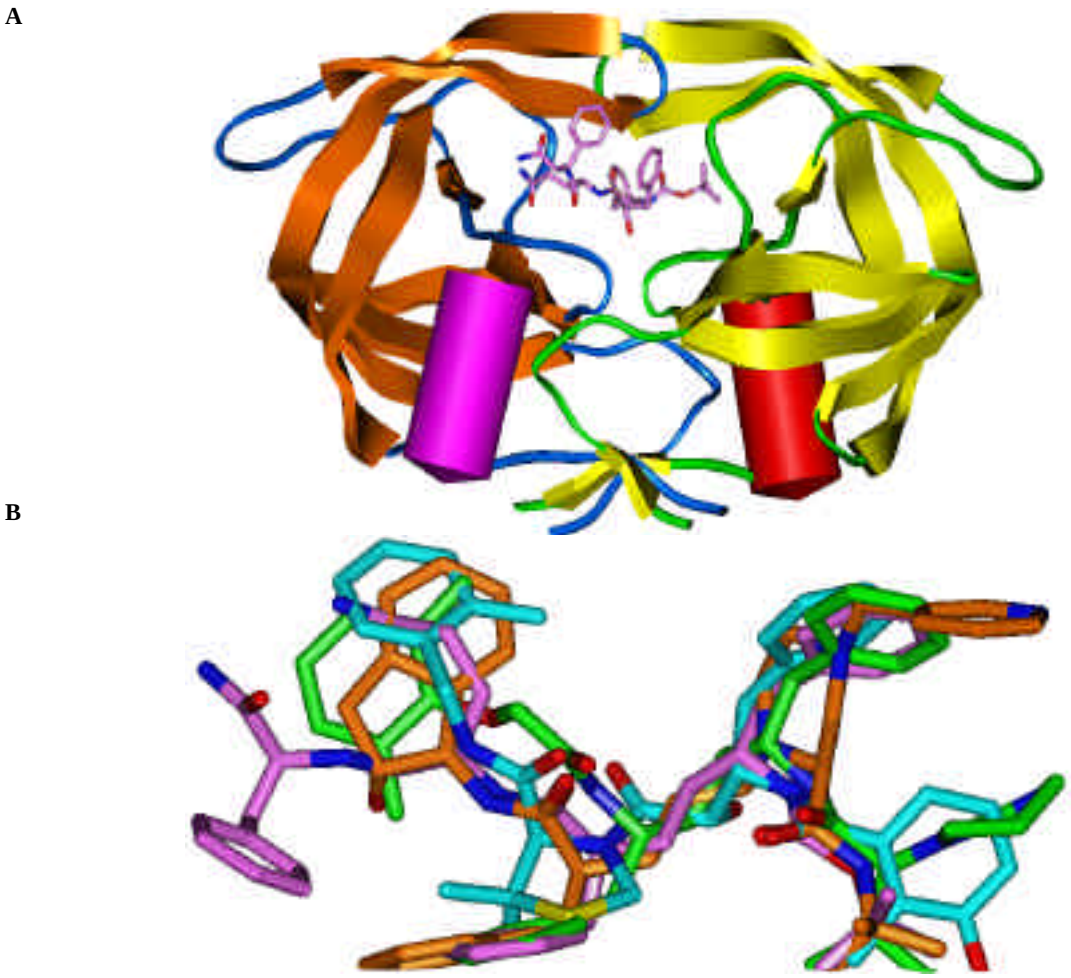


Fig. (1). A. Schematic representation of HIV-1 protease as found in its complex with QF-34 [76]. Each monomer has been colored differently to illustrate the dimeric nature of the enzyme. The inhibitor is shown as sticks with carbon atoms colored in pink. B. Superimposition of indinavir (orange), lopinavir (green), JE-2147 (cyan), and QF-34 (pink), as found in the crystal structures of their respective complexes with HIVp (PDB codes 1K6C, 1MUI, 1KZK, and 1IZH, respectively).

inhibitor is bound to the enzyme [21], are positioned very close together and in a nearly coplanar arrangement. The hydrolysis mechanism (Fig. (**2a**)) involves activation of a water molecule by these aspartates and nucleophilic attack of the water oxygen to the amide carbonyl of the bond to be cleaved. Breakdown of the resulting tetrahedral intermediate by successive proton transfers leads to the amino and carboxylate products.

3. INHIBITOR DESIGN STRATEGIES THAT LED TO CURRENTLY MARKETED HIVP INHIBITORS

The main strategy in the development of synthetic HIVp inhibitors (PIs) has been to replace the scissile P1-P1' amide bond by a non-hydrolyzable isostere with tetrahedral geometry, as shown in Fig. (**2b**). The crystal structures of HIVp-PI complexes have revealed the central OH group of the hydroxyl-containing transition-state isosteres positioned within hydrogen bonding distance between the carboxylic groups of the two catalytic Asp25 residues, close to the position occupied by the water molecule in the active site of all uncomplexed aspartyl proteases. Interestingly, the stereochemical requirements of this OH group (i.e. *R*- or *S*-configuration) have been found to depend greatly on inhibitor length [3].

Initial inhibitor designs were guided by studies on substrate specificity which showed that the S1 and S2 subsites were almost invariably occupied by the side chains of hydrophobic amino acids (Phe, Tyr, Leu, Met) and the S2' subsite almost exclusively by Gln or Glu. Since the minimal-length substrate was a heptapeptide the first inhibitors tended to be rather large peptide-like compounds spanning S5 to S3' [22]. The inherently poor biopharmaceutic properties of peptides precluded the development of peptide-based inhibitors into effective drugs, and prompted the structure-based design of smaller ligands with inhibitory properties. It was also reasoned that incorporation of the side chains of Phe and Pro into reduced amide or hydroxyethylamine isosteres would result in higher selectivity toward HIVp relative to mammalian proteases (e.g. pepsin and cathepsin D) given that Phe-Pro and Tyr-Pro are unusual sites of attack for the mammalian enzymes.

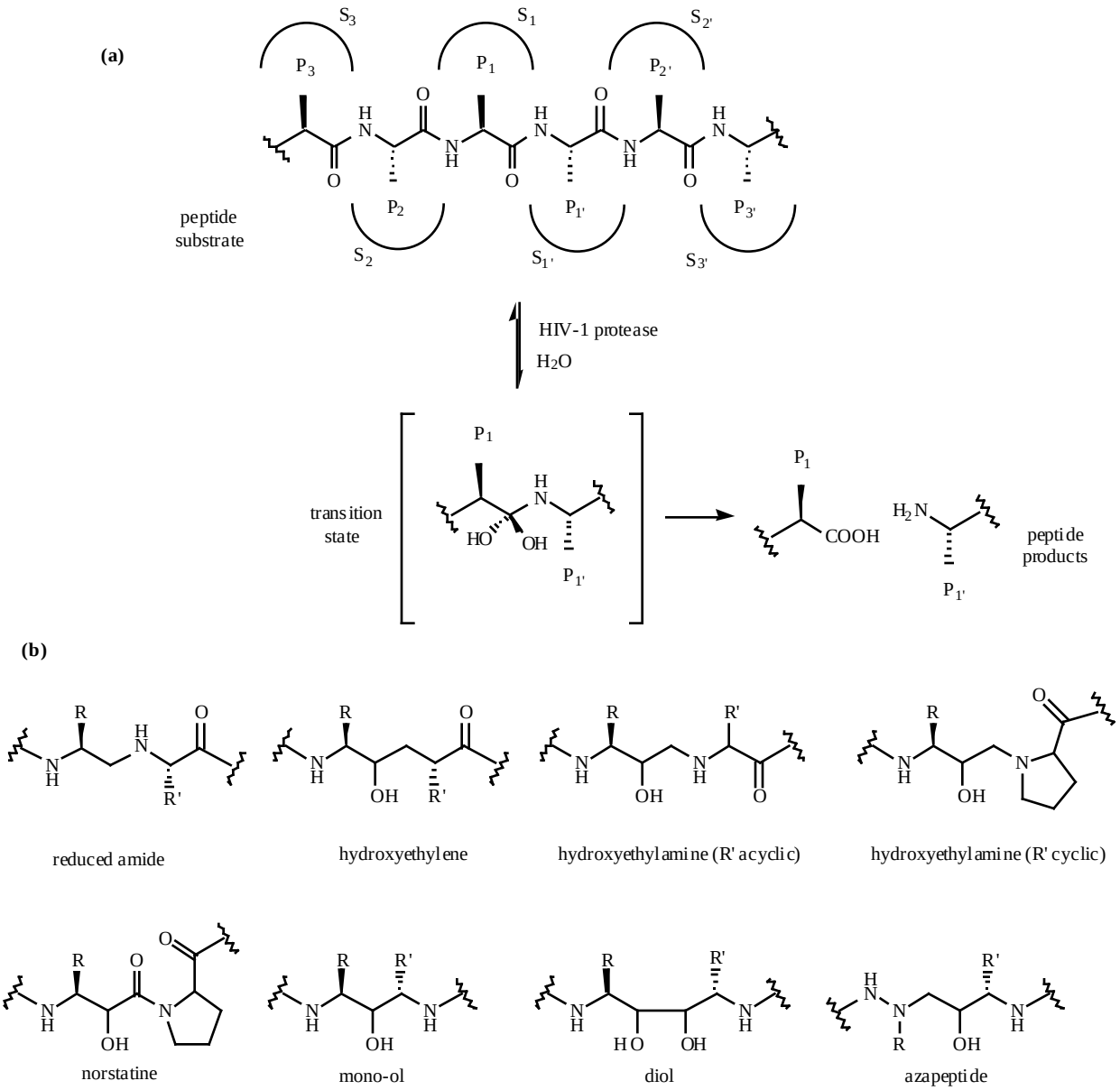


Fig. (2). (a) Schematic of the HIVp binding site with peptide substrate bound showing the standard nomenclature of ligand substituents and enzyme subsites, and hydrolytic mechanism. (b) Examples of non-hydrolyzable isosteres of the peptide bond cleaved by HIV-1 protease.

Later studies showed that tri- and tetrapeptide mimetics could also be potent PIs [23]. Furthermore, incorporation of non-natural amino acid side chains into a variety of peptidomimetic scaffolds (Fig. (**2b**)) was shown to lead to inhibitors that could be more potent than those based on natural substrate sequences [2-4,9,12]. It was also noted that the dependence of the inhibition constant on inhibitor length decreased as the size of the P1' substituent increased. More importantly, by examining a number of HIVp-PI complexes it was discovered that the binding strength of the inhibitor was not a direct function of the total number of contacts or the total number of subsite interactions. These analyses helped define a minimal inhibitor model, which consisted of a core structure containing four side chains to be lodged at S1/S1' and S2/S2' subsites (Fig. (**2**)). While chemical diversity in P2 and P2' substituents was mostly aimed at improving the binding affinity of the ligand, optimization of important pharmaceutical properties, such as aqueous solubility and oral bioavailability, had to rely on additional modifications on the P3 and P3' substituents. Since an undesired outcome of this strategy was a substantial increase in size, it soon became clear that it was more efficient to design groups in P2/P2' that simultaneously provided good binding and superior biopharmaceutical properties [9].

The compounds incorporating the hydroxyethylamine moiety were found to be much more potent than the reduced amide isosteres, and cyclization of the N atom in a *cis*-decahydroisoquinoline ring system by Hoffman-LaRoche scientists led to saquinavir (Invirase®), the first PI to be administered to humans (Fig. (**3**)). The crystal structure of

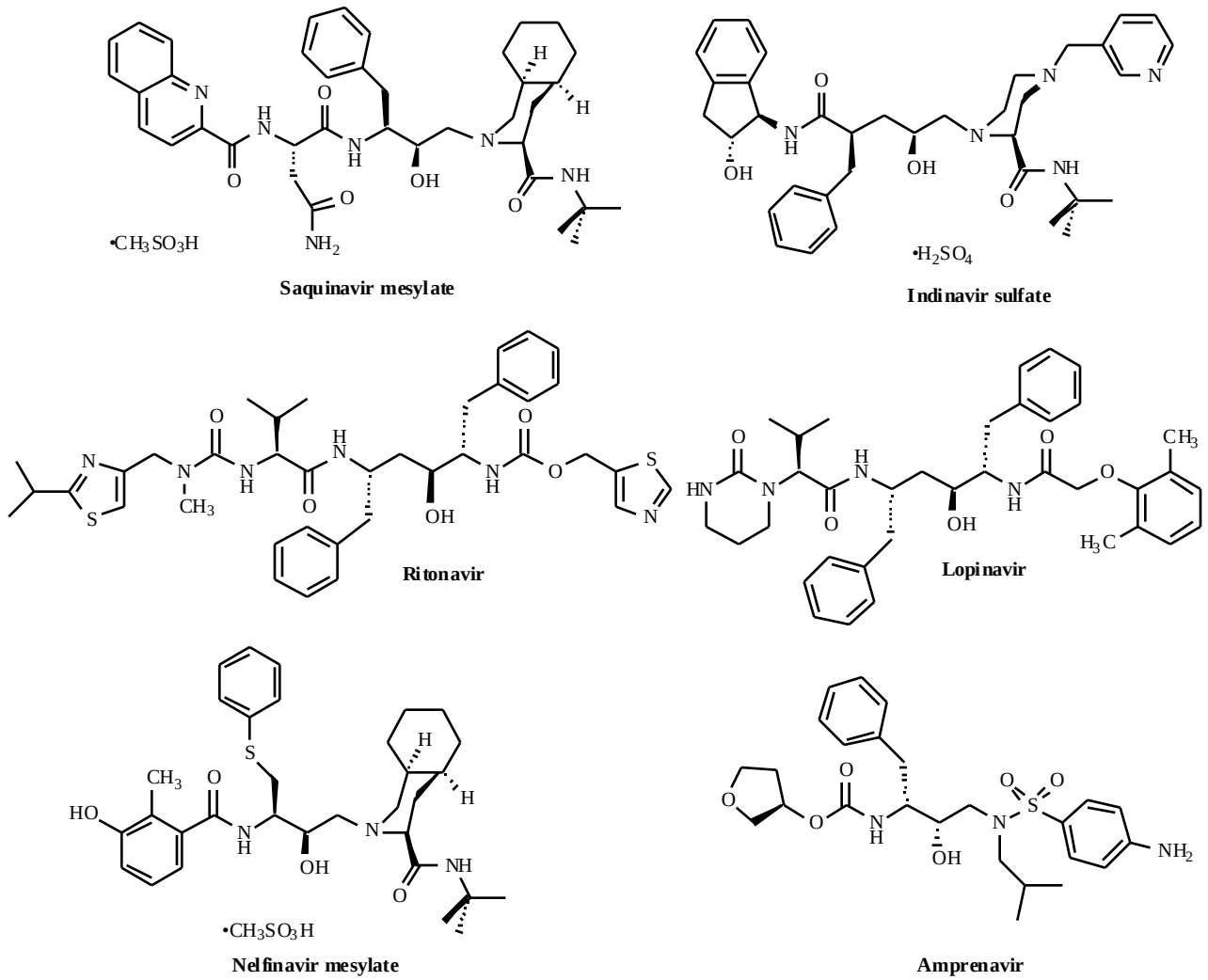


Fig. (3). Structures of protease inhibitors approved and marketed in the US and Europe (saquinavir mesylate - Hoffman-La Roche AG, indinavir sulfate - Merck and Co. Inc, ritonavir and lopinavir - Abbott Laboratories, nelfinavir mesylate - Pfizer Inc. and Roche Holding AG, and amprenavir - GlaxoSmithKline Plc).

the HIVp-saquinavir complex reveals the usual extended conformation of the inhibitor and the expected hydrogen bonding interactions with the enzyme [24]. The decahydro-isoquinoline moiety occupies the S1' subsite and part of the S3' binding pocket, and the bulky *t*-butylamide substituent fills S2' and makes a hydrogen bond to the flap water.

Following a similar approach, but using a hydroxyethyl-ene isostere as a dipeptide mimic, systematic changes in the substituents that fill the enzyme recognition subsites helped Merck researchers to define the relationships between structural modifications and both efficiency and bioavail-ability. Guided by X-ray crystal structure determinations, molecular modeling techniques and computational chemistry calculations [25], the orally bioavailable L-735,524 was designed [26] and finally approved by the FDA under the name indinavir (Crixivan®), a highly HIV selective and orally bioavailable PI (Fig. (**3**)).

Taking advantage of the fact that the active site of HIVp, as opposed to that of mammalian pepsins, is two-fold (C2) symmetric, pseudo-C2-symmetric mono-ol (e.g. A-74704) and C2- symmetric diol inhibitors (e.g. A-77003) were designed at Abbott in an attempt to achieve higher specificity for retroviral proteases [27,28]. Co-crystallization of A-74704 with HIVp indeed revealed that the inhibitor bound in a highly symmetrical fashion with the central OH located between the two Asp25 residues and the central carbonyls hydrogen bonding to the flap water molecule [27]. In the case of the diols, however, only the most potent *S,S* diastereoisomer (A-76928) was found to bind symmetrically [28]. When other related diols were unexpectedly shown by SmithKline Beecham workers not only to bind HIVp asymmetrically but also to be potent inhibitors of monomeric porcine pepsin [29], symmetric inhibitors ceased to be seen as advantageous. Improvement of the aqueous solubility of A-77003 through the incorporation of heterocyclic groups at one or both ends of the molecule and the discovery of the enhanced potency of deshydroxy diols led to a new series of asymmetric compounds [30] including ABT-538 (Figure **3**), which entered clinical trials and was approved in 1996 under the name ritonavir (Norvir®). Further drug design based on the X-ray crystal structure of the

complex between HIVp and ritonavir led to ABT-378 [31], later known as lopinavir (Fig. (**3**)), which has a diminished interaction with Val 82 relative to ritonavir and has also been co-crystallized with HIVp [32]. Lopinavir is approved in a formulation (Kaletra®) involving coadministration with ritonavir.

In a collaborative effort between Lilly and Agouron, an ortho-substituted benzamide was used to replace the decahydroisoquinoline ring of saquinavir to produce LY-289,612. Noting that the P1 and P3 substituents of this compound made van der Waals contacts with each other in its complex with HIVp, it was reasoned that a larger P1 group such as S-naphthyl could span both S1 and S3 subsites. The resulting compound was a potent inhibitor and the crystal structure indeed showed that the S-naphthyl group occupied the adjacent S1 and S3 subsites. In doing so, the complex deviated from C2 symmetry and the S1 and S1' binding pockets were different in size [12]. Further departing from the peptidomimetic concept, the P2 group was replaced by Agouron chemists with a 2-methyl-3-hydroxybenzamide leading to AG-1254, which was further modified to yield AG-1343 [33]. This drug was then marketed as nelfinavir (Viracept®) (Fig. (**3**)), and became the first PI to be approved for the treatment of pediatric AIDS.

An HIVp inhibitor with a low molecular weight more in consonance with those of the majority of marketed drugs emerged from a structure-based drug design program launched at Vertex Pharmaceuticals. VX-478 was the lead compound in a new class of N,N-disubstituted (hydroxyethyl)aminosulfonamides and still represents one of the smallest and least stereochemically complex of all PIs. As novel structural features it includes a sulfonamide group in the P2' position and a 3(*S*)tetrahydrofuryloxy group in the P2 position. VX-478 was later developed by Glaxo and became known as amprenavir (Agenerase®) (Fig. (**3**)). The structure of its complex with HIV-1 protease, solved at 1.9 Å resolution [34], showed the hydroxyl group located between the two Asp25 residues and the flap water hydrogen bonded to the P1 carbonyl group and one of the sulfonyl oxygens. Amprenavir is a potent, competitive inhibitor of HIV-1 and HIV-2 proteases that combines aqueous solubility with synthetic accessibility, despite the presence of three asymmetric centers.

4. MEDICAL PROBLEMS ASSOCIATED WITH USE OF CLINICALLY APPROVED PIS

4.1. Pharmacological Resistance to PIs

The inability of HIV RT to correct transcription errors during nucleic acid replication accounts for the remarkable mutability of this virus [35]. As a consequence, within a few cell culture passages in the presence of different anti-HIV drugs or in treated patients, HIV variants with decreased susceptibility to one or more of the inhibitors are inevitably selected, with PIs being no exception [36]. Thus, a 100-fold resistant double-mutant (G48V/L90M) emerges under the selective pressure of increasing concentrations of saquinavir [37] wheras *in vitro* resistance to amprenavir is characterized by a protease containing the single mutation I50V or an M46I/I47V/I50V triple mutation [38]. For indinavir, variable patterns of multiple substitutions have been reported consistently involving alteration of residues M46 (to I or L) and/or V82 (to A, F, or T) [39]. *In vitro* selection with ritonavir produces a resistant variant with five substitutions (M46I/L63P/A71V/V82F/I84V) [40], relatively similar to the pattern identified in indinavir-exposed treatment failures (L10R, M46I, L63P, V82T, and I84V) [39]. After serial passages of HIV-1 in the presence of increasing concentrations of nelfinavir, D30N (later identified in patients as a major cause of failure to nelfinavir treatment) [41] and M46I+I84V/A variants with 7- and 30-fold reductions in susceptibility, respectively, were isolated [42].

Mutations conferring resistance to PIs are definitely not restricted to amino acids located in the active site of the enzyme [43]. Changes outside the active site (M46I, A71V, L63P, L10I, etc) were thought of as "compensatory mutations" aimed at improving the catalytic efficiency of the enzyme rather than decreasing inhibitor binding. However, recent fluorescence inhibition assays and high-sensitivity isothermal titration calorimetry experiments have shown that non-active site mutations are themselves responsible for very large drops in inhibitor binding affinity [44]. An up-to-date compilation of the most common HIV-1 mutations selected by PIs (as well as other classes of antiretroviral agents) is available on the Internet at http://hiv-web.lanl.gov [45].

The amino acid substitutions predicted to give rise to resistance from *in vitro* studies have been shown to be present in patients with no previous record of PI treatment [46]. These mutations tend to accumulate over the course of the increasing selective pressure in an ordered, stepwise fashion [47], and there tends to be significant overlap in the identity of the mutations selected with different inhibitors, which can give rise to high levels of cross-resistance [48]. On the other hand, the fact that early mutants still retain susceptibility to other PIs with nonoverlapping resistance profiles has supported the use of dual PI therapy to increase the duration of viral suppression.

The resistant strains show significant increases both in the protease inhibition constant (K_i) and in the concentration required to reduce by 90% the replication of viruses containing the identical enzyme (IC_{90}). However, no correlation was apparent between the calculated mutant/wild-type ratios of K_i and IC_{90} when a panel of 19 different inhibitors (including the first five marketed PIs) was studied [49]. This finding appears to suggest that measurements of enzyme inhibition with mutant proteases may be poorly predictive of the antiviral effect in resistant viruses. To further cloud the picture, selection of viral variants with increasing concentrations of lopinavir [50] and other PIs have revealed not only a sequential appearance of mutations in the protease gene but also mutations in some of the proteolytic cleavage sites in the p7/p1/p6 region of the *gag* polyprotein. Mutation of these *gag* sites appears to be required for the growth of highly resistant HIV-1 selected by these compounds.

4.2. Pharmacokinetics and Potential for Drug Interactions of PIs

Estimation of the PI concentrations required for *in vivo* activity using data from *in vitro* assays is complicated by the

fact that their antiviral potency can be attenuated by binding to serum proteins, particularly to albumin and α_1-acid glycoprotein (AGP) [51]. As a consequence, it has become customary to assay the *in vitro* activity of current PIs' in the presence of human serum ("shift" assays). Among the approved inhibitors, indinavir and lopinavir show the smallest degree of impact of added serum.

Oral absorption of currently approved PIs can be strongly influenced by the pharmaceutical formulation as well as by the presence of food [52]. In this latter respect, nelfinavir, ritonavir, and especially saquinavir, are recommended to be taken with meals whereas indinavir is better taken 1 hour before or two hours after meals. Amprenavir is taken twice a day, without regard to food. Adherence to the recommended regimen is extremely important as the appearance of resistance mutations can be delayed in patients if sufficiently high and sustained plasma levels (greater than or equal to IC_{90} throughout an average dosing interval) are achieved.

Differences in first-pass hepatic metabolism (to which saquinavir is the most susceptible) also have a strong influence on the oral bioavailability of PIs, which are metabolized by cytochrome P450 enzymes (CYP). Consequently, their plasma concentrations can be altered by the concomitant use of inhibitors (e.g. ketoconazole, fluoxetine) or inducers (e.g. rifampin, rifabutin) of these drug metabolizing enzymes. PIs themselves act as CYP inhibitors highlighting its potential for incurring interactions with other drugs (e.g. astemizole, terfenadine, triazolam, ergot alkaloids) by increasing their blood levels. When an *in vitro* model based on human liver microsomes was used to evaluate the inhibitory potency of ritonavir, indinavir, nelfinavir, and saquinavir on six human cytochromes, ritonavir was identified as a potent inhibitor of CYP3A, CYP2C9, CYP2C19, and CYP2D6 isoforms [53]. Indinavir was also a potent CYP3A inhibitor, while nelfinavir and saquinavir were less potent. None of the PIs studied, however, had important inhibitory potency against CYP1A2 or CYP2E1. In this respect, the possibility exists of pharmacokinetically enhancing ("boosting") the activity of a PI through CYP inhibition by concurrent administration of another PI, most notably ritonavir [54], as in the approved ritonavir-lopinavir association (Kaletra®). This approach has proved successful in increasing dosing intervals and minimizing large differences between peaks and troughs in plasma concentrations which translates into both lower viral loads in patients and a reduction in the eventual emergence of resistance.

On the other hand, hepatic enzyme induction by ritonavir and nelfinavir has also been reported. CYP induction is determined typically by measuring both CYP mRNA and protein levels, as well as microsomal activity, in primary cultures of human hepatocytes. Using a pregnane X receptor (PXR or SXR, the receptor for xenobiotic substances) reporter gene assay, ritonavir was recently shown to produce marked PXR activation and CYP3A4 induction [55]. Because of these metabolism-related issues, a dose escalation regimen is recommended during the first two weeks of therapy with this drug.

4.3. Adverse Effects of Protease Inhibitors

All approved PIs have gastrointestinal side effects ranging from nausea (more frequent with ritonavir, as well as vomiting) to loose stools or diarrhea (dose-limiting for nelfinavir), and headache occurs with indinavir and saquinavir. Indinavir causes fewer gastrointestinal problems but it may precipitate in the renal tubules giving rise to nephrolithiasis [52].Cases of worsening glycemia control in patients with pre-existing diabetes, and cases of new-onset diabetes including diabetic ketoacidosis have also been reported with the use of all clinically employed PIs (FDA's MEDWATCH program: http://www.fda.gov/medwatch/). Increased recognition has been given in recent years to serum lipid abnormalities and fat redistribution (lipodystrophy).

A recent study has shown that ritonavir treatment is able to increase plasma triglyceride and cholesterol levels in mice through increased fatty acid and cholesterol biosynthesis in adipose tissue and liver. This PI induced accumulation of the activated forms of sterol regulatory binding proteins (SREBP) 1 and 2 in the nucleus of hepatocytes and adipocytes, resulting in elevated expression of lipid metabolism genes [56].

5. RATIONALE FOR COMBINATION THERAPY

Enhanced antiretroviral activity with no associated increase in cellular toxicity was achieved when RT nucleoside inhibitors started to be co-administered with PIs. The first combinations shown to work well included 2 nucleoside analogs (e.g. zidovudine and lamivudine or stavudine and lamivudine) and a PI [57]. Among the many alternatives available today are the use of double PI combinations (with the possible advantage of twice-daily dosing) or the combined use of one PI with a nonnucleoside RT inhibitor such as nevirapine (Viramune®) or efavirenz (Sustiva®). Although some of the reported combinations can significantly delay selection of mutations, proper human treatment is complicated by pharmacokinetic effects, as summarized above.

It is clear that the cross-resistance profile of the different PIs is a key factor to be evaluated in combination therapy, as well as the possibility of synergistic action rather than simply additive effects, as recently shown *in vitro* for lopinavir and saquinavir over an entire range of drug combination ratios tested [58]. It must also be noted that the full potential of combination therapy with the increasingly large drug arsenal has yet to be realized as only a limited number of the possible combinations and dosing regimens have been fully tested. An up-to-date list of recommended combinations leading to highly active antiretroviral therapy (HAART) for both children and adults can be found at http://www.aidsinfo.nih.gov/guidelines [59].

6. SECOND-GENERATION INHIBITORS

The emergence of viral resistance, difficulties in compliance with the arduous treatment protocols, and adverse side effects have exposed the urgent need for a second generation of HIV protease inhibitors with (i) activity against recombinant mutant laboratory strains and clinical

isolates of HIV-1, (ii) efficacy in cell-based assays in the presence of biologically relevant protein, (iii) improved pharmacokinetic parameters in animal models, and (iv) less severe side effects.

There are several strategies for the design of new protease inhibitors that may inhibit drug-resistant HIV-1 variants. One approach is to use accumulated knowledge of the three-dimensional structures of mutant HIV proteases to design inhibitors that bind tightly to key mutant enzymes. Another strategy is to design compounds that are more flexible in their interactions with the active site, so that the inhibitors can adapt to the structural changes in a variety of mutant enzymes.

6.1. Further Modifications on Known Leads

Fosamprenavir (formerly known as VX-175 and GW433908; Fig. (**4**)) is the calcium phosphate ester prodrug of amprenavir and appears to be hydrolyzed to amprenavir and inorganic phosphate by alkaline phosphatase as it is absorbed through the gut epithelium. The increased water solubility relative to the parent compound eliminates the need for a complex formulation and results in a smaller sized tablet compared to amprenavir. This represents a significant delivery and dosing advantage over amprenavir, since patients need to take only two tablets twice a day instead of eight tablets twice a day, which is expected to make a positive contribution to compliance. Data from phase I studies in healthy volunteers have demonstrated that, whether administered as tablets or suspension, the pharmacokinetics of fosamprenavir is only slightly affected by food [60].

Structure-based incorporation of a novel 3(*R*),3a(*S*),6a(*R*)-bis-tetrahydrofuranyl (bis-THF) moiety as a P2 substituent into a scaffold based on the (*R*)-(hydroxyethyl)sulfonamide isostere present in amprenavir led to TMC-114 (Fig. (**4**)), which showed an EC_{50} of 4.7 nM against wild-type HIV and an EC_{50} of less than 10 nM against 94% of 261 randomly selected isolates resistant to at least one protease inhibitor

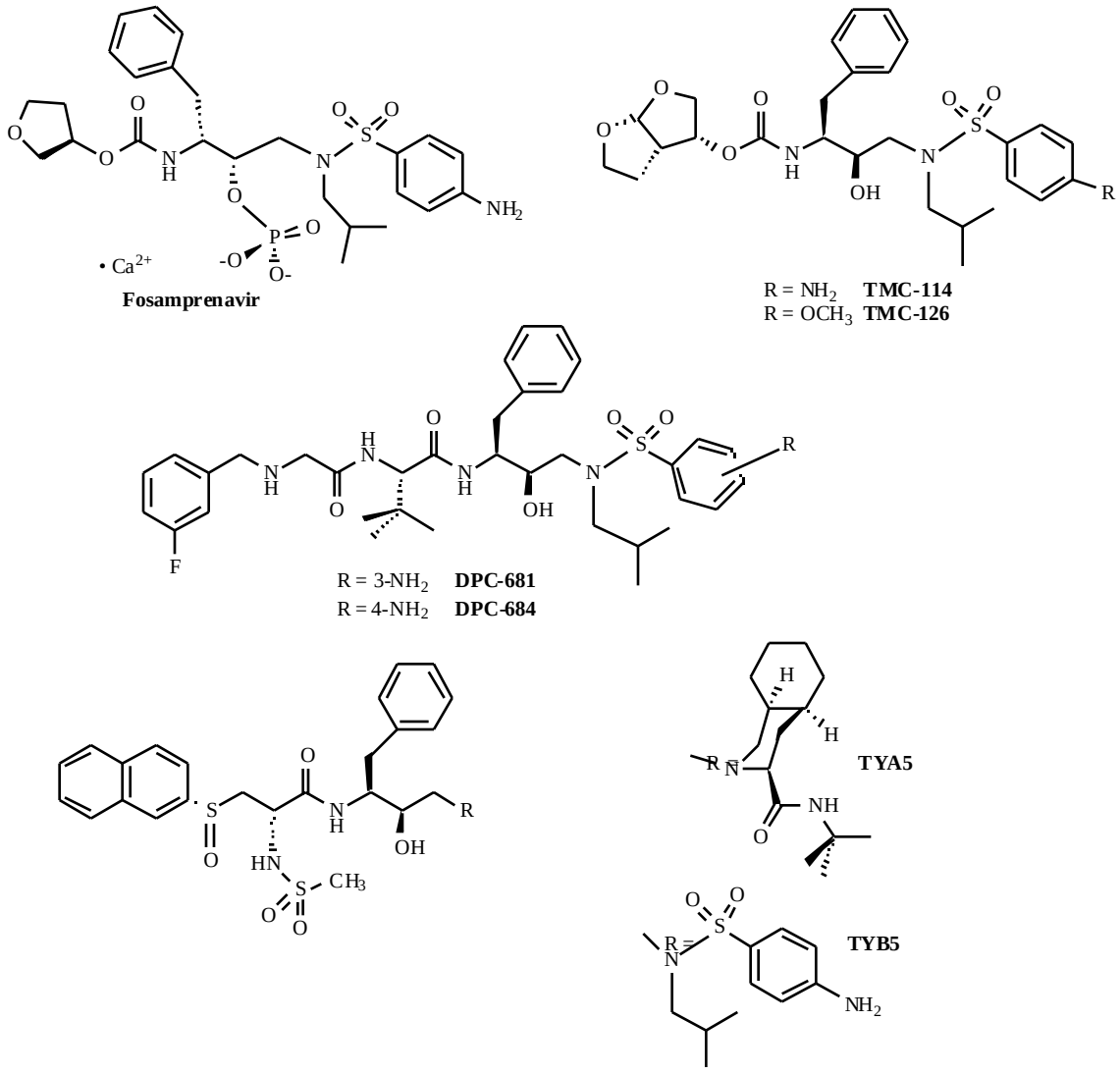


Fig. (4). Examples of second-generation sulfonamide-containing HIV-1 protease inhibitors, all of which (with the exception of TYA5) are related to amprenavir.

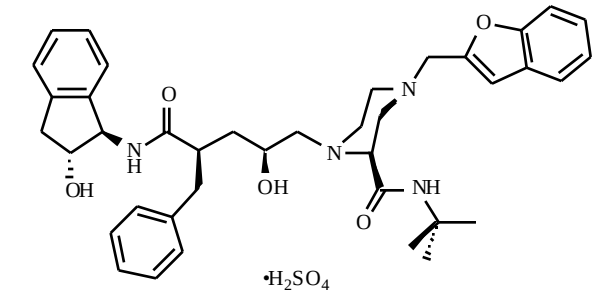
[61]. This remarkable potency is thought to arise from the fact that the bis-THF oxygens can make direct hydrogen-bonding interactions with main chain NH groups from Asp29 and Asp30. Since passaging experiments also showed a very slow development of resistance [62] and survival of only poorly replicating mutants R41T and K70E [63], TMC-114 was selected for clinical development by Tibotec-Virco NV (Mechelen, Belgium), later acquired by Johnson & Johnson. The related TMC-126 (also known as UIC-PI [64] and UIC-94003 [65]) contains a *p*-methoxyphenyl in place of the *p*-anilino group present in the P2' position of TMC-114 (Fig. (**4**)). Both compounds produce very rapid drops in viral load and are highly active against HIV mutants cross-resistant to currently marketed PIs. Upon selection in the presence of TMC-126 of HIV-1 strains already containing L10F, M46I, I50V, A71V, and N88D mutations, the novel active-site mutation, A28S, appeared [65]. The resistant strains, however, emerged more slowly and evolved using genetic pathways different from those seen with other inhibitors.

DPC-681 and DPC-684 (Fig. (**4**)) are two experimental sulfonamide PIs from DuPont Pharmaceuticals under development by Bristol Myers Squibb that derive from the Searle compound SC-52151 (telinavir), which showed no antiviral activity in a Phase I clinical trial due to extensive protein binding [66]. Both compounds are potent inhibitors of wild-type HIV (IC_{90}s = 4 to 40 nM) and resistant variants including that with the D30N mutation in the protease. Remarkably, DPC-681 and DPC-684 had mean IC_{50} values of <20 nM against a panel of chimeric viruses constructed from 30 clinical samples (from patients who failed PI-containing regimens) that harboured between 5 and 11 mutations, including positions 10, 32, 46, 47, 50, 54, 63, 71, 82, 84, and 90 [67]. Unfortunately, electrocardiographic changes first observed in dogs and later in humans precluded further development of the clinical candidate DPC-684.

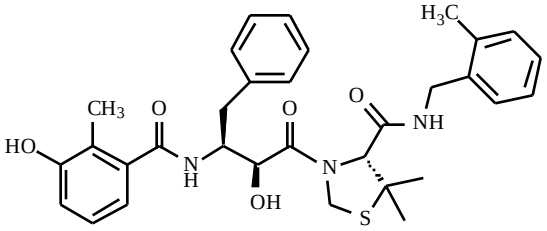
Scientists at Lilly demonstrated the feasibility of replacing the asparagine present in many PI as the P2 ligand (Fig. (**2**)) with appropriately substituted D-aspartic acid and D-cysteine analogs in which the nitrogen was capped by either an acetyl or methanesulfonyl group [68]. Some of the resulting compounds were not only highly potent but also orally bioavailable. Expansion of this concept and incorporation into inhibitor design of other structural fragments from known inhibitors (such as the decahydroisoquinoline unit used in saquinavir and nelfinavir or the sulfonamide unit used in amprenavir) have recently led to novel nanomolar PIs such as TYA5 and TYB5 (Fig. (**4**)), which are also active against some multidrug resistant clinical isolates [69]. Interestingly, the isosteric replacement of the only amide bond in the latter compound by an (*E*)-alkene was not detrimental for HIVp inhibition but led to significant loss of anti-HIV activity.

Further exploration of Merck's hydroxylaminepentanamide transition-state isostere series of HIV protease inhibitors, which initially resulted in the identification of indinavir (Fig. (**3**)), yielded MK-944a (L-756,423) as a clinical backup (Fig. (**5**)). This compound competitively inhibits HIVp with a K_i value of 0.049 nM, is effective in the presence of α_1-acid glycoprotein or human serum albumin, has a longer half-life than indinavir sulfate in several animal models (rats, dogs, and monkeys) and should cause fewer kidney problems [70].

JE-2147 (also named AG1776 and KNI-764), is a tetrapeptide mimetic (Fig. (**5**)) built on a P1 allophenyl-norstatine-P1' dimethylthiazolidinecarboxylic acid backbone which has modified phenolic rings with different linker



MK-944a (L-756,423)



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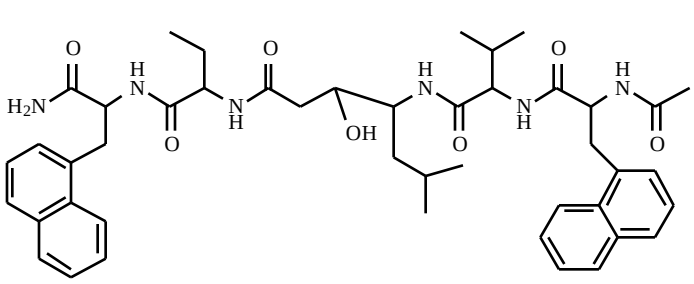

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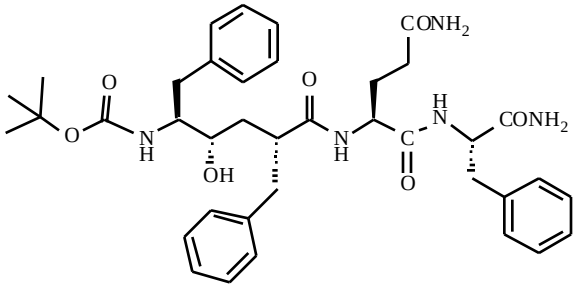


QF-34

Fig. (5). Examples of second-generation HIV protease inhibitors with improved pharmacokinetic profiles (MK-944a) or possessing activity against HIVp-2 and/or a range of clinical isolates usually resistant to approved drugs.

lengths as the P2 and P2' substituents. Although JE-2147 lacks the P3 substituent found in the parent compound, the kynostatin KNI-272 [4,71], the two inhibitors have been shown to display comparable potencies (IC_{50} = 30 and 27 nM, respectively) in a cell-based assay [72]. Besides, JE-2147 is almost as effective against many multi-drug-resistant strains as it is against wild-type HIVp. The L10F, M46I, I47V, and I84V mutations arise in cell culture in the presence of JE-2147, and reduce the binding affinity of JE-2147 for HIVp (28-fold increase in IC_{50}). Although the mutations raised against JE-2147 occur individually in response to other drugs, this particular pattern of mutations is unique to JE-2147 and, of all of them, I47V appears to have the greatest contribution to the reduction in the level of binding. Recently, the 1.09 Å resolution structure of HIVp in complex with JE-2147 has allowed refinement of anisotropic displacement parameters, which reportedly provide a basis for interpreting atomic motion in HIVp and understanding the mechanisms of drug resistance [73].

Comparison of the crystal structures of the statine-based inhibitor LP-130 (Fig. (**5**)) in complex with retroviral proteases from HIV-1, feline immunodeficiency virus, and equine infectious anemia virus showed extensive similarities in the interactions between this nanomolar-range multiple inhibitor and the active-site residues of these enzymes [74]. The ability of LP-130 to accommodate to different types of residues forming the distinct binding sites appears to arise from an absence of the strong, specific interactions that are usually sought after in order to improve PIs' potencies. On the other hand, the flexibility inherent in its central portion comprising the P2-P2' moieties allows the naphthylalanine substituent at the P3/P3' positions to rearrange and establish distinct favorable interactions with different sets of residues, which accounts for the variation in K_i values. Further elaboration of this idea led to the development of the pseudopeptide QF34 (Fig. (**5**)), a subnanomolar PI shown to be equipotent towards HIV-1 and HIV-2 proteases [75]. Since most of the sequence variation between these two targets is located in positions critical for the development of PI resistance, QF34 was suggested to have the potential to inhibit multi-resistant HIVp species. Kinetic analyses using constructed HIVp species harboring the typical (signature) mutations that confer resistance to commercially available PIs has indeed showed that QF34 can effectively inhibit a wide variety of mutant enzymes. Interestingly, the recently solved crystal structure of the HIVp-QF34 complex has revealed an unusual binding mode, such that the inhibitor avoids contact sites that are mutated upon resistance development [76]. Given the flexibility of the central portion comprising the P2-P2' moieties, it is assumed that the P3' substituent can rearrange and establish new interactions in the face of a mutation, as shown previously for LP-130. These results appear to suggest a promising route for the design of second-generation PIs that are active against a variety of resistant HIVp variants.

6.2. Incorporation of the Flap Water Molecule into Inhibitor Design

A recurrent feature that appeared in all of the early HIVp-PI complexes was a buried water molecule bridging the two NH groups of Ile50 present in each of the flaps and two hydrogen bond acceptor atoms of the inhibitor (Fig. (**6a**)). This water molecule ('flap water') is thus tetrahedrally coordinated and shielded from bulk solvent. The following two strategies have been successful in incorporating into the structure of the inhibitor the binding features of this flap water molecule.

6.2.1. 4-hydroxycoumarins and 4-hydroxy-2-pyrones

The oral anticoagulant warfarin was shown to possess inhibitory effects on HIV-1 replication and spread [77], and the structurally related phenprocoumon was independently identified as a weak HIVp competitive inhibitor. When HIVp was co-crystallized with this latter 4-hydroxycoumarin at 2.5 Å resolution [78], the structure of the complex revealed the C-4 hydroxyl group located within hydrogen bonding distance to the two catalytic Asp25 residues and the coumarin lactone replacing the ubiquitous flap water found in complexes of HIVp with peptide-derivative inhibitors (Fig. (**6b**)).

4-hydroxy-3-(3-phenoxypropyl)-2*H*-1-benzopyran-2-one (PD-99560), which lacks both amide bonds and chiral centers, was identified at Parke-Davis as a low micromolar non-peptide HIVp competitive inhibitor [79]. Elaboration of this initial hit and realization of the beneficial effect of replacing the coumarin nucleus with a pyrone system lacking the rigid, fused _-phenyl ring (Fig. (**6c**)) led to a series of 4-hydroxy-5,6-dihydropyrones [80], among which PD-178390 was selected for development. No further clinical progress of this compund has been reported, probably as a consequence of the merging of the company with Pfizer.

The same 4-hydroxy-5,6-dihydropyrone pharmacophore was used at Pharmacia & Upjohn to develop a novel class of non-peptidic HIVp inhibitors which were expected to share the high oral bioavailability and low clearance of warfarin and analogues. Dozens of derivatives were perseveringly produced in a series of iterative cycles of structure-based design and optimization, and PNU-140690 [81] was finally chosen as a clinical candidate with activity against HIV-1 and HIV-2 under the name tipranavir (Fig. (**7**)) [82]. One of the appealing features of tipranavir, which is currently being developed by Boehringer Ingelheim, is that it retains sustained antiviral activity against clinical isolates already resistant to other protease inhibitors [83]. It is presently in Phase III trials and is being studied in twice-daily dosing in combination with ritonavir (Norvir®).

6.2.2. Cyclic Ureas and Conceptually Related Inhibitors

In a renowned and very elegant example of structure-based drug design, the strategy followed at Du Pont Merck for water displacement and high affinity binding in the HIVp active site was to use a 7-membered cyclic urea ring [84]. This is a highly preorganized scaffold that incorporates a diol functionality (Fig. (**6d**)), with the urea nitrogens providing suitable handles for introducing complementary groups into the S2 and S2' subsites [85]. The optimal stereochemistry in this series was shown to be 4*R*,5*S*,6*S*,7*R* although the potency of the 4*R*,5*S*,6*R*,7*R* diastereomer was of comparable magnitude [86]. The X-ray and solution NMR structures of

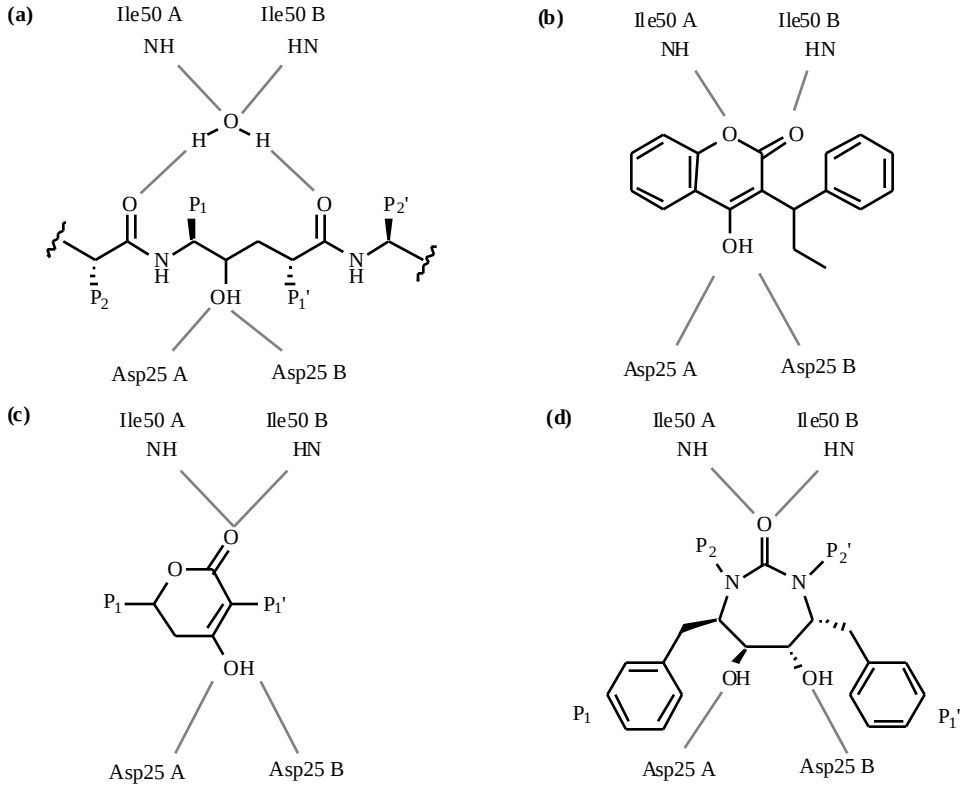
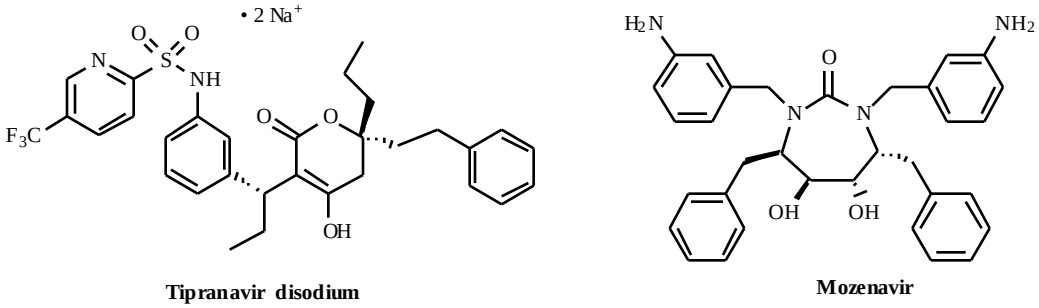


Fig. (6). (a) Hydrogen bonds (dotted lines) between the flap water and both the protein and a typical inhibitor. Examples of molecular scaffolds that incorporate the functionality of this structural water molecule into an HIVp inhibitor: (b) phenprocoumon, (c) a 5,6-dihydro-4-hydroxy-2-pyrone, and (d) a cyclic urea.



Tipranavir disodium **Mozenavir**

Fig. (7). Examples of HIVp inhibitors in clinical development that incorporate the functionality of the flap water molecule into their structure.

several complexes in this series have confirmed the displacement of the structural flap water and have provided additional details of the interactions, for example that both Asp25 are protonated in these complexes [87]. The use of *p*-hydroxymethylbenzyl groups led to the development of DMP-323, a potent PI that entered clinical trials but was withdrawn as a result of high blood level variability in humans. The related DMP-450 [88] containing *m*-aminomethylbenzyl groups qualified as a second clinical candidate that was developed first by Bristol-Myers Squibb and later by Triangle Pharmaceuticals as once-daily mozenavir (Fig. (**7**)). Progress was interrupted, however, due to disappointing results, including reduced activity against virus resistant to indinavir and ritonavir. Indeed, both I84V and V82A had been reported as escape mutations to mozenavir in cell cultures, and it is known that the double mutation L10F/I84V confers even greater resistance [89]. Further developments in this series have led to some other very potent PIs, such as the symmetric SD-146, but they could not proceed any further due to their extreme insolubility in water and oils [90].

None of the other inhibitors conceptually related to the cyclic ureas, such as the 6-membered tetrahydropyrimidinones [91], the cyclic sulfamides [92], the cyclic sulfones based on a 7-membered ring thiepane dioxide [93], the cyclic cyanoguanidines [94], and sulfonamide-substituted cyclo-octylpyranones [95] appear to have made it into the clinic.

6.3. Azapeptides

Replacement in a peptide of the Cα group of one or more amino acids by a nitrogen atom leads to azapeptides [96]

possessing properties that closely resemble those of the parent peptides. Incorporation of an azapeptide into inhibitor design has led to atazanavir, initially developed by Novartis as CGP-73547 [97] and later by Bristol-Myers Squibb as BMS-232632 (Fig. (**8**)) [98]. Atazanavir (Zrivada®), currently in Phase III clinical trials, combines satisfactory oral bioavailability with good antiviral activity against wild-type and several resistant mutant strains of HIV. Thus, nelfinavir-, saquinavir-, and amprenavir-resistant strains of HIV-1 have been shown to remain sensitive to this compound, whereas sensitivity is reduced only 6- to 9-fold in indinavir- and ritonavir-resistant viruses [99]. In selection experiments, the first mutation in the viral protease was N88S (a position in a helix that lies behind the substrate binding pocket and previously reported to confer resistance to the hydroxyethylurea inhibitor SC-55389A [100]) and the change I84V also appeared to be important. Atazanavir-resistant viruses, however, remain sensitive to saquinavir, while showing various levels of cross-resistance to nelfinavir, indinavir, ritonavir, and amprenavir (0.1- to 71-fold decreases in sensitivity). More recently, both the I50L substitution and the I50L/A71V combination were found to confer reduced susceptibility to atazanavir but these mutations were not associated with loss of susceptibility to other PIs [101]. This profile makes atazanavir a valuable addition for use in combination therapy but what really makes it stand out from existing PIs is the possibility of once-daily dosing and the lack of associated dyslipidaemias in patients [102], although it may increase plasma levels of bilirubin and cause prolongation of the cardiac QT interval in some individuals.

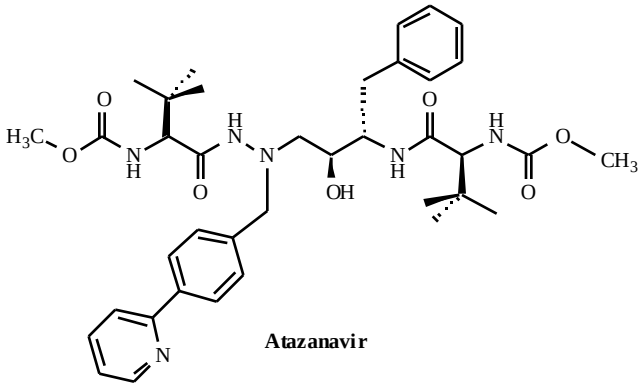


Fig. (8). Molecular structure of atazanavir, the first azapeptide that has been clinically developed as an HIVp inhibitor.

7. LESSONS FROM THERMODYNAMIC AND KINETIC STUDIES

Binding of many inhibitors to HIVp has been shown to be entropically driven with the dominant binding force being the increase in solvent entropy that accompanies the burial of a significant hydrophobic surface [11]. This entropy of desolvation is opposed by the conformational entropy loss upon binding, which probably contributes to the lower binding affinity of the substrates relative to the inhibitors [103]. Peptide substrates, on the other hand, are more flexible in solution than most synthetic inhibitors and they can probably adapt better to backbone rearrangements or subtle conformational changes induced by mutations on the protease. In addition to closure of the twofold related flaps, other domain motions associated with substrate binding have been identified that result from rigid body rotations at primarily hydrophobic surfaces. Mutations at interdomain interfaces, which may be spatially removed from the active site, can disfavor the 'closed' conformation (Fig. (**1**)) and increase the off-rate of competitive inhibitors thereby leading to resistance [103]. Regarding the enthalpy of formation of intermolecular interactions upon complexation, it is opposed by the unfavorable binding enthalpy of rearranging the flap region (Fig. (**1**)) and also by the enthalpy of desolvation.

These enthalpy-entropy compensatory changes that take place in wild-type and mutant proteases upon binding both substrates and inhibitors are very relevant from a drug design standpoint but they are not easy to predict [11]. One example is the potent inhibitor JE-2147 (Figure **5**, K_i = 41 ± 18 pM), which can establish no more hydrogen bonds with the protease than can the inhibitors of Fig. (**3**), yet it binds with an enthalpic term that is up to 10 kcal mol^{-1} more favorable [104]. Another example is provided by amprenavir (Fig. (**3**)) and the related TMC-126 (Fig. (**4**)): whereas the former binds to wild-type HIVp with high affinity ($5.0 \cdot 10^9$ M^{-1} or 200 pM) in a process equally favored by enthalpic and entropic contributions, the latter binds with much higher affinity ($2.6 \cdot 10^{11}$ M^{-1} or 3.9 pM) in a process in which enthalpic contributions overpower entropic contributions by nearly a factor of 4. On the other hand, the mutation I50V lowers the binding affinities of amprenavir and TMC-126 by a factor of 147 and 16, respectively, and similar ratios are observed for the V82F/I84V dual mutation [105]. This implies that the affinity of TMC-126 for the amprenavir-resistant HIVp mutants is still higher than that of amprenavir for the wild-type enzyme, in accord with the inhibition results from *in vitro* and *in vivo* studies.

Optimization of inhibitors has usually been guided by a compound variable (K_D or K_i) rather than by the independent variables representing the association (k_{on}) and dissociation (k_{off}) rates for the interaction between the enzyme and an inhibitor. But insofar as the association rate is concentration-dependent, slow association rates can be compensated by high intracellular concentrations whereas dissociation rates can be slowed down only by optimizing the interaction. By using a surface plasmon resonance-based biosensor [106], a group has recently published highly relevant kinetic data regarding the interaction between HIVp and 58 structurally diverse transition-state analogue inhibitors [107]. Association and dissociation rate constants, as well as affinities, were determined (Table **1**) and displayed as k_{on}-k_{off}-K_D maps. Interestingly, different classes of inhibitors were found to fall into distinct clusters in these maps. Significant changes in association and dissociation rates were found as a result of modifying the P1/P1' or P2/P2' side chains of a linear lead compound. Similarly, cyclic urea and cyclic sulfamide inhibitors displayed different kinetic features and the affinities of both classes of cyclic compounds were limited by fast dissociation rates.

Results such as these highlight that similar affinities within a series can result from combinations of association and dissociation rates differing 2-3 orders of magnitude

Table 1. Association and Dissociation Rate Constants (k_{on}, k_{off}) and Equilibrium Constants (K_d) for the Interaction between Inhibitors and HIV-1 Protease [107].

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (nM)	K_i (nM)
indinavir	$1.53 \cdot 10^{6} \pm 2.42 \cdot 10^{5}$	$1.58 \cdot 10^{-3} \pm 1.29 \cdot 10^{-4}$	1.07 ± 0.108	0.31
nelfinavir	$6.63 \cdot 10^{5} \pm 3.04 \cdot 10^{5}$	$6.68 \cdot 10^{-4} \pm 5.88 \cdot 10^{-5}$	1.64 ± 0.480	0.54
ritonavir	$3.92 \cdot 10^{6} \pm 1.11 \cdot 10^{6}$	$2.16 \cdot 10^{-3} \pm 2.98 \cdot 10^{-4}$	0.608 ± 0.075	0.59
saquinavir	$8.17 \cdot 10^{5} \pm 1.61 \cdot 10^{5}$	$2.27 \cdot 10^{-4} \pm 3.04 \cdot 10^{-5}$	0.315 ± 0.07	0.23
amprenavir	$4.43 \cdot 10^{6} \pm 1.25 \cdot 10^{6}$	$4.88 \cdot 10^{-3} \pm 1.45 \cdot 10^{-3}$	1.13 ± 0.111	0.23
DMP-323	$2.52 \cdot 10^{10} \pm 9.99 \cdot 10^{9}$	83.3 ± 22.1	3.83 ± 1.25	0.27

(Table **1**). The lack of gross changes in affinity may lead to the erroneous conclusion that chemical modifications are not having a strong influence in the interaction when it may be true that the underlying kinetic constants are varying widely.

8. DIMERIZATION INHIBITORS

The homodimeric nature of HIVp suggests that enzyme inhibition could be accomplished in an alternate way by preventing the self assembly of the biologically active dimer in infected cells. A large part of the dimerization interface is made up by an interdigitation of the N- and C-terminal portions of each monomer (Fig. (**1**)). Since this region appears to be highly conserved among HIV-1 isolates, it stands out as an attractive target that is quite distinct from the substrate binding cavity. In fact, the proof of concept for the feasibility of this hypothesis was obtained as early as 1991 when peptides corresponding to the N- and C-termini of HIVp were shown to effectively inhibit enzyme activity [108,109], albeit at micromolar concentrations. In one of the first examples of dissociative inhibition, kinetic analysis showed that the tetrapeptide Ac-Thr-Leu-Asn-Phe-COOH, corresponding to the COOH-terminal segment of the enzyme, was able to bind to the inactive protomers and prevent their association into the active dimer [109]. Further interference with dimer assembly has been achieved, among others [110], by contiguous peptide sequences containing the N- and C-terminal regions of HIVp linked with a 3.5 Å tether composed of three glycine residues [111] and by covalently crosslinked N-terminal HIVp peptides [112].

Peptides with affinity for the interfacial surface of HIVp's constituent monomers have been sought also using a genetic selection approach. By fusing a non-catalytically active D25N HIVp variant to the DNA-binding domain of the repressor protein from bacteriophage λ, a hybrid repressor was generated that allowed high-throughput screening of a library of $5 \cdot 10^{8}$ peptides with 9 random amino acid residues. Less than one peptide in 10^{6} was found to be able to disrupt HIVp monomer association and in those identified there was an abundance of nonpolar residues, especially valine, alanine, and glycine [113]. Addition of a cysteine residue to the C-terminus of one of these peptides and crosslinking with the homobifunctional reagent 1,6-hexane-*bis*-vinyl-sulfone (Fig. (**9**)) led to a 40-fold more potent dissociative inhibitor (dissociative inhibition constant of 0.8 μM).

Similar low-micromolar, noncompetitive or dissociative inhibition constants have been reported for a few nonpeptidic natural products and derivatives, most significantly some triterpenes (e.g. ursolic acid, Fig. (**9**)), identified through a pharmacophore-based computer search of the Cambridge Structural Database, and several simplified pentaester derivatives of didemnaketals A (Fig. (**9**)) and B (first isolated from a marine ascidian belonging to the genus *Didemnum*), the most potent of which is shown in Fig. (**9**) [114].

9. OVERALL PERSPECTIVE AND FUTURE PROSPECTS

The initial optimism surrounding the development and clinical introduction of PIs, especially in combination with additional antiretroviral agents, was soon tempered by their toxicity profiles, their potential for drug interactions, and by the emergence of resistance. Although HAART has had a major beneficial impact on life expectancy and has significantly reduced the number of hospitalizations for HIV-related problems and the incidence of major AIDS-related opportunistic infections, major drawbacks still remain: complicated drug regimens have a negative effect on patient compliance, drug side-effects are cumulative and long-term, and the outcome of therapy is highly dependent on viral load and initial counts of CD4+ lymphocytes. The patient's nonadherence to the medication schedule can result in incomplete suppression of viral replication and the selection of resistant strains. The P-glycoprotein efflux system can be important in limiting oral absorption and reducing brain entry of many of these drugs, including indinavir, nelfinavir, and saquinavir [52]. Another therapeutic challenge is finding ways to eliminate HIV from sanctuaries (e.g. the central nervous system and the testicles) [115], and from infected resting CD4+ cells which contain an integrated provirus that produces few or no viral particles and cannot be eliminated with current antiretroviral drugs.

Parallel to the list of successful PIs and drug candidates is an impressively longer list of compounds that did not make it into the clinic. Most often, the therapeutic utility of many of the compounds synthesized has been compromised by poor pharmacokinetic parameters. Improved pharmacokinetic properties have been usually achieved by reducing both the peptide nature of the inhibitors, as well as their size. Development of new PIs is further complicated by the

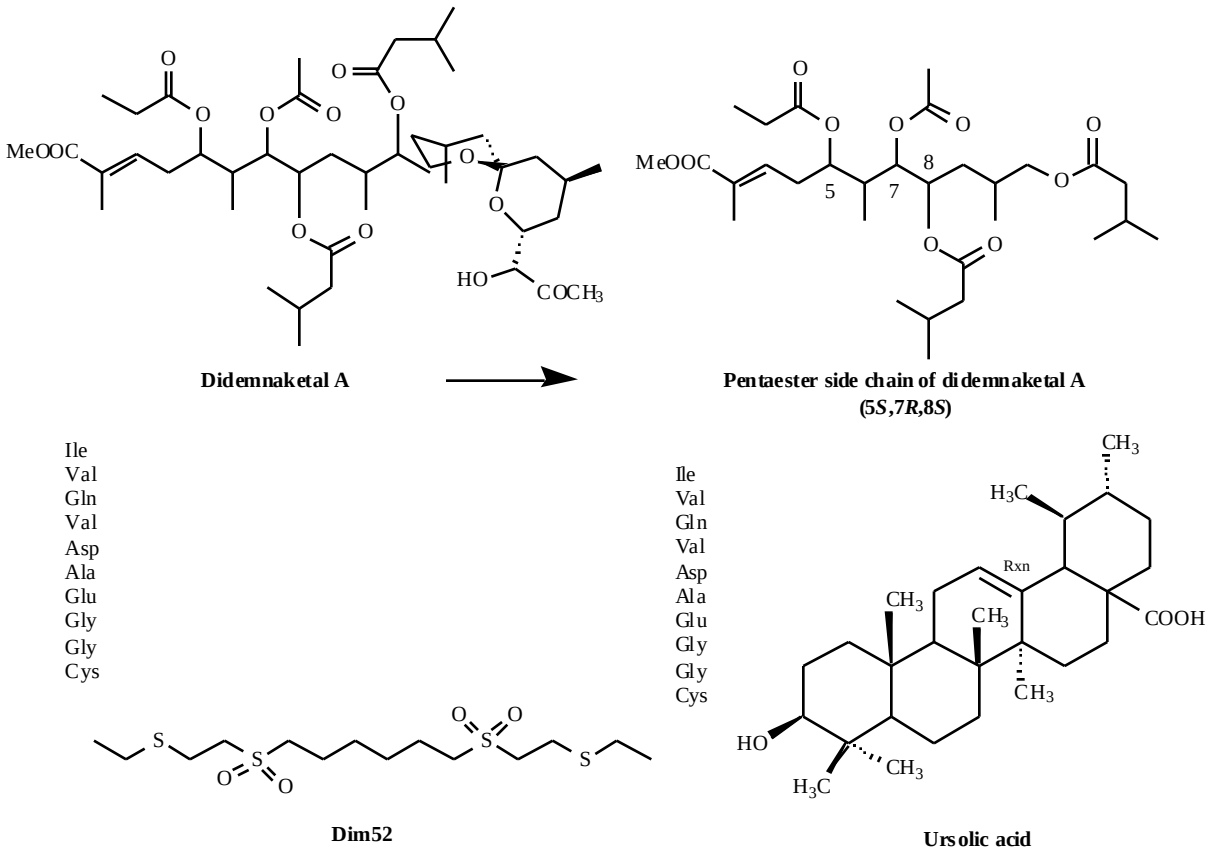


Fig. (9). Examples of compounds reported to be able to prevent HIVp monomer association or promote dimer dissociation.

need to test agents in combination, avoiding extended periods of monotherapy (the sequential addition of single drugs to an ineffective regimen is likely to be associated with a poor therapeutic outcome).

The second-generation PIs are expected to be not only potent and orally bioavailable, but also more tolerable and able to overcome cross-resistance in patients. That simultaneous fulfillment of all of these requirements is no easy task is attested by the large number of compounds that did not progress into phase I/II clinical studies or were dropped at later stages. Thus, despite the wealth of structural, kinetic, and thermodynamic knowledge gained from the complexes of both wild-type and mutant HIVp with inhibitors and the vast number of compounds synthesized, this relatively small target keeps posing problems that are not easy to solve. Thus, although there are reasons for moderate optimism the reality is sobering and highlights our intrinsic limitations in medicinal chemistry enterprises.

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