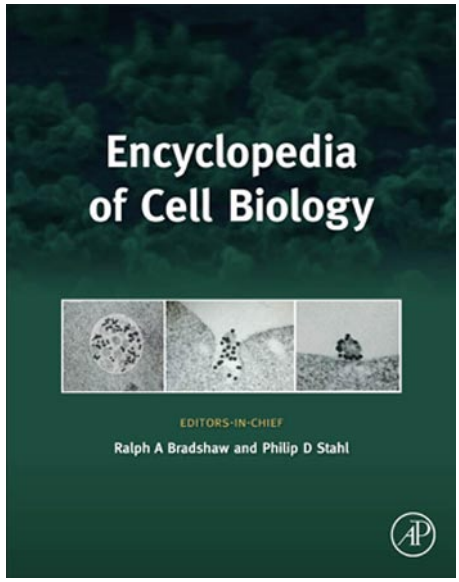

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PROTEIN SYNTHESIS/DEGRADATION: PROTEIN DEGRADATION – PATHOLOGICAL ASPECTS

Contents

Inhibitors of HIV Protease

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Glossary

Allosteric site A site other than a protein's (usually enzyme's) active site, which affects its activity when an effector molecule binds to it.

Aspartic protease An enzyme cleaving peptide bonds in proteins that has an active site consisting of two closely spaced, coplanar aspartate residues that activate a water molecule for the hydrolysis reaction. Enzymes belonging to the AA clan of aspartic peptidases, typified by pepsin found in gastric juice, are present in most organisms higher than bacteria, as well as in retroviruses, where they are called retropepsins and function as symmetric homodimers.

Bioavailability The fraction of an administered dose of a drug that reaches the systemic circulation, usually less than 100% in oral administration.

Diastereomer A stereoisomer of an optically active compound, in which the configuration of one or several chiral centers has been inverted (e.g., from *R* to *S*). Diastereomers should not be confused with (a pair of) enantiomers, in which the configuration of all chiral centers has been inverted. Consequently, enantiomers are mirror symmetric while diastereomers are not, and for this reason display different physical and chemical properties.

HIV (-1,-2) Human immunodeficiency virus (type 1 or 2), the causative agent of AIDS. The most common and virulent variant is designated HIV-1, whereas HIV-2 is more closely related to simian immunodeficiency virus and is less virulent.

HTLV Human T-cell leukemia virus, the first retrovirus to be associated with human disease (lymph node leukemia).

Integrase A virally encoded enzyme responsible for the incorporation of the double-stranded DNA copy of the retroviral genome into host cell genome. After integration, the infection is permanent and the infected cell cannot be cured.

Lipodystrophy A pathological condition characterized by abnormal or degenerative conditions or distribution of the body's fat.

M-PMV Mason–Pfizer monkey virus, a retrovirus similar to HIV, causing acquired immunodeficiency syndrome (AIDS)-like syndrome in macaque (rhesus) monkeys.

Nucleophile A chemical entity (such as a water molecule or hydroxyl group) rich in electrons, that is capable (usually after additional activation) of attacking another chemical group (such as the C atom of a peptide bond (O=)C–N) with depleted electron density, during a chemical transformation, such as hydrolysis.

Pepstatin A universal inhibitor of aspartic proteases, originally isolated from cultures of various species of *Actinomyces*.

Retrovirus A virus with a single-stranded mRNA genome, which is reverse-transcribed into DNA in a process that inverts the normal flow (DNA→RNA) of genetic information.

Reverse transcriptase (RT) A virally encoded enzyme that produces a double-stranded DNA copy of a retroviral RNA genome. RT is an RNA-dependent DNA polymerase, with ribonuclease activity.

RSV Rous sarcoma virus, a retrovirus causing sarcoma in chickens. The first retrovirus to be described, over a century ago, by Peyton Rous. The currently preferred name is avian sarcoma virus (ASV).

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Introduction

The emergence of the acquired immunodeficiency syndrome (AIDS) epidemic in the early 1980s and the subsequent identification of the human immunodeficiency virus (HIV) as its causative agent brought into focus the need to accelerate research on retroviruses, in order to assist the efforts to create anti-HIV drugs. No such drugs were available during the decade after the first reports of the new disease started appearing and initially the disease itself was invariably lethal since the retrovirus, which infects T4 leukocytes, devastates the immune system and leads to its complete failure. With lack of any treatment options, the outlook was obviously very grim.

Retroviruses have been known for over a century, since the identification of an infectious agent causing cancer in chickens, later named after its discoverer Rous sarcoma virus (RSV) (Rous, 1911). However, the exact nature of the agent, of its life cycle and the mode of infectivity were not established until much later. It is now known that the genetic material of retroviruses consists of single-stranded RNA of positive polarity (mRNA) that becomes transcribed into DNA in a reverse (or retro) transcription reaction that is catalyzed by a retrovirus-specific and virally encoded reverse transcriptase (RT). Another retroviral enzyme, integrase (IN), incorporates the resulting double-stranded viral DNA into the host genome, thus making the genetic material of the retrovirus (provirus) a permanent part of the infected cell.

One of the unusual characteristics of many viruses, including all retroviruses, is that their proteins are not translated as individual final units, but rather as one or more large polyproteins that need to be processed (cleaved) into the mature viral enzymes and structural proteins. In retroviruses, the enzyme responsible for such an activity is retroviral protease (PR). Analyzing the retrotransposon and retroviral genomic sequences, including human T-cell leukemia virus (HTLV) (closely related to HIV) and RSV, Toh *et al.* (1985) found a single copy of a signature sequence D(S/T)G (aspartate, serine or threonine, and glycine) in the translated proteins that could be matched with the active-site motif of aspartic proteases from the pepsin family. Pepsin and similar cell-derived aspartic proteases, however, contain this motif in two copies in their pseudo-twofold-symmetric active site, each contributed by a separate domain of a single polypeptide chain. To reconcile these puzzling observations, Toh *et al.* postulated the presence of a pepsin-like protease in retroviruses, with the caveat that the retroviral proteases would need to be symmetric homodimers composed of two identical subunits. This hypothesis was consistent with an earlier speculation by Tang *et al.* (1978) that cell-derived aspartic proteases have arisen from smaller proteins by gene duplication and divergent evolution. The postulate about the homodimeric nature of retroviral proteases (retropepsins) was experimentally confirmed when the crystal structure of RSV protease was determined (Miller *et al.*, 1989a).

Without a functional protease (which could be inactivated by mutation or by inhibitors) the retrovirus is still capable of replicating, but only immature viral particles can be formed, which are noninfectious, i.e., cannot infect other T4 cells or other patients. Although inhibition of HIV PR does not cure the infection *per se*, it can be hoped that with prolonged treatment, inhibition of HIV PR will allow sufficient time for the elimination of the pool of the infected T4 cells.

Structure and Enzymatic Mechanism of HIV Protease

The structure of HIV-1 (type 1) PR itself, in unliganded state, was independently determined in 1989 by three groups (Navia *et al.*, 1989; Wlodawer *et al.*, 1989; Lapatto *et al.*, 1989). As in the case of RSV PR, HIV-1 PR is also a homodimer of two protein chains, 99 residues each. The protein fold of one subunit topologically resembles a truncated version of a single domain of pepsin, with the inter-subunit interface formed by four intertwined β strands from all termini (residues 1–4 and 95–99 from each subunit). The N-terminal strand is followed by two more β strands comprising residues 9–15 and 18–25, the latter including the catalytic Asp25. The next strand consists of residues 30–35 and is followed by a broad loop 36–42. Two more β strands, 43–49 and 52–58 form a flexible 'flap' loop that acts as a gating element for the active site. The second half of the HIV-1 PR subunit is topologically related to the first half and consists of β strands 52–66, 69–78, and 83–85. A prominent helix comprising residues 86–94 (absent in the N-terminal fold) has a clear counterpart in pepsins.

In the catalytic mechanism of aspartic proteases, a nucleophilic water molecule is activated by hydrogen bonding between the two (hemiprotonated) aspartate carboxylic groups. Such a catalytic water molecule was also found in the active sites of RSV PR (Miller *et al.*, 1989a) and HIV-1 PR (Wlodawer *et al.*, 1989) and its involvement in the enzymatic mechanism was gleaned from structural data (Jaskólski *et al.*, 1991).

Inhibitors of Retroviral Proteases

Strategies aimed at deactivation of HIV PR are focusing on relatively small chemical molecules that could inhibit its catalytic function. In a broad classification, the inhibitors can be covalent (irreversible) or non-covalent (reversible). Covalent inhibitors, such as molecules with a reactive oxirane (epoxy) group (Ro *et al.*, 1999) that attaches itself to the active-site aspartate, are not good candidates, even though they would block the enzyme permanently, because they can also modify many important host proteins. Non-covalent inhibitors typically compete with substrates for the active site, but bind more strongly and are resistant to cleavage. In the simplest approach, a competitive inhibitor is designed using as template the amino acid sequence of a good peptidic substrate (with some optimization), and replacing the scissile bond by a non-cleavable surrogate. Non-peptidic inhibitors use a similar strategy but the sequences of chemical moieties designed for docking in the respective enzyme binding sites do not have amino acid or peptidic character.

Whereas the structures of ligand-free retroviral proteases were crucial for delineating their structural and evolutionary relationship to pepsins, they were by themselves not sufficient to guide the design of potent inhibitors of HIV-1 PR.

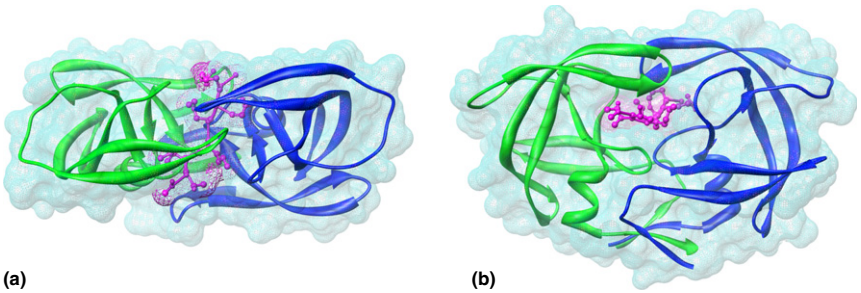

Figure 1 Two canonical views of HIV-1 PR complexed with MVT-101, the first inhibitor cocrystallized with this enzyme. The main-chain traces of the two subunits of the enzyme are colored green and blue, and the ball-and-stick model of the inhibitor is in magenta. The molecular surface of the protein is shown in light blue, and the surface of the inhibitor in pink. In (a) the homodimeric enzyme is viewed along its twofold axis. In (b) this axis is vertical. Figure prepared by Dr. Jiri Vondrasek.

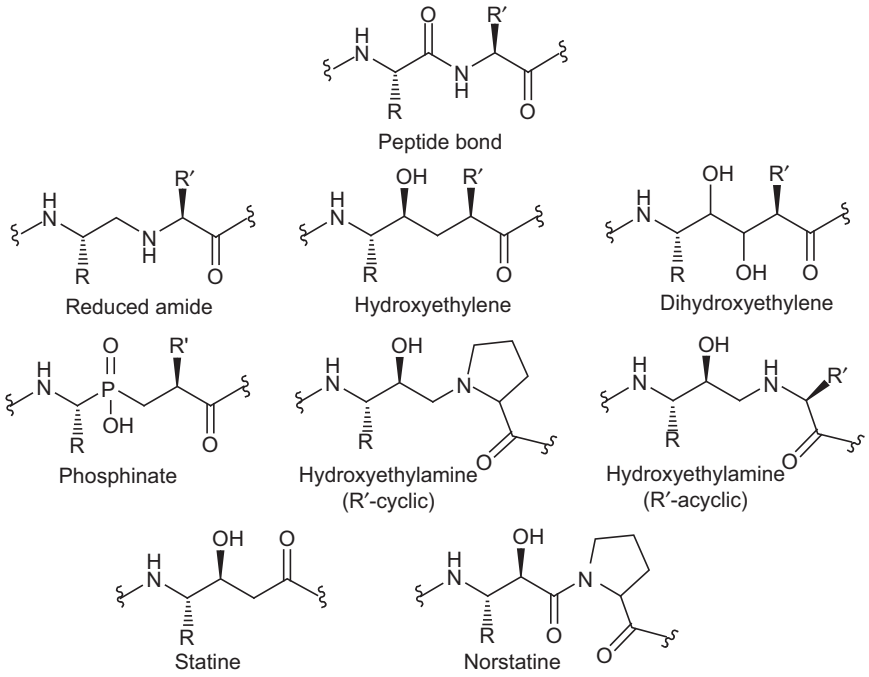

Figure 2 Chemical formulas of the peptide bond and its selected non-cleavable replacements.

Nevertheless, a model of an inhibitor complex was created on the basis of the structure of the apoenzyme of RSV PR superimposed on rhizopuspepsin, a fungal pepsin-like enzyme, in complex with a peptidic inhibitor with a reduced peptide replacing the cleavage site, His-Pro-Phe-His-Phe-ψ[CH-NH]Phe-Val-Tyr (where ψ denotes the reduced bond between the two Phe residues). Analysis of this model allowed successful delineation of seven subsites for the interactions between the side chains of the inhibitor and HIV-1 PR (Weber *et al.*, 1989).

Progress toward full understanding of the details of the interactions between the inhibitors of HIV-1 PR and the enzyme was boosted by the elucidation of the crystal structures of numerous complexes, now most likely going into thousands (although it is not possible to ascertain the exact count). The first such structure involved a complex with MVT-101 (Miller *et al.*, 1989b), a peptidic inhibitor created on the basis of a good natural substrate of HIV-1 PR with the sequence Ac-Thr-Ile-Met-Met-Gln-Arg.amide (K_m 1.4 mM) (Figure 1). In an approach established previously for the synthesis of peptidic inhibitors of human renin (which is a pepsin-like aspartic protease involved in the blood pressure control cascade), both methionines in this inhibitor were replaced by norleucine isosteres, and the scissile peptide between them was replaced by a reduced analog. The inhibition constant K_i for MVT-101 was 0.78 μM, indicating that this compound acted as a comparatively weak inhibitor and could not be considered as a good drug candidate, although it was powerful enough to form a stable complex with the protease during crystallization experiments.

Binding of MVT-101 led to quite substantial rearrangements of the structure of the enzyme, with the tips of the flaps, locked over the active-site-bound inhibitor, moving as much as 7 Å away from their positions in the free protease (where they were most likely fixed by crystal packing). In addition, in the complex with MVT-101 and all other peptidic inhibitors, a tightly bound water molecule was found at the interface

between the locked flap arms and the central part of the bound inhibitor, in variance with analogous pepsin-like complexes, where essentially only one flap is long enough to gate access to the active site. Although the HIV PR dimer is symmetric in the absence of a ligand with unique directionality, its binding induces some asymmetry which, if not transmitted to the surface during crystallization, leads to apparent twofold orientational disorder of the inhibitor that was noted in a number of structures. This binding, in turn, allowed delineation of the pockets that would accommodate the side chains of MVT-101. In the convention of Schechter and Berger (1967), in which the N-terminal (N-Pn…P1-) and C-terminal (-P1′…Pn′-C) substrate/inhibitor residues (linked by the P1-P1′ scissile bond/analog) are docked in the corresponding Sn…S1…S1′…Sn′ subsites of the enzyme, subsite S3 of HIV-1 PR included Arg8′, Asp29, and Gly48; subsite S2 was lined by Ala28, Ile47, Ile50′, and Ile84; and subsite S1 included Leu23′, Asp25′, Ile50, Pro81′, Val82′, and Ile84′. (Primed residues refer to the second subunit of the homodimer.) The subsites on the other side of the non-scissile P1-P1′ bond were generally similar with few exceptions. Subsite S1′ included Leu23′, Gly27, Asp25, Ile50′, Pro81, Val82, and Ile84; subsite S2′ consisted of Val32′, Ile47′, Gly48′, and Ile50; and subsite S3′ was surrounded by Arg8, Gly27′, Asp29′, Gly48′, and Val82.

A large number of structures of complexes with other peptide-based inhibitors that included non-scissile peptide mimetics of different chemistry (**Figure 2**) followed, providing a wealth of information about the plasticity of the enzyme subsites. The structure of a complex with acetyl-pepstatin, in which the scissile bond was replaced by the unnatural amino acid statine, elucidated the binding of this standard inhibitor of cell-derived aspartic proteases to HIV-1 PR (Fitzgerald *et al.*, 1990). Another early structure included a complex with JG-365, a hydroxyethylamine-containing peptide analog (Swain *et al.*, 1990). The structural data on a large number of inhibitors and inhibitor complexes of HIV PR were previously summarized in detailed reviews (Wlodawer and Erickson, 1993; Fitzgerald, 1993; Wlodawer and Vondrasek, 1998; Qiu and Liu, 2011).

First generation of HIV-1 PR Inhibitors Approved as AIDS Drugs

A complete summary of the status of the inhibitors of HIV-1 PR that have been approved for clinical use by the food and drug administration (FDA) can be found in Wikipedia. The first such inhibitor, approved on 6 December 1995, was saquinavir (**Figure 3**), developed by Hoffmann-La Roche. This compound, originally designated as Ro 31–8959, was the subject of intensive biochemical, biological, and structural studies (Craig *et al.*, 1991). It has a molecular weight (MW) of 671. In common with JG-365, saquinavir utilizes hydroxyethylamine as a non-cleavable peptide isostere. However, early crystallographic studies (Krohn *et al.*, 1991) suggested that the more potent version of this compound should be the *S* diastereomer of the –C*(OH)– chiral center, rather than the *R* form found in JG-365. Another major difference between these two compounds is the replacement of Pro, located in the P1′ position of JG-365, by DIQ ((*S*,*S*,*S*)-decahydroisoquinoline-3-carbonyl) in saquinavir. The structure of a saquinavir complex reported in 1991 (Krohn *et al.*, 1991) was deposited in the Protein Data Bank only in 1996 and released in 1997 (PDB ID 1HXB), but a number of other structures using the native and mutant forms of HIV-1 PR became available since then, including the atomic-resolution (0.97 Å) structure (**Figure 4(a)**) of a complex with the V82A mutant (PDB ID 2NMZ; (Tie *et al.*, 2007)). The main problem with the original formulation of the drug (sold under the name of Invirase) was poor bioavailability, as low as 3–4%. However, the later reformulation (brand name Fortovase) increased the bioavailability very significantly.

Three more inhibitors of HIV-1 PR gained FDA approval in 1996 and 1997 (Wlodawer and Vondrasek, 1998). Abbott Laboratories (now AbbVie) developed ritonavir (brand name Norvir), a relatively large (MW 721) peptidomimetic inhibitor (**Figure 3**) that was designed as a result of testing the concept of making the inhibitors fully, or nearly, C_2-symmetric. Even though ultimately such symmetric inhibitors turned out to be more difficult to manipulate for improved solubility and bioavailability, the concept resulted in the inclusion of a $-CH_2-C(OH)-$ group as a non-cleavable linker between two phenylalanines that occupy the S1 and S1′ subsites of the enzyme. Ritonavir has much better bioavailability than saquinavir, but it quickly became apparent that it had an unanticipated ability to act as a very potent inhibitor of cytochrome P450 (particularly of its isoform Cyp3A4), slowing down the removal of other pharmaceutical agents from circulation (Lea and Faulds, 1996). For that reason, ritonavir is currently used only as a booster for other protease inhibitors (see below).

Another peptidomimetic inhibitor of HIV-1 PR is indinavir (**Figure 3**; brand name Crixivan), developed by Merck (Vacca *et al.*, 1994). Slightly smaller (MW 614) than either saquinavir or ritonavir, it utilizes a hydroxyethylamine insert as a non-cleavable group. The compound is highly selective for HIV-1 PR (K_i 0.52 nM) and HIV-2 PR (K_i 3.3 nM) while it shows no inhibitory activity against any mammalian (including human) aspartic proteases.

An inhibitor that finally shed any peptidic character is viracept (**Figure 3**; brand name Nelfinavir). Although this drug utilizes the same DIQ group at its 'C terminus' as saquinavir, it does not retain any actual peptide bonds. Viracept was developed by Agouron Pharmaceuticals (Kaldor *et al.*, 1997), a company, now part of Pfizer, especially created with the task of introducing structure-based drug design (Appelt *et al.*, 1991). Viracept is smaller (MW 568) than the previous approved inhibitors of HIV-1 PR and was the first protease inhibitor approved for the treatment of pediatric AIDS.

The next inhibitor to reach the market, amprenavir (**Figure 3**; brand name Agenerase), was originally discovered at Vertex Pharmaceuticals, another venture capital company explicitly created to apply rational structure-based drug design in practice. The company was later acquired by GlaxoSmithKline, which became the distributor of this drug. Amprenavir is smaller still (MW 506) than the previous drugs but it was withdrawn from the market in 2004 after introduction of its prodrug form, fosamprenavir (brand name Lexiva (US) or Telzir (Europe)), in which a main-chain hydroxyl group was modified by phosphorylation. The prodrug is converted to amprenavir by host enzymes, thus slowing the release (and excretion) of the active form.

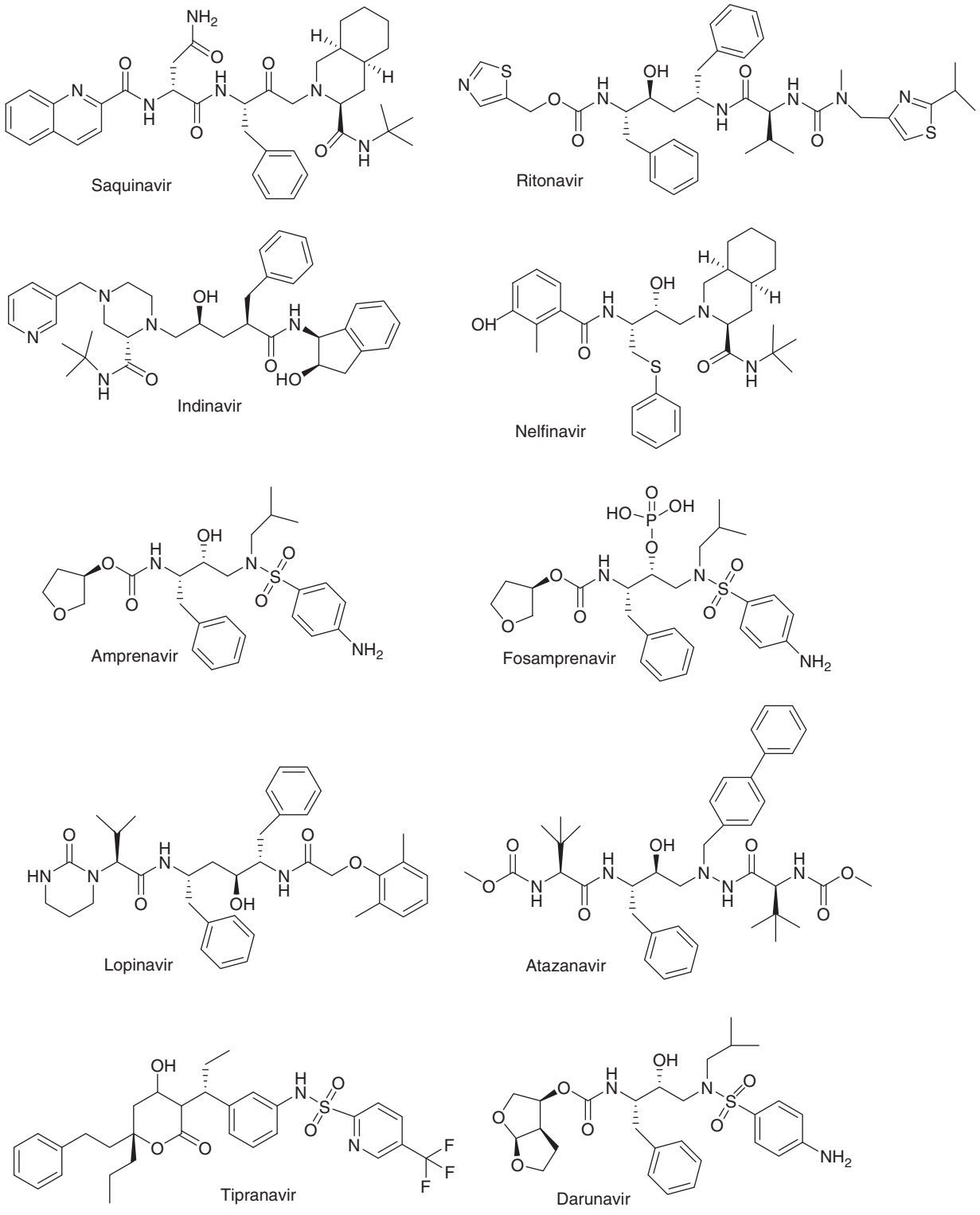

Figure 3 Chemical formulas of the 10 FDA-approved inhibitors of HIV-1 PR.

Emergence of Resistance to Clinical Inhibitors of HIV-1 PR

Since the RT enzymes of retroviruses lack proofreading mechanism, the error rates in transcription are very high, estimated at 1:10 000 for HIV-1 (Katz and Skalka, 1990). Such an elevated level of mutations indicates that every possible variant of the retroviral genome has already been transcribed in an infected patient treated with protease inhibitors (or, for that matter, with any other anti-AIDS therapy). Thus, development of resistance is

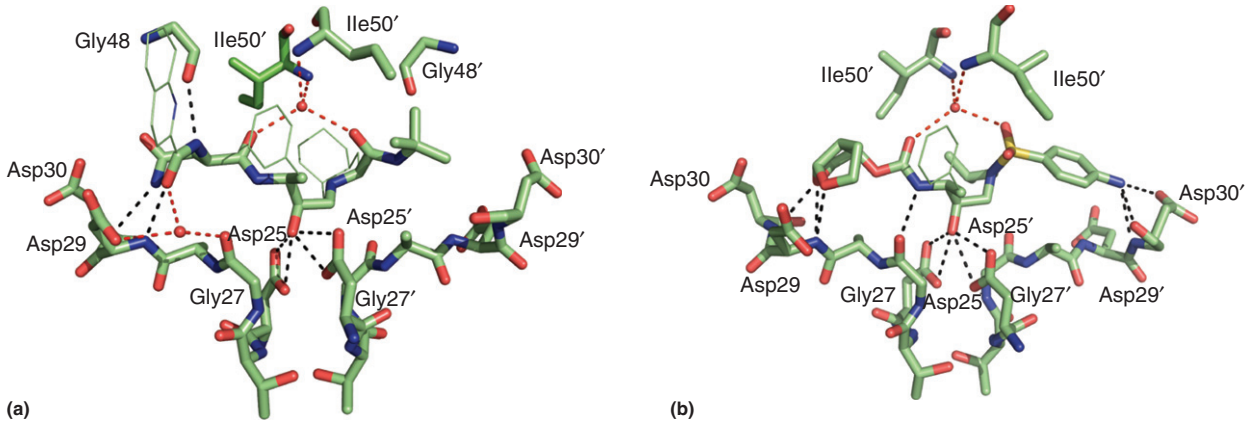

Figure 4 Hydrogen-bond interactions between HIV-1 PR and selected inhibitors. Hydrogen bonds are indicated by dashed lines. Interactions mediated by water molecules are shown in red. (a) Hydrogen-bond interactions between HIV-1 PR and saquinavir, based on the atomic-resolution structure of the complex (PDB ID 2NMZ). (b) Hydrogen-bond interactions between HIV-1 PR and darunavir, seen in an atomic-resolution crystal structure (PDB ID 2HS1). Figure prepared by Dr. Jerry Alexandratos.

just a matter of days, since what is required is only selection of preexisting variants of the virus, rather than mutagenesis subsequent to exposure of the virus to challenging drugs. This problem was noticed early on and became clearly evident in the early clinical studies. Indeed, Condra *et al.* (1995) showed that patients subjected to experimental monotherapy using only indinavir, developed resistance not only to this particular inhibitor, but also to other drugs that inhibit HIV-1 PR, including saquinavir, amprenavir, and ritonavir. The authors stated that "These observations reflect the common nature of the inhibitors' target and suggest that combination therapy with multiple protease inhibitors may yield multiply resistant variants. In addition, initial therapy with any one inhibitor may limit the benefit of subsequent treatment with the remaining compounds." It is significant that these observations were published even before the approval of the first of these drugs by the FDA, thus the need for multidrug therapy combining different classes of targets (such as RT and IN) and for the development of novel protease inhibitors that would be less effective in eliciting viral resistance, was apparent already early on.

One of the most common mutations in HIV-1 PR exposed to the first-generation inhibitors is replacement of Val82 by either threonine or alanine. The structure of a complex of HIV-1 PR with ritonavir was used at Abbott to guide synthesis of a new inhibitor, lopinavir (**Figure 3**; Sham *et al.*, 1998; Hurst and Faulds, 2000) that would not interact with residue 82 at all despite its comparatively larger size (MW 629 – still smaller, though, than the parent compound ritonavir). The problem with lopinavir, however, was its very short half-life in plasma, no longer than 2–4 h. However, combination of therapeutic amounts of lopinavir with subtherapeutic quantities of ritonavir extended the half-life of the drug very significantly. The form of lopinavir approved by the FDA is such a combination (brand name Kaletra).

Atazanavir (**Figure 3**; brand name Reytaz), developed at Bristol-Myers Squibb, is another FDA-approved inhibitor of predominantly peptidic character (Robinson *et al.*, 2000), but with unexpectedly long half-life in plasma, allowing for once-a-day dosing. Although not explicitly designed to combat resistance, it is nevertheless quite successful in this regard, especially if boosted with ritonavir. An advantage of atazanavir over other inhibitors of HIV-1 PR is that it causes fewer side effects, in particular it is less likely to cause lipodystrophy and elevate cholesterol levels.

Tipranavir (**Figure 3**; brand name Aptivus), developed at Boehringer-Ingelheim, is a non-peptidic protease inhibitor originally synthesized at Upjohn as part of a structure-based discovery program (Thaisrivongs and Strohbach, 1999). It is administered in combination therapy with ritonavir to treat particularly difficult HIV infections. Tipranavir is successful in inhibiting the replication of viruses that are resistant to other protease inhibitors and it is recommended for patients not responding to other treatments. Development of resistance to tipranavir itself requires several simultaneous mutations of the HIV-1 PR sequence. In one study, as many as 10 mutations were required to decrease tipranavir's inhibitory potency by two orders of magnitude (Doyon *et al.*, 2005).

The most recent among the FDA-approved inhibitors of HIV-1 PR and most often used in current clinical practice is darunavir (**Figure 3**; brand name Prezista), developed at Tibotec/Johnson and Johnson. It is a non-peptidic compound with MW 548 and the K_d value of 4.5 pM, the latter significantly lower than for other inhibitors of HIV-1 PR (Koh *et al.*, 2003). It is also commonly administered in combination with ritonavir, in order to increase its plasma half-life. The hydrogen bonded interactions between darunavir and HIV-1 PR are shown in **Figure 4(b)**.

Roads Not Taken – Inhibitors of HIV-1 PR That Did Not Become Drugs

Whereas the clinically relevant inhibitors of HIV-1 PR represent a triumph of rational drug design, many other compounds have been created as either intermediate steps of their development, or as testing grounds for the utilization of innovative chemical principles. Thus, for example, many fully twofold symmetric inhibitors were found to be quite potent, although they never became drugs (Kempf *et al.*, 1994). Some of the symmetric designs included an additional retropepsin-specific feature, namely a mimic of the water molecule that mediates the interactions

between the flap loops and the central region of the peptidic inhibitors or substrates. A way to accomplish this dual goal was to utilize cyclic urea derivatives as the central part of the inhibitors. Several very potent inhibitors, such as DMP-450 and DMP-323 resulted from these approaches, but for a variety of reasons, some of them commercial rather than scientific, they did not progress to become clinical drugs (Lam *et al.*, 1994).

One of the earliest attempts to use structural information for rational design of HIV-1 PR inhibitors was a computational experiment that led to the identification of haloperidol, a compound related to a known antipsychotic agent bromoperidol, as a potential inhibitor of this enzyme (DesJarlais *et al.*, 1990). This was an interesting early discovery of a non-peptidic compound which, however, did not lead to its practical application as an antiviral agent, mainly because the required concentration of haloperidol would exceed by several orders of magnitude the doses of bromoperidol administered in psychiatric treatment.

Considerable efforts went into modification of the successful inhibitors of HIV-1 PR that would lead to easier and more efficient synthesis. An example is provided by the modification of amprenavir, in which the non-cleavable isostere of the peptide bond was replaced by 1,2,3-triazole. Such compounds could be produced by fast and high-yield azide-alkyne click chemistry and were shown to be excellent inhibitors of both the native and mutant enzymes (Brik *et al.*, 2005).

A very attractive chemical procedure that used solid-state synthesis of 4-aryl-1,4-dihydropyridines as precursors of C_2-symmetric cage compounds (Hilgeroth *et al.*, 1999, 2002) with inhibitory properties against HIV PR, has never been developed beyond preliminary tests. Also other novel compounds that were not only non-peptidic, but even non-carbon, called carboranes, have not been developed in practice, although they appeared promising in preliminary studies (Cigler *et al.*, 2005).

Where Will the HIV-1 PR Inhibitors Be in the Future?

Detailed studies of not only inhibitor complexes cocrystallized with active HIV-1 PR, but also of substrate peptides bound to inactive enzyme (usually variants with the catalytic Asp25 mutated to asparagine) led to the definition of the concept of 'substrate envelope' – the minimum volume occupied by parts of the majority of efficiently processed substrates of the enzyme (Prabu-Jeyabalan *et al.*, 2002). The postulate was that an inhibitor that does not extend much beyond such an envelope is unlikely to be resistance-prone, since any mutations interfering with its binding would also prevent efficient processing of legitimate substrates. Based on this principle, a number of new inhibitors with nanomolar potencies against patient-derived wild-type viruses from HIV-1 clades A, B, and C, as well as their multidrug resistance (MDR) variants, were synthesized and tested (Parai *et al.*, 2012). In that work, introduction of cyclic and acyclic P2 carbamates with multiple hydrogen-bond donor and acceptor centers resulted in the creation of highly potent inhibitors of HIV-1 PR.

Another important concept applicable to the design of 'resistance-resistant' inhibitors is targeting the protein backbone. It was speculated that inhibitors that maximize their interactions in the HIV-1 PR active site, particularly through multiple hydrogen bonds with the protein backbone of wild-type HIV-1 protease, would maintain those contacts with mutant proteases as well. Targeting the sequence-independent protein backbone rather than the sequence-specific side chains, should therefore curtail the emergence of drug-resistant variants of HIV, as mutations that alter the backbone conformation would most likely reduce or abrogate the catalytic capacity as well (Ghosh *et al.*, 2012).

Fragment screening is a method of searching for compounds capable of even weak binding to the target proteins, with the aim of ultimately increasing their avidity through connecting two or more such 'fragments' covalently. This approach has also been applied to HIV PR, mostly with the aim of finding molecules that would bind to allosteric sites distant from the active site, interfering with such obligatory property as dimerization. Binding of components of suitable cocktails (i.e., chemical libraries) of such test compounds could be monitored by crystallography or, for example, by surface plasmon resonance. Although these studies yielded some interesting preliminary results, they did not lead so far to practical applications (Bauman *et al.*, 2012).

Most of the approaches to inhibitor design treat the target enzyme as a rigid object. However, consideration of the inherent flexibility of protein chains is becoming increasingly important and has already been utilized in the design of inhibitors of HIV PR (Lukman *et al.*, 2014). Another novel concept involved replacement of some non-exchangeable hydrogen atoms in the inhibitor molecule by deuterium. Deuterated compounds are metabolized more slowly, thus allowing prolonged maintenance of their effective blood levels. Such an approach was tested on a modification of atazanavir named CTP-518 (GlaxoSmithKline/Concert Pharmaceuticals). Whereas preclinical studies demonstrated that this modification retained the antiviral potency and increased the half-life and plasma trough levels, the results of Phase I clinical trials, initiated in 2009, have not been reported.

Interference with dimerization is a very attractive approach to HIV PR inhibition because it would prevent the formation of a viable enzyme in the first place (Boggetto and Reboud-Ravaux, 2002). Dimerization inhibitors would have to be extremely potent because the dissociation constant of HIV-1 PR is very low, which also hinders structural studies of this protein in monomeric form. The latter obstacle could be circumvented by looking at similar enzymes from other retroviruses (such as Mason–Pfizer monkey virus (M-PMV)), which can be crystallized as monomers (Khatib *et al.*, 2011; Gilski *et al.*, 2011). Interestingly, it appears that darunavir is capable of acting not only as a very potent competitive inhibitor of HIV-1 PR, but is also able to interfere with the dimerization equilibrium of the enzyme (Hayashi *et al.*, 2014).

Whereas it is not certain which of the novel approaches to the design of inhibitors of HIV-1 PR will lead to new and more potent AIDS drugs, the multitude of experimental approaches bodes well for the future success in creating compounds with novel mode of action and lower potential for triggering resistance. The last word has clearly not yet been said.

See also: Intracellular Infectiology: Infectious Agents: HIV – The Cell Biology of Virus Infection and Replication

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