features of PIs are mostly inherent to their chemical natures: (1) high pill burden, (2) frequent dosing regimens, and (3) various side effects including lipodystrophy and dyslipidemia, although PIs remain an essential component of combination chemotherapy for both drug-naive and treatment-experienced patients with AIDS. It is worth noting that NRTIs are associated with critical adverse effects including mitochondrial toxicity. The role of PIs in HAART has been important and even PI-only regimens have been considered.

As soon as the first PIs were administered in humans, it was found that all PIs are inhibitors of the CYP3A4 system that is the major enzyme catabolizing most PIs and numerous other drugs. It catabolizes more than 50% of marketed drugs and is also frequently involved in drug–drug interactions (Overington *et al.*, 2006). RTV is by far the strongest inhibitor of CYP3A4, and SQV the weakest. Indeed, coadministration of low-dose RTV boosts the exposure of most PIs, which facilitates flexible dosing, including once daily dosing (*vide infra*).

V. "Boosting": A Critical Modification of Clinical Efficacy of PIs

Soon after the development of the first PIs, a problem inherent to this class of inhibitors was recognized, that is, poor pharmacokinetics, low maximum concentration (C_{max}), low plasma trough levels (C_{trough}), and short plasma half-life. The isoenzyme CYP3A4, a subunit of the cytochrome *P450* hepatic enzyme system, is mostly responsible for such poor pharmacokinetic parameters. RTV is by far the most potent inhibitor of the isoenzyme CYP3A4, and it was soon learned that concomitant administration of small doses of RTV with a PI allows "boosting" of the most important phamacokinetic parameters of almost all PIs (Kempf *et al.*, 1997). The unexpected but highly beneficial interactions between RTV and the other PIs have simplified otherwise complex regimens by reducing the frequency and number of pills to be administered, and in many cases by making dosing independent of food intake. Indeed, when boosted, PIs such as fosamprenavir and atazanavir can be taken only once a day. Moreover, boosting of certain PIs such as IDV or APV appears to make such PIs more effective against PI-resistant HIV-1 variants probably by elevating their plasma levels (Condra *et al.*, 2000). However, cautions should be used since plasma levels of PIs may decrease after long durations of treatment. For example, Gisolf *et al.* (2000) have reported that plasma levels of SQV even with RTV boosting dropped by 40% after a 10-month period of therapy. Thus, if there is any suspicion of reduced efficacy of boosted PI treatment after months of therapy, it is recommended that plasma levels of PI be examined and dose adjustments be made.

Until recently, only a limited set of data was available regarding the comparison of the clinical efficacy of each "boosted" PI-containing regimen. However,

there has recently been a wide range of settings where "boosted" PIs are being examined to compare clinical features of each member of PIs with or without other classes of antiretroviral agents. Indeed, the Department of Health and Human Services (DHHS) Guidelines (issued in October, 2006) (DHHS, 2006) recommends that first-line antiretroviral therapy be initiated with either an efavirenz-based regimen or the one containing twice-daily lopinavir/RTV, twice-daily fosamprenavir/RTV, or once-daily atazanavir/RTV. For example, the results of the KLEAN study involving antiretroviral-naive patients with HIV-1 infection have shown that lopinavir/RTV soft-gel capsules (SGC) *bid* and fosamprenavir/RTV *bid* with abacavir/lamivudine fixed dose combination (FDC) performed similarly with regard to virological and immunologic effects as well as adverse effects such as lipid elevations. Moreover, none of the patients with virological failure on either regimen had major PI resistance amino acid substitutions in their HIV-1 (Eron *et al.*, 2006). There are also new data showing that lopinavir/RTV SGC, when used as a once-a-day regimen combined with tenofovir and emtricitabine *qd*, maintains high levels of antiviral activity comparable to the twice-daily regimen in drug-naive patients (Johnson *et al.*, 2006). The results of the RESIST study have shown that tipranavir/RTV delivers better outcomes over comparator "boosted" PIs in highly treatment-experienced patients, although the role of the TPV/RTV combination in salvage therapy is likely to be modest due to its safety, pharmacokinetic issues (twice-daily regimen, etc.), and the near-future increasing availability of other more attractive antiretroviral drugs (Hicks *et al.*, 2006). Taken together, continuing evaluations of "boosted" PI-including regimens should certainly merit to improve the efficacy of HAART.

VI. Viral Resistance to PIs

As described above, HAART has had a major impact on the AIDS epidemic in industrially advanced nations; however, we have also encountered a number of challenges in bringing about the optimal benefits of the currently available therapeutics of AIDS to individuals receiving HAART (De Clercq, 2002; Siliciano *et al.*, 2004; Simon and Ho, 2003). Such limitations and flaws of HAART are worsened by the development of drug-resistant HIV-1 variants (Carr, 2003; Fumero and Podzamczer, 2003; Grabar *et al.*, 2006; Hirsch *et al.*, 2004; Little *et al.*, 2002). Table I illustrates mutations conferring high and intermediate resistance to currently approved PIs.

A. Emergence of Drug Resistance to PIs

A variety of drug resistance mechanisms are at play with PIs. The most important ones from a purely drug-binding standpoint are mutations in the

TABLE 1 Mutations Associated with Resistance to Currently Approved Protease Inhibitors

	Mutations conferring high/intermediate resistance								
Nelfinavir	D30N		M46I/L			V82A/ T/F	I84V	N88S	L90M
Saquinavir				G48V		V82A	I84V		L90M
Indinavir			M46I/L			V82A/ T/F/S	I84V		L90M
Ritonavir		L33F	M46I/L			V82A/ T/F/S	I84V		L90M
Fosamprenavir[a]			M46I/L	I47V	I50V		I84V		L90M
Lopinavir		L33F	M46I/L	I47V	I50V	V82A/ T/F/S	I84V		L90M
Atazanavir		L33F	M46I	G48V	I50V	V82A	I84V	N88S	L90M
Tipranavir		L33F	M46I			V82A/ T/F/L	I84V		L90M
Darunavir[b]		L33F			I50V		I84V		

[a]Fosamprenavir is the prodrug of amprenavir; the latter is no longer manufactured.
[b]The mutations that are found in those with a diminished virological response to DRV/r in the POWER studies include V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V (De Meyer, 2006). However, it is not known yet as to which amino acid mutations are the major ones responsible for the apparent resistance to DRV/RTV.

active site of the protease enzyme that lead to loss of binding and hence diminished anti-HIV-1 activity of the inhibitor. Such mutations are necessary but not sufficient for the emergence of high-level resistance in the clinical setting. The reason for this appears to be that active site mutations alone result in only suboptimal resistance, which is consistent with biochemical studies on drug-resistant mutant proteases (Condra *et al.*, 1995; Mitsuya and Erickson, 1999; Yin *et al.*, 2006). The structures of protease, complexed with an inhibitor, allow us to attempt to rationalize the structural effects of drug resistance-conferring mutations on the interactions between the enzyme and inhibitor (Ghosh *et al.*, 2006a,b; Mitsuya and Erickson, 1999). In some instances, these hydrogen bonds are mediated by bridging water molecules. The enzyme also contains a number of well-defined pockets (or subsites) in its active site region into which inhibitor's side chains protrude, resulting in tight binding interactions between the enzyme and the inhibitor. Since a similar pattern of hydrogen bonds are believed to be made for both substrates and peptidomimetic inhibitors, the specificity should reside in the pattern of largely nonpolar subsite interactions between the inhibitor and the enzyme side-chain atoms. Thus, mutations of specificity-determining residues that would directly interfere with inhibitor binding, but not with substrate processing, constitute an obvious mechanism

for resistance to PIs. Other resistance pathways might include nonactive site mutations that indirectly interfere with inhibitor binding through long-range structural perturbations of the active site, mutations that result in an enzyme with enhanced catalysis of stability, and cleavage site mutations that lead to enhanced processing by mutant enzymes (*vide infra*). Combinations of different mutations may also lead to additive, synergistic, and compensatory effects.

Mutations have been observed in nearly half of all possible positions in a monomer's 99 amino acids in response to drug-selection pressure (Ghosh *et al.*, 2006a,b; Mitsuya and Erickson, 1999; Molla *et al.*, 1996). Many of these mutations may presage the emergence of mutants in the clinical setting (Condra *et al.*, 1995). They can be classified as either active site or nonactive site mutations according to whether they occur inside or outside the inhibitor-binding subsites and directly contact the inhibitor (*vide infra*). Certain amino acid substitutions, common or not, exist in virus in a normal environment apparently having no substantial impact on fitness, are seen without regard to antiviral therapy in certain viral isolates, and termed polymorphism (e.g., lysine or glutamate at RT codon 122) (Kavlick and Mitsuya, 2001). Such polymorphisms might also coincidentally convey fitness on the virus in an altered environment, for example, improving virus fitness under selective drug pressure by conferring some degree of resistance. It can be stated that such a virus possesses *natural resistance* and can therefore negatively impact anti-HIV-1 therapy. When HIV-1 variants resistant to an NRTI, 2′-β-fluoro-2′,3′-dideoxyadenosine (FddA or lodenosine), *in vitro* (HIV-$1_{FddA}{}^{R}$), all clones derived from HIV-$1_{FddA}{}^{R}$ contained Pro119Ser and Leu214Phe substitutions, and an infectious clone containing Pro119Ser and Leu214Phe generated by site-directed mutagenesis confirmed phenotypic resistance to FddA (Tanaka *et al.*, 1997). However, clonal subpopulations of the wild-type HIV-1 (HIV-1_{LAI}) used in the selection also possessed the Pro119Ser (23%) and Leu214Phe (69%) substitutions (Tanaka *et al.*, 1997). These data suggest that certain natural viral polymorphisms may confer resistance and that such virus can expand under selective drug pressure.

For the latest information on genotypic and phenotypic HIV-1 drug resistance, visit http://hivdb.stanford.edu/pages/genotype-phenotype.html or http://www.hiv.lanl.gov/content/hiv-db/mainpage.html

B. Primary and Secondary Mutations

Drug resistance mutations that surround the active site usually interfere with the binding of a PI to the protease and are referred to primary mutations (Table I; Fig. 4). Because of their locations near the substrate-binding cleft, these mutations affect processing of the natural substrates as well and, therefore, often confer a fitness cost to HIV-1. Primary mutations that

interfere with PI binding, located distant to the active site, have also been documented (i.e., Leu90Met). However, most mutations that are not within the substrate-binding cavity do not affect inhibitor binding *per se* and do not confer resistance by itself (without other primary mutations), but compensate for the deleterious effects on enzymatic activity caused by primary mutations. These compensatory mutations are referred to as secondary mutations. Thus, high-level drug resistance to PIs requires the stepwise accumulation of multiple primary and secondary mutations to generate a protease capable of discriminating inhibitors from natural substrate and yet maintaining adequate catalytic efficiency needed for virus replication.

C. Active Site Mutants

The first described resistance mutation for HIV-1 protease was a Val82Ala mutation that was selected using a symmetric diol inhibitor (Otto *et al.*, 1993). Since then, resistance mutations have been observed in each of the unique specificity pockets, S3, S2, S1 and, by symmetry, S1′, S2′, and S3′. However, only a subset of all residues that constitute a particular subsite mutates in response to a particular drug. The structural effects of mutations on drug binding have been modeled using the crystal structures of the appropriate wild-type enzyme–inhibitor complexes, and used to rationalize the effects of specific mutations on drug-binding affinities (Markowitz *et al.*, 1995; Otto *et al.*, 1993).

Most of the subsite mutations, such as Ile84Val and Val82Ala (or Ile or Phe), affect hydrophobic and van der Waals interactions and can be considered to be "packing" mutants somewhat analogous to hydrophobic mutations in a protein core (Markowitz *et al.*, 1995). Crystallographic analyses of HIV-1 protease/inhibitor complexes show that most of the surface of an inhibitor and its immediate protein environment are solvent inaccessible (see more about Val82Ala substitution in Section VI). Some mutations, such as Val82Ile, are more effective when combined with second active site mutations such as Val32Ile (Kaplan *et al.*, 1994). Other mutations can affect electrostatic interactions. While numerous crystal structures of wild-type HIV protease-inhibitor complexes have been published, crystal structures of mutant HIV protease-inhibitor complexes have also recently emerged in the literature.

D. Nonactive Site Mutants

While the precise structural mechanism of drug resistance can often be pinpointed for active site mutations that directly affect inhibitor binding, the evaluation of nonactive site mutants is more challenging and there may be several different mechanisms at work. Some mutations might act in concert with active site mutations by compensating for a functional deficit caused by the latter. For example, the defective Arg8Glu mutation is found

almost exclusively in combination with one or more mutations outside the active site region, such as Met46Ile (Ho *et al.*, 1994; see Fig. 4 for the site of Met-46). Mutations of Met-46 to Ile, Leu, or Phe are often found in the presence or absence of other active site mutations, such as Val82Ile, Ala or Phe, and Ile84Val. Met-46 is in the flap of HIV protease and recent molecular dynamic simulations on flap movement have shown that the Met46Ile mutant exhibits a markedly different dynamical behavior than the wild-type enzyme (Collins *et al.*, 1995), and presumably alters enzyme kinetics. However, a role for Met-46 in polyprotein–substrate recognition is also possible.

Other nonactive site mutants may indirectly alter the structure of the active site region. Many of these nonactive site mutations are found in multiple combinations with one or more active site mutations. In some cases, the introduction of nonactive site mutations alone does not lead to a marked or even measurable reduction in inhibitor binding, in contrast to the case for all known active site mutations. However, the fact that certain mutations are only observed in the presence of drug means that they must by definition provide the virus with some selective replication advantage. At least one engineered HIV-1 protease mutant, Gly48Tyr, exhibited greater catalytic efficiency than the wild-type enzyme toward artificial peptide substrates (Lin *et al.*, 1995).

E. Cleavage Site Mutants

Additional mutations in the HIV-1 genome have been found that do not lie within the protease enzyme, but are instead located near the cleavage sites of Gag-Pol and Gag substrates. These mutations are also secondary mutations that compensate for the reduced catalytic efficiency caused by primary protease mutations (Cote *et al.*, 2001; Doyon *et al.*, 1996; Tamiya *et al.*, 2004; Zhang *et al.*, 1997). Since active site mutations are thought to alter the rate of one or more cleavages that must occur during viral maturation, one can think that compensating mutations in the cleavage sites on the Gag or Gag-Pol polyproteins might render them better substrates for particular mutant enzymes. Studies by Doyon *et al.* (1996) have identified a mutation in the p1/p6 Gag polyprotein cleavage site (Leu449Phe) substitution that can synergize with the Ile84Val mutant to produce a virus with 350- and 500-fold decreased sensitivity to substrate-based PIs BILA 1906 BS and BILA 2185 BS, respectively. The mutation altered the p1/p6 cleavage site from Phe-Leu to Phe-Phe. Indeed, a synthetic peptide containing the Phe-Phe cleavage site was cleaved at higher catalytic efficiency by the Ile84Val HIV mutant protease than the corresponding peptide with the wild-type sequence. Cleavage site mutations at the p7/p1 cleavage site have also been

observed in breakthrough virus isolated from patients on IDV therapy (Zhang *et al.*, 1997).

F. Noncleavage Site Mutants

In addition to the cleavage site mutations described above, mutations can also be seen in noncleavage sites in Gag-Pol polyproteins. For instance, multiple amino acid substitutions (e.g., Leu75Arg, His219Gln/Pro, and Val390Asp/Val390Ala) have been identified at noncleavage sites of Gag proteins, which emerge on long-term exposure to a PI(s) and are indispensable for HIV-1 replication in the presence of such a PI(s). These mutations affect Gag functions without affecting Gag's cleavage sensitivity to protease (Gatanaga *et al.*, 2002). For instance, His219Gln and His219Pro represent polymorphic amino acid residues; however, these substitutions confer on HIV-1 replication advantage in a unique way (Gatanaga *et al.*, 2006). Both human $CD4^+$MT-2 and H9 cell lines contain high levels of cyclophilin-A, and the cyclophilin-A content of virions generated in these cells is far greater than that in human peripheral blood mononuclear cells. Such high cyclophilin-A-containing virions limit the replication of HIV-1 containing wild-type His-219. The His219Gln and His219Pro substitutions reduce cyclophilin-A incorporation into virions and potentiate viral replication.

G. Insertions in Gag-Pol Polyproteins

The addition of certain amino acids can also contribute to the development of viral resistance. Winters *et al.* (1998) identified a 6-base pair insert between codons 69 and 70 of the RT gene in HIV-1 isolated from multiple NRTI-treated patients and conducted elegant site-directed mutagenesis studies, showing that the insert alone confers on HIV-1 reduced susceptibility to multiple NRTIs. Peters *et al.*(2001) have also identified duplication of a proline-rich motif, Ala-Pro-Pro (APP) in the PTAP motif of the Gag protein in HIV-1 variants isolated from patients with AIDS receiving NRTI(s) including ddI, d4T, AZT, 3TC, and have shown that this addition could improve the viral assembly and packaging at membrane locations, resulting in increased infectivity and viral resistance to NRTIs.

Tamiya *et al.* (2004) have also identified unique insertions (TGNS, SQVN, AQQA, SRPE, APP, and/or PTAPPA) near the p17/p24 and p1/p6 Gag cleavage sites in addition to the known multiple amino acid substitutions within the protease in full-length molecular infectious multidrug-resistant (MDR) HIV-1 clones generated from HIV-1 variants isolated from patients with AIDS who had received long-term antiviral therapy. Such inserts mostly compromise the enzymatic functions of the wild-type protease; however, they restore the Gag processing by the mutant protease and enable PI-resistant HIV-1 variants to remain replication competent.

VII. PIs with Activity Against Drug-Resistant HIV-1

A. Mutations That Allow Discrimination of PIs from Natural Substrates

Each PI tends to select for particular primary mutation(s) ("signature" mutations) and subsequent secondary mutations both *in vitro* and *in vivo* (Markowitz *et al.*, 1995; Otto *et al.*, 1993; Yusa and Harada, 2004). Nonetheless, many primary mutations are capable of causing cross-resistance to multiple PIs, even though they tend only to emerge during therapy with specific inhibitors (Hertogs *et al.*, 2000; Kemper *et al.*, 2001; Tisdale *et al.*, 1995; Watkins *et al.*, 2003). All PIs are shorter than the natural substrates and contain hydrophobic moieties that interact with S2-S2′ subsites (Fig. 3 for darunavir). Thus, although PIs may be chemically unique from each other, they occupy a similar space within the protease-binding cavity, which explains at least in part how individual mutations may cause PI cross-resistance.

Structural analysis of primary mutations has formed the basis for our current understanding of PI resistance at the molecular level. One such mutation is Val82Ala, originally described after selection with RTV or IDV (Condra *et al.*, 1995; Deeks *et al.*, 1998; Molla *et al.*, 1996). This mutation is capable of conferring HIV resistance to a number of PIs, particularly early generation compounds. Crystal structures of protease containing Val82Ala complexed with either natural substrates or a PI were compared

FIGURE 3 Darunavir bound in the hydrophobic cavity within protease. Hydrophobic cavity within protease with darunavir (DRV/TMC114; PDB ID 1S6G) is shown. Brown and green regions are lipophilic while the blue regions are hydrophilic (determined using MOLCAD). The S2 and S2′ subsites are indicated. The figure was generated using Sybyl 7.0 (Tripos, Inc.).

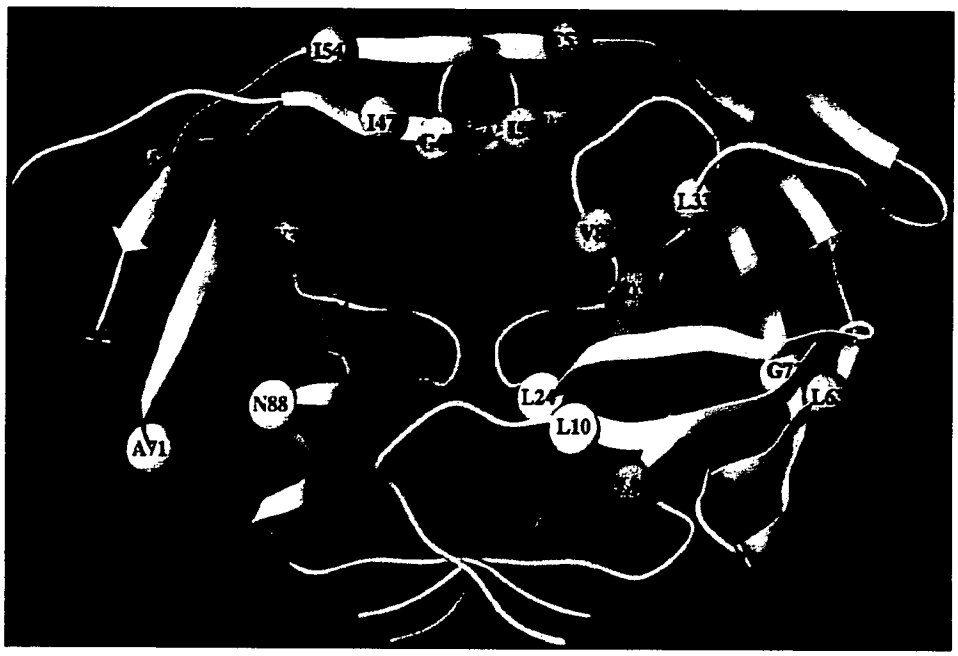

FIGURE 4 Locations of amino acid substitutions associated with drug resistance to PIs. Structure of protease homodimer with positions of amino acid residues associated with clinical resistance to current PIs is indicated. Primary and secondary mutations are indicated with red and white spheres, respectively. The protease monomers are shown in green and orange ribbons. Mutations are shown on only one monomer for clarity. The figure was generated using Maestro version 7.5.

(these enzymes also contained Asp25Asn, a mutation that inactivated the enzyme to prevent cleavage of the substrate but did not appear to affect hydrogen bonding between protease and ligand) (Prabu-Jeyabalan *et al.*, 2003). Val82Ala results in significant changes in the crystal structures of protease complexed to a PI, including changes in the flap position and subsequent disruption of hydrogen bonding, as well as the loss of van der Waals interactions between mutant protease and a PI. On the other hand, crystal structures between natural substrate peptides complexed to either wild-type protease or Val82Ala mutant protease have not demonstrated significant changes. Molecular interactions between protease and the longer natural substrates consist mainly of extensive backbone–backbone hydrogen bonds as well as more extensive van der Waals interactions (Prabu-Jeyabalan *et al.*, 2000, 2002, 2003). This suggests that the Val82Ala mutation, which decreases the size of the side chain, has little effect on substrate binding, but has a much greater detrimental effect on PI binding.

Others have shown that MDR protease with mutations at multiple positions (amino acids Leu-10, Met-36, Met-46, Ile-54, Leu-63, Ala-71, Val-82, Ile-84, and Leu-90) has an expanded active site cavity (Logsdon *et al.*, 2004).

Again, the binding of PIs to this MDR protease was noticeably different than binding to wild-type protease. Although the crystal structure of this MDR protease with natural substrates was not assessed, this work provides further insight into the structural effects of multiple protease resistance mutations.

To further understand the difference between substrate binding and PI binding to protease, an analysis of the structures of eight different inhibitors complexed to protease has been conducted. King *et al.* (2004a) demonstrated that despite the chemical differences of the PIs, all compounds occupied a similar volume within the active site cavity that is termed the "inhibitor envelope." If the inhibitor envelope was compared with the "substrate envelope," the space within the protease that is occupied by a natural substrate, the inhibitors protrude from the substrate envelope in very distinct locations. At these positions, PIs may have van der Waals interactions with amino acid positions such as Gly-48, Ile-50, Val-82, and Ile-84. It is known that mutations at these residues result in PI primary resistance (Table I, Fig. 4), and therefore, these mutations likely disrupt PI and protease molecular associations. In contrast, these same mutations have little effects on natural substrates that do not make molecular interactions at these amino acid positions (King *et al.*, 2004a).

B. Development of PIs with Activity Against Drug-Resistant HIV

Of the currently approved PIs, APV fits predominantly within the substrate envelope (King *et al.*, 2004b). The mutation profile for APV is also different compared with that for other PIs, providing more evidence that PIs that have greater resemblance to natural substrates will be less affected by primary mutations selected by first generation PIs. A conceptually intriguing structure-based design of PIs targeting active site protein backbone or "backbone binding" has resulted in inhibitors with impressive drug resistance profiles (Ghosh *et al.*, 2006c). Structural analysis revealed that while mutations occur throughout the protease, the backbone conformation is surprisingly conserved, especially in the active site (Ghosh *et al.*, 2006a). Because mutations of backbone atoms of the protease cannot occur, disruption of these bonds is more difficult compared with hydrogen bonds that many PIs form between amino acid sidechains, which can be affected by substitution mutations. Further development of inhibitors successfully exploiting these elements has resulted in PIs with significant activity against MDR HIV.

C. Design Rationale of Darunavir

APV contains an interesting tetrahydrofuranyl (3*S*-THF) urethane moiety as the P2 ligand (Fig. 2) (Kim *et al.*, 1995). The importance and potency enhancing effect of the 3(*S*)-THF ring was shown in inhibitors

containing both hydroxyethylene as well as hydroxyethylamine isostere (Ghosh *et al.*, 1993). The THF ring oxygen forms a weak hydrogen bond with the residue in the S2-region of the protease active site upon drug binding. Incorporation of a stereochemically defined 3(*R*), 3a(*S*), 6a(*R*)-*bis*-tetrahydrofuranyl (*bis*-THF) urethane in the hydroxyethylamine isostere led to two PIs, TMC-126 and darunavir (DRV, TMC-114) (Fig. 2) (Ghosh *et al.*, 1998, 2001, 2002). TMC-126 differs from darunavir due to a replacement of 4-aminobenzenesulfonamide with 4-methoxybenezenesulfonamide.

The critical *bis*-THF ligand was designed and developed to make extensive interactions with the residues at the S2-region and specifically target and maximize "backbone-binding" interactions (Ghosh *et al.*, 1994, 1996, 1998, 2006b). Structural analysis indeed revealed that the *bis*-THF derivative effectively hydrogen bonds to the NH of the Asp-29 and Asp-30 as shown (Fig. 5) (Koh *et al.*, 2003; Tie *et al.*, 2004). Both drugs have increased activity against both wild-type as well as clinical isolates containing multi-PI-resistant mutations (De Meyer *et al.*, 2005; Koh *et al.*, 2003; Yoshimura *et al.*, 2002). Furthermore, based on the analysis of DRV, the larger *bis*-THF rings of TMC-126 and DRV protrude slightly more from the substrate envelope

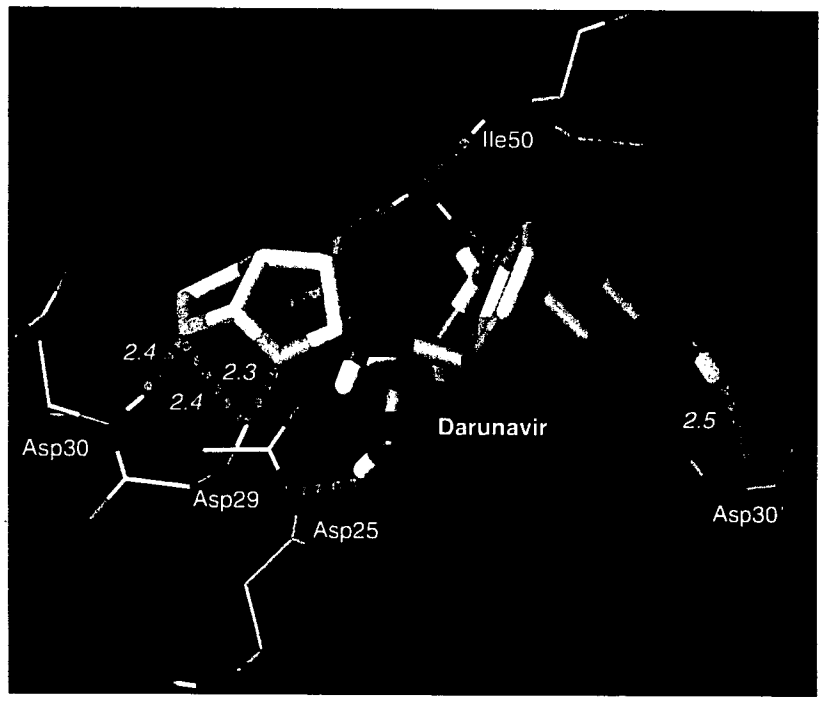

FIGURE 5 Hydrogen bond interactions of darunavir with protease catalytic sites. Hydrogen bond interactions of darunavir with Asp-29 and Asp-30 in the S2 subsite, and Asp-30′ in the S2′ subsite. The hydrogen bonds are shown in green broken lines. The figure was generated using Maestro version 7.5.

compared with the THF ring of APV (King *et al.*, 2004b). Although the THF ring of APV is able to form hydrogen bonds with the backbone carboxylate oxygen of Asp-30, the interaction is relatively weak. In contrast, *bis*-THF makes strong hydrogen bonds with the main chain atoms of Asp-29 and Asp-30 in the S2 subsite of the protease. Furthermore, the *bis*-THF moiety fills the hydrophobic pocket in the S2-region more effectively than the THF ring of APV. This may account for a tenfold increase in activity of TMC-126 and DRV against a panel of clinical isolates with various combinations of protease resistance mutations (Koh *et al.*, 2003; Surleraux *et al.*, 2005b).

Attempts to design compounds capable of further exploiting these critical interactions with the main chains of Asp-29 and Asp-30 in the S2 subsite are currently underway (Ghosh *et al.*, 2005). On the opposite side of these same inhibitor molecules, it has been reported that the P2′ substituents such as the 4-aminobenzenesulfonamide of APV and DRV, or the 4-methoxybenezenesulfonamide of TMC-126, also introduce hydrogen bonding with the backbone carboxyl of Asp-30′ in the S2′ subsite (Fig. 3) (Surleraux *et al.*, 2005b). Compounds designed to optimize this interaction have also demonstrated potent *in vitro* activity against PI-resistant HIV-1 variants (Surleraux *et al.*, 2005a). Maximization of hydrogen bond interactions between the protease backbone and TMC-126 or DRV results in highly favorable enthalpic contributions that drive inhibitor binding. This differs from first generation PIs (i.e., NFV, SQV, and IDV) that have unfavorable enthalpic interactions with protease. Binding of these PIs to protease was entropically driven as a result of the burial of hydrophobic residues of these compounds. Thus, mimicking the backbone hydrogen bonding of natural substrates in at least two separate subsites of protease has yielded more thermodynamically adaptable PIs capable of overcoming protease resistance conferred by amino acid substitutions (King *et al.*, 2004b; Ohtaka and Freire, 2005).

Katlama and her colleagues compared the efficacy and safety of multiple doses of DRV plus low-dose RTV (DRV/r) with investigator-selected control PI(s) (CPI[s]) in a phase IIb randomized POWER 1 clinical trial (Katlama *et al.*, 2007). This involved 318 patients with one or more primary PI mutation and HIV-1 RNA>1000 copies/ml, receiving optimized background therapy, plus DRV/r 400/100 mg *qd*, 800/100 mg *qd*, 400/100 mg *bid* or 600/100 mg *bid*, or CPI(s). DRV/r 600/100 mg *bid* demonstrated the highest virological and immunological responses (Katlama *et al.*, 2007). Adverse event incidence was similar between treatments: headache and diarrhea were more common with CPI(s). All patients receiving DRV/r were switched for the ongoing open-label phase of the trial.

D. HIV-1 Resistance to Darunavir

As mentioned above, APV selects for a unique pattern of protease resistance mutations compared with first generation PIs and this holds true for the structurally similar TMC-126 and DRV. TMC-126 resistance

appears to be mediated by a novel mutation, Asp28Ser, along with subsequent acquisition of Ile50Val (Yoshimura *et al.*, 2002). Although Ile50Val has been demonstrated to confer primary resistance on APV in clinical isolates, Ala28Ser has not been described yet as a common protease resistance mutation, likely because of the effect this mutation has on the catalytic efficiency of protease (Hong *et al.*, 1998; Yoshimura *et al.*, 2002).

Computational modeling analysis does not indicate that TMC-126 has hydrogen bond interactions with either the backbone or side chain of Ala-28, which suggests that the reduction in potency of TMC-126 is due to either steric hindrance caused by the larger serine of A28S, or possibly due to unfavorable solvation energy effects during binding (Yoshimura *et al.*, 2002). Although the pharmacokinetic properties of TMC-126 were not suitable for further clinical development, the related compound DRV exerts significant activity against multi-PI-resistant clinical HIV-1 isolates and has favorable pharmacokinetics (Arasteh *et al.*, 2005; De Meyer *et al.*, 2005; Koh *et al.*, 2003); DRV has been approved as a prescription drug for treatment of those who do not respond to any other existing antiviral regimens in June 2006. Despite the chemical similarities of TMC-126 and DRV, Ala28Ser has not been described yet after DRV selection (Koh and Mitsuya, unpublished data). The reasons for this are unclear at this point. Instead, *in vivo* resistance with strains harboring Arg41Thr and Lys70Glu has been identified (De Meyer *et al.*, 2005). Isolates harboring these two mutations were found to have 8-to 10-fold resistance to DRV, 20-fold resistance to SQV, and 6-fold resistance to lopinavir. Otherwise, resistance remained less than 4-fold for all other first generation PIs. The molecular mechanisms that allow Arg41Thr and Lys70Glu to confer DRV resistance is also currently unknown, as site-directed mutants carrying one or both of these mutations show no reduction in sensitivity to other PIs tested (De Meyer *et al.*, 2005). Nonetheless, there appears to be a higher genetic barrier to the emergence of resistance to both TMC-126 and DRV, and both drugs have been shown to maintain potent activity against multi-PI-resistant strains, suggesting that their unique interactions with HIV protease can provide the framework for developing subsequent generations of PIs. It is of note that another *bis*-THF containing PI, brecanavir (GW640385), has shown activity against both wild-type and drug-resistant HIV (Ward *et al.*, 2005). *In vitro* selection of HIV-1 with brecanavir resulted in the emergence of the novel A28S mutation seen initially with TMC-126 (Yates *et al.*, 2004). Unfortunately, in late 2006 clinical trials of brecanavir were terminated due to the difficulty of formulation.

Profiles of HIV-1 resistance to DRV have now been gradually accumulated. De Meyer *et al.* (2005) selected the wild-type HIV-1_{LAI} with DRV in test tubes. Selection of resistant HIV-1 with other PIs was readily possible and resulted in the emergence of strains carrying known PI resistance-associated mutations. The concentrations of the current PIs could be readily increased to 1 μM, still allowing for virus replication. In contrast, DRV concentration could not be increased beyond 200 nM even after prolonged

exposure. In the presence of 100 nM TMC-114, virus strains had acquired Arg41Thr and Lys70Glu mutations, which were not concluded to be responsible for an apparent modest viral resistance to DRV.

However, when a mixture of multiple HIV-1 isolates resistant to multiple PIs was employed, highly DRV-resistant HIV-1 variants have been selected (Koh *et al.*, 2007). By 39 passages (in the presence of 1.0 μM DRV), the virus had acquired ~170-fold IC_{50} increases than that against HIV-1_{NL4-3}. The virus relatively well propagated and was found to contain 12 mutations including Leu10Ile, Ile15Val, Lys20Arg, Leu24Ile, Val32Ile, Met36Ile, Met46Leu, Leu63Pro, Lys70Gln, Val82Ala, Ile84Val, and Leu89Met in the protease-encoding region. The virus at passage 30 with 0.3 μM DRV (HIV-$1_{DRV\text{-}P30}$) was titrated and examined for its susceptibility to DRV and other PIs using p24 assay. HIV-$1_{DRV\text{-}P30}$ was found highly resistant to DRV (110-fold greater IC_{50} than that against HIV-1_{NL4-3}) (Koh *et al.*, 2007). Altogether, these data suggest that DRV generally would not easily permit HIV-1 to develop significant resistance; however, HIV-1 could develop high levels of DRV-resistance, probably when superinfection with multi-PI-resistant HIV-1 variants and ensuing homologous recombinations occur.

Indeed, De Meyer *et al.* have found mutations including Val11Ile, Val32Ile, Leu33Phe, Ile47Val, Ile50Val, Ile54Leu/Met, Gly73Ser, Leu76Val, Ile84Val, and Leu89Val in HIV-1 strains isolated from those with a diminished virological response to DRV/r in the POWER studies (De Meyer, 2006). However, it is not known yet as to which amino acid mutations are major ones responsible for the apparent resistance to DRV.

E. Tipranavir and Darunavir

The approval of TPV as well as DRV (Fig. 2) for clinical use in drug-experienced patients demonstrated the success of structure-based drug design in the development of novel compounds. All previously clinically approved PIs were classified as peptidomimetics, due to the fact that they share structural similarity to the tetrahedral intermediate formed during hydrolytic cleavage of a peptide bond of the natural substrate (Randolph and DeGoey, 2004). In contrast, TPV was developed from a class of compounds known as dihydropyrones that are structurally similar to coumadin and were found to inhibit protease (Chrusciel and Strohbach, 2004). Traditional peptidomimetics all utilize a ubiquitous water molecule within the protease activity site in order to form hydrogen bonds with the flap domain of the enzyme. A key characteristic of TPV and other nonpeptidic PIs (NPPIs) in development is the absence of this water molecule as seen in crystal structures of NPPIs with protease. Instead, NPPIs contain a suitable chemical moiety that directly forms hydrogen bonds to the flap region without the need for water molecules. It is hypothesized that this allows

more entropically favorable interactions for the binding of these inhibitors to protease (Chrusciel and Strohbach, 2004).

The unique binding motif and structure of TPV has been thought to provide increased flexibility that would allow it to adjust to amino acid changes within the active site (Larder *et al.*, 2000; Turner *et al.*, 1998). Indeed, TPV exerts antiviral activity against a wide range of HIV variants resistant to multiple PIs (Back *et al.*, 2000; Larder *et al.*, 2000; Poppe *et al.*, 1997; Rusconi *et al.*, 2000). Nonetheless, resistance to TPV can occur although the acquisition of up to 10 mutations is required to achieve high levels of resistance (Doyon *et al.*, 2005). The mutations Leu33Phe and Ile84Val appear to be key substitutions responsible for the development of TPV resistance. Leu33Phe is a secondary mutation not present in the active site of protease and does not appear to affect TPV binding to protease. It has been suggested that this mutation protects the protease from autocatalysis (Schake, 2005). Ile84Val appears to directly diminish TPV binding by altering hydrophobic interactions that the drug has with this amino acid residue (Schake, 2004). Despite this, the potent activity of TPV against multi-PI-resistant HIV appears to stem from the hydrogen bond network it forms with the most invariant region of the protease active site that includes the catalytic Asp-25, as well as the backbone residues of Asp-29, Asp-30, Gly-48, and Ile-50 (Fig. 6) (Muzammil *et al.*, 2005; Schake, 2004).

At present, no randomized comparison data of TPV versus DRV are available, although there are a few reports on the benefits of TPV and DRV in those who have failed combination therapy with other PIs (Farthing *et al.*, 2006; Hill and Moyle, 2006). Such data show that both TPV and DRV are of useful option in patients who have failed other PIs; it is still to be determined how well they work against HIV-1 variants previously exposed to specific PI options. However, considering that TPV had been labeled a "last hope" PI option for people who have tried and failed other PIs, it appears that DRV—with its activity in patients no longer responding to TPV—certainly offers a significant advantage.

More profiles and the mechanisms of HIV-1 resistance to NPPIs such as TPV and DRV should be fully disclosed when more *in vitro* and clinical data involving more HIV-1 isolates and more individuals will continue to refine our knowledge of the molecular mechanisms of resistance, and provide us with novel insights for the prevention and treatment of drug-resistant HIV-1. A number of ongoing studies should further define the efficacy and safety of TPV and DRV in other patient populations and it is possible, in the near future, that these drugs could serve as a first-line therapeutic in HAART as well.

VIII. Conclusions

Successful antiviral drugs, in theory, exert their virus-specific effects without disturbing cellular metabolism or function. However, at present,

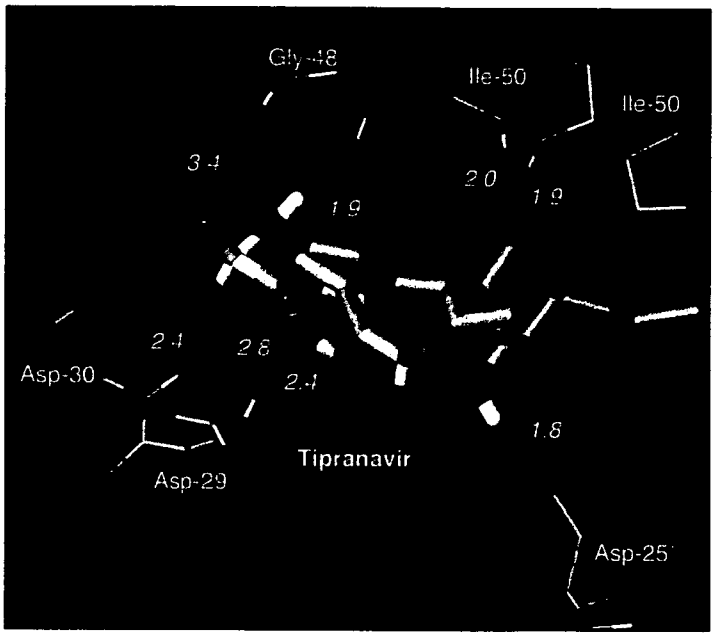

FIGURE 6 Hydrogen bond interactions of TPV with protease. Hydrogen bond interactions of TPV with Asp-29 and Asp-30 in the S2 subsite, with a catalytic aspartate (Asp-25′), and with flap residues Gly-48, Ile-50, and Ile-50′. The hydrogen bonds are shown in green dashed lines. The figure was generated using Maestro version 7.5.

no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or side effects, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives. Thus, the identification of new class of antiretroviral drugs which have a unique mechanism(s) of action and produce no or minimal side effects remains an important therapeutic objective.

A variety of novel anti-HIV-1 agents that target different steps in the HIV replication cycle are currently in preclinical trials and will undoubtedly improve our ability to manage HIV-1 infection when they are duly introduced into clinics. However, as has been the case with RTIs and PIs, the development of drug resistance will likely limit the effectiveness of these drugs as well. Thus, a key element in future drug design strategies will be to understand how drug resistance mutations affect the interaction of the drug with its target, and to develop compounds with the adaptability to inhibit these variants along with wild-type HIV-1. New generation RTIs and PIs have already shown promise in accomplishing this task, by utilizing knowledge of the molecular, biochemical, structural, and thermodynamic nature of drug resistance. This should serve as a model in the design of more effective anti-HIV-1 therapeutics.

Acknowledgments

This work was supported in part by the Intramural Research Program of Center for Cancer Research, National Cancer Institute, National Institutes of Health and in part by a Grant for Promotion of AIDS Research from the Ministry of Health, Welfare, and Labor of Japan, and the Grant to the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re emerging Infectious Diseases (Renkei Jigyo: No. 78, Kumamoto University) of Monbu-kagakusho (HM), and by a grant from the National Institutes of Health (GM53386, AKG).

References

Arasteh, K., Clumeck, N., Pozniak, A., Lazzarin, A., De Meyer, S., Muller, H., Peeters, M., Rinehart, A., and Lefebvre, E. (2005). TMC114/ritonavir substitution for protease inhibitor(s) in a non-suppressive antiretroviral regimen: A 14-day proof-of-principle trial. *AIDS* 19(9), 943–947.

Back, N. K., van Wijk, A., Remmerswaal, D., van Monfort, M., Nijhuis, M., Schuurman, R., and Boucher, C. A. (2000). *In-vitro* tipranavir susceptibility of HIV-1 isolates with reduced susceptibility to other protease inhibitors. *AIDS* 14(1), 101–102.

Carr, A. (2003). Toxicity of antiretroviral therapy and implications for drug development. *Nat. Rev. Drug Discov.* 2(8), 624–634.

Chrusciel, R. A., and Strohbach, J. W. (2004). Non-peptidic HIV protease inhibitors. *Curr. Top. Med. Chem.* 4(10), 1097–1114.

Collins, J. R., Burt, S. K., and Erickson, J. W. (1995). Flap opening in HIV-1 protease simulated by 'activated' molecular dynamics. *Nat. Struct. Biol.* 2(4), 334–338.

Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., *et al.* (1995). *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* 374 (6522), 569–571.

Condra, J. H., Petropoulos, C. J., Ziermann, R., Schleif, W. A., Shivaprakash, M., and Emini, E. A. (2000). Drug resistance and predicted virologic responses to human immunodeficiency virus type 1 protease inhibitor therapy. *J. Infect. Dis.* 182(3), 758–765.

Cote, H. C., Brumme, Z. L., and Harrigan, P. R. (2001). Human immunodeficiency virus type 1 protease cleavage site mutations associated with protease inhibitor cross-resistance selected by indinavir, ritonavir, and/or saquinavir. *J. Virol.* 75(2), 589–594.

De Clercq, E. (2002). Strategies in the design of antiviral drugs. *Nat. Rev. Drug Discov.* 1(1), 13–25.

De Meyer, S. (2006)."8th International Congress on Drug Therapy in HIV Infection, Glasgow, UK, 12–16 November 2006."

De Meyer, S., Azijn, H., Surleraux, D., Jochmans, D., Tahri, A., Pauwels, R., Wigerinck, P., and de Bethune, M. P. (2005). TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. *Antimicrob. Agents Chemother.* 49(6), 2314–2321.

Deeks, S. G., Grant, R. M., Beatty, G. W., Horton, C., Detmer, J., and Eastman, S. (1998). Activity of a ritonavir plus saquinavir-containing regimen in patients with virologic evidence of indinavir or ritonavir failure. *AIDS* 12(10), F97–F102.

Department of Health and Human Services (DHHS). (2006). "Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents. October 10, 2006."

Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., and Lamarre, D. (1996). Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J. Virol.* 70(6), 3763–3769.

Doyon, L., Tremblay, S., Bourgon, L., Wardrop, E., and Cordingley, M. G. (2005). Selection and characterization of HIV-1 showing reduced susceptibility to the non-peptidic protease inhibitor tipranavir. *Antiviral Res.* 68(1), 27–35.

Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., Wang, X. C., Norbeck, D. W., Plattner, J. J., Rittenhouse, J. W., Turon, M., and Wideburg, N. (1990). Design, activity, and 2.8 Å crystal structure of a C2 symmetric inhibitor complexed to HIV-1 protease. *Science* 249, 527–533.

Eron, J., Jr., Yeni, P., Gathe, J., Jr., Estrada, V., DeJesus, E., Staszewski, S., Lackey, P., Katlama, C., Young, B., Yau, L., Sutherland-Phillips, D., Wannamaker, P., *et al.* (2006). The KLEAN study of fosamprenavir-ritonavir versus lopinavir-ritonavir, each in combination with abacavir-lamivudine, for initial treatment of HIV infection over 48 weeks: A randomised non-inferiority trial. *Lancet* 368(9534), 476–482.

Farthing, C., Ward, D., and Hicks, C. (2006). Tipranavir/r demonstrates superior and durable treatment responses compared with comparator PI/r in highly treatment experienced patients: Week 96 RESIST 1 and 2 results [Abstract 1385]. *In* "46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2006."

Fitzgerald, P. M., and Springer, J. P. (1991). Structure and function of retroviral proteases. *Annu. Rev. Biophys. Biophys. Chem.* 20, 299–320.

Fumero, E., and Podzamczer, D. (2003). New patterns of HIV-1 resistance during HAART. *Clin. Microbiol. Infect.* 9(11), 1077–1084.

Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M. F., Nagashima, K., Gorelick, R. J., Mardy, S., Tang, C., Summers, M. F., and Mitsuya, H. (2002). Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. *J. Biol. Chem.* 277(8), 5952–5961.

Gatanaga, H., Das, D., Suzuki, Y., Yeh, D. D., Hussain, K. A., Ghosh, A. K., and Mitsuya, H. (2006). Altered HIV-1 Gag protein interactions with cyclophilin A (CypA) on the acquisition of H219Q and H219P substitutions in the CypA binding loop. *J. Biol. Chem.* 281(2), 1241–1250.

Ghosh, A. K., Thompson, W. J., McKee, S. P., Duong, T. T., Lyle, T. A., Chen, J. C., Darke, P. L., Zugay, J., Emini, E. A., Schleif, W. A., Huff, J. R., and Anderson, P. S. (1993). 3-Tetrahydrofuran and pyranyl urethanes as high affinity P_2-ligands for HIV-1 protease inhibitors. *J. Med. Chem.* 36, 292–294.

Ghosh, A. K., Thompson, W. J., Fitzgerald, P. M., Culberson, J. C., Axel, M. G., McKee, S. P., Huff, J. R., and Anderson, P. S. (1994). Structure-based design of HIV-1 protease inhibitors: Replacement of two amides and a 10 pi-aromatic system by a fused bis-tetrahydrofuran. *J. Med. Chem.* 37(16), 2506–2508.

Ghosh, A. K., Kincaid, J. F., Walters, D. E., Chen, Y., Chaudhuri, N. C., Thompson, W. J., Culberson, C., Fitzgerald, P. M., Lee, H. Y., McKee, S. P., Munson, P. M., Duong, T. T., *et al.* (1996). Nonpeptidal P2 ligands for HIV protease inhibitors: Structure-based design, synthesis, and biological evaluation. *J. Med. Chem.* 39(17), 3278–3290.

Ghosh, A. K., Krishnan, K., Walters, D. E., Cho, W., Cho, H., Koo, Y., Trevino, J., Holland, L., and Buthod, J. (1998). Structure based design: Novel spirocyclic ethers as nonpeptidal P2-ligands for HIV protease inhibitors. *Bioorg. Med. Chem. Lett.* 8(8), 979–982.

Ghosh, A. K., Shin, D., Swanson, L., Krishnan, K., Cho, H., Hussain, K. A., Walters, D. E., Holland, L., and Buthod, J. (2001). Structure-based design of nonpeptide HIV protease inhibitors. *IL Farmaco* 56, 29–35.

Ghosh, A. K., Pretzer, E., Cho, H., Hussain, K. A., and Duzgunes, N. (2002). Antiviral activity of UIC-PI, a novel inhibitor of the human immunodeficiency virus type 1 protease. *Antiviral Res.* 54, 29–36.

Ghosh, A. K., Swanson, L. M., Cho, H., Leshchenko, S., Hussain, K. A., Kay, S., Walters, D. E., Koh, Y., and Mitsuya, H. (2005). Structure-based design: Synthesis and biological evaluation of a series of novel cycloamide-derived HIV-1 protease inhibitors. *J. Med. Chem.* 48(10), 3576–3585.

Ghosh, A. K., Schiltz, G., Perali, R. S., Leshchenko, S., Kay, S., Walters, D. E., Koh, Y., Maeda, K., and Mitsuya, H. (2006a). Design and synthesis of novel HIV-1 protease inhibitors incorporating oxyindoles as the P2′-ligands. *Bioorg. Med. Chem. Lett.* 16(7), 1869–1873.

Ghosh, A. K., Sridhar, P. R., Leshchenko, S., Hussain, A. K., Li, J., Kovalevsky, A. Y., Walters, D. E., Wedekind, J. E., Grum-Tokars, V., Das, D., Koh, Y., Maeda, K., *et al.* (2006b). Structure-based design of novel HIV-1 protease inhibitors to combat drug resistance. *J. Med. Chem.* 49 (17), 5252–5261.

Ghosh, A. K., Sridhar, P., Kumaragurubaran, N., Koh, Y., Weber, I. T., and Mitsuya, H. (2006c). Bis-tetrahydrofuran: A privileged ligand for darunavir and a new generation of HIV-protease inhibitors that combat drug-resistance. *Chem. Med. Chem.* 1, 937–950.

Gisolf, E. H., van Heeswijk, R. P., Hoetelmans, R. W., and Danner, S. A. (2000). Decreased exposure to saquinavir in HIV-1-infected patients after long-term antiretroviral therapy including ritonavir and saquinavir. *AIDS* 14(7), 801–805.

Grabar, S., Weiss, L., and Costagliola, D. (2006). HIV infection in older patients in the HAART era. *J. Antimicrob. Chemother.* 57(1), 4–7.

Hertogs, K., Bloor, S., Kemp, S. D., Van den Eynde, C., Alcorn, T. M., Pauwels, R., Van Houtte, M., Staszewski, S., Miller, V., and Larder, B. A. (2000). Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: A survey of over 6000 samples. *AIDS* 14(9), 1203–1210.

Hicks, C. B., Cahn, P., Cooper, D. A., Walmsley, S. L., Katlama, C., Clotet, B., Lazzarin, A., Johnson, M. A., Neubacher, D., Mayers, D., and Valdez, H. (2006). Durable efficacy of tipranavir-ritonavir in combination with an optimised background regimen of antiretroviral drugs for treatment-experienced HIV-1-infected patients at 48 weeks in the Randomized Evaluation of Strategic Intervention in multi-drug reSistant patients with Tipranavir (RESIST) studies: An analysis of combined data from two randomised open-label trials. *Lancet* 368(9534), 466–475.

Hill, A., and Moyle, G. (2006). Relative week 48 antiviral efficacy of TMC114/r and tipranavir/r versus control PI in the POWER and RESIST trials [Abstract H-1386]. *In* "46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 2006."

Hirsch, H. H., Kaufmann, G., Sendi, P., and Battegay, M. (2004). Immune reconstitution in HIV-infected patients. *Clin. Infect. Dis.* 38(8), 1159–1166.

Ho, D. D., Toyoshima, T., Mo, H., Kempf, D. J., Norbeck, D., Chen, C. M., Wideburg, N. E., Burt, S. K., Erickson, J. W., and Singh, M. K. (1994). Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. *J. Virol.* 68(3), 2016–2020.

Hong, L., Hartsuck, J. A., Foundling, S., Ermolieff, J., and Tang, J. (1998). Active-site mobility in human immunodeficiency virus, type 1, protease as demonstrated by crystal structure of A28S mutant. *Protein Sci.* 7(2), 300–305.

Hornak, V., Okur, A., Rizzo, R. C., and Simmerling, C. (2006). HIV-1 protease flaps spontaneously open and reclose in molecular dynamics simulations. *Proc. Natl. Acad. Sci. USA* 103(4), 915–920.

Jacobsen, H., Ahlborn-Laake, L., Gugel, R., and Mous, J. (1992). Progression of early steps of human immunodeficiency virus type 1 replication in the presence of an inhibitor of viral protease. *J. Virol.* 66, 5087–5091.

Johnson, M. A., Gathe, J. C., Jr., Podzamczer, D., Molina, J. M., Naylor, C. T., Chiu, Y. L., King, M. S., Podsadecki, T. J., Hanna, G. J., and Brun, S. C. (2006). A once-daily lopinavir/ritonavir-based regimen provides noninferior antiviral activity compared with a twice-daily regimen. *J. Acquir. Immune Defic. Syndr.* 43(2), 153–160.

Kaplan, A. H., Michael, S. F., Wehbie, R. S., Knigge, M. F., Paul, D. A., Everitt, L., Kempf, D. J., Norbeck, D. W., Erickson, J. W., and Swanstrom, R. (1994). Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased

sensitivity to an inhibitor of the viral protease. *Proc. Natl. Acad. Sci. USA* 91(12), 5597–5601.

Katlama, C., Esposito, R., Gatell, J. M., Goffard, J. C., Grinsztejn, B., Pozniak, A., Rockstroh, J., Stoehr, A., Vetter, N., Yeni, P., Parys, W., and Vangeneugden, T. (2007). Efficacy and safety of TMC114/ritonavir in treatment-experienced HIV patients: 24-week results of POWER 1. *AIDS* 21(4), 395–402.

Kavlick, M. F., and Mitsuya, H. (2001). The emergence of drug resistant HIV-1 variants and its impact on antiretroviral therapy of HIV-1 infection. *In* "The Art of Antiretroviral Therapy" (E. D. Clercq, Ed.). American Society for Microbiology, Washington, DC.

Kemper, C. A., Witt, M. D., Keiser, P. H., Dube, M. P., Forthal, D. N., Leibowitz, M., Smith, D. S., Rigby, A., Hellmann, N. S., Lie, Y. S., Leedom, J., Richman, D., *et al.* (2001). Sequencing of protease inhibitor therapy: Insights from an analysis of HIV phenotypic resistance in patients failing protease inhibitors. *AIDS* 15(5), 609–615.

Kempf, D., Norbeck, D., Codacovi, L., Wang, X., Kohlbrenner, W., Wideburg, N., Paul, D., Knigge, M., Vasavanonda, S., Craig-Kennard, A., Saldivar, A., Rosenbrook, W., Jr., *et al.* (1990). Structure-based, C_2 symmetric inhibitors of HIV protease. *J. Med. Chem.* 33, 2687–2689.

Kempf, D. J., Marsh, K. C., Kumar, G., Rodrigues, A. D., Denissen, J. F., McDonald, E., Kukulka, M. J., Hsu, A., Granneman, G. R., Baroldi, P. A., Sun, E., Pizzuti, D., *et al.* (1997). Pharmacokinetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. *Antimicrob. Agents Chemother.* 41(3), 654–660.

Kim, E. E., Baker, C. T., Dwyer, M. D., Murcko, M. A., Rao, B. G., Tung, R. D., and Navia, M. A. (1995). Crystal structure of HIV-1 protease in complex with VX-478, a potent and orally bioavailable inhibitor of the enzyme. *J. Am. Chem. Soc.* 117, 1181–1182.

King, N. M., Prabu-Jeyabalan, M., Nalivaika, E. A., and Schiffer, C. A. (2004a). Combating susceptibility to drug resistance: Lessons from HIV-1 protease. *Chem. Biol.* 11(10), 1333–1338.

King, N. M., Prabu-Jeyabalan, M., Nalivaika, E. A., Wigerinck, P., de Bethune, M. P., and Schiffer, C. A. (2004b). Structural and thermodynamic basis for the binding of TMC114, a next-generation human immunodeficiency virus type 1 protease inhibitor. *J. Virol.* 78 (21), 12012–12021.

Koh, Y., Nakata, H., Maeda, K., Ogata, H., Bilcer, G., Devasamudram, T., Kincaid, J. F., Boross, P., Wang, Y. F., Tie, Y., Volarath, P., Gaddis, L., *et al.* (2003). Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* 47, 3123–3129.

Koh, Y., Towata, T., Ghosh, A., and Mitsuya, H. (2007). *In* "14th Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA, February 25–28, 2007."

Larder, B. A., Hertogs, K., Bloor, S., van den Eynde, C. H., DeCian, W., Wang, Y., Freimuth, W. W., and Tarpley, G. (2000). Tipranavir inhibits broadly protease inhibitor-resistant HIV-1 clinical samples. *AIDS* 14, 1943–1948.

Lin, Y., Lin, X., Hong, L., Foundling, S., Heinrikson, R. L., Thaisrivongs, S., Leelamanit, W., Raterman, D., Shah, M., Dunn, B. M., and Tang, J. (1995). Effect of point mutations on the kinetics and the inhibition of human immunodeficiency virus type 1 protease: Relationship to drug resistance. *Biochemistry* 34(4), 1143–1152.

Little, S. J., Holte, S., Routy, J. P., Daar, E. S., Markowitz, M., Collier, A. C., Koup, R. A., Mellors, J. W., Connick, E., Conway, B., Kilby, M., Wang, L., *et al.* (2002). Antiretroviral-drug resistance among patients recently infected with HIV. *N. Engl. J. Med.* 347(6), 385–394.

Logsdon, B. C., Vickrey, J. F., Martin, P., Proteasa, G., Koepke, J. I., Terlecky, S. R., Wawrzak, Z., Winters, M. A., Merigan, T. C., and Kovari, L. C. (2004). Crystal structures of a multidrug-