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Review

Protease inhibitor resistance in HIV-infected patients: Molecular and clinical perspectives

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Abstract

The problem of HIV-1 drug resistance has established a need for new compounds that retain activity against mutated resistant viral isolates. Fortunately, a number of new compounds have recently been developed that possess excellent activity against HIV-1 strains that contain as many as eight relevant drug-resistance mutations in the viral protease (PR) gene. These newer protease inhibitors (PI) are characterized by higher genetic barrier for drug resistance, meaning that higher numbers of mutations are required for resistance to develop in comparison with older members of the PI family of drugs. Thus, different PIs can be used sequentially in HIV therapy in a manner that can overcome previous drug resistance and potentially forestall the development of additional resistance mutations in the viral PR. All currently used PIs, in general, require ritonavir to be used as a pharmalogical boosting agent. There is a need to develop novel PIs, that will not require such boosting, and that are also characterized by potent antiviral activity and a high genetic barrier for resistance.

Keywords: Protease inhibitors; HIV; Cross-resistance; Mutations; Genetic barrier; Salvage therapy

Contents

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1. Introduction

Human immunodeficiency virus (HIV) protease inhibitors (PIs) were introduced into clinical practice in 1995. Their potency and efficacy became rapidly evident and translated into major clinical benefits for HIV-infected people: more prolonged viral control, better viral suppression and reduced morbidity and mortality (Gulick et al., 1997; Hammer et al., 1997). These results earned them an essential place as part of antiretroviral drug (ARV) combination regimens. Research on the discovery of novel PIs continues to be intense as this class offers the most numerous options for treatment of HIV infection.

On the other hand, resistance to PIs has also emerged and represents a limitation in the treatment of HIV infection that can lead to disease progression (Deeks et al., 1999; Ledergerber et al., 1999; Lucas et al., 1999; Mocroft et al., 2003). Important considerations concerning the issue of PI resistance include patient adherence to therapy, how the resistance patterns of different PIs are distinct but still overlap, how prone PIs are to selection for resistance, and how frequently drug resistance is transmitted. The current availability of numerous PI drugs and their resistance pathways permit clinicians to use them in a variety of circumstances, either as initial therapy or as salvage therapy, in the treatment of the chronically infected patient. Efforts are being made to simplify therapy and optimize patient compliance while maintaining excellent possibilities for long-lasting viral suppression. Recent clinical trials of monotherapy with potent ARVs include the use of the ritonavir-enhanced PIs (PI/r). Clinical trials of boosted PIs offer significant improvements for the treatment of both wild-type and ARV-resistant virus, encourage patient compliance and significantly prolong the efficacy of ARVs for many patients. However, all comparisons to date between any given PI administered without ritonavir (RTV) boosting versus with use of RTV have revealed increased toxicities when boosting is employed (Cohen, 2005). This notwithstanding, the use of RTV boosting has served to limit the emergence of PI drug resistance. This is important as the transmission of drug resistant HIV has increased, as ARV coverage has expanded and patients are treated for longer periods, a fact that has led to changes in recommendations by expert international committees.

Within this context, this review attempts to integrate the subject of resistance to PIs from both a molecular and clinical perspective in order to highlight the implications on the use of this drug class for treatment of HIV infection.

2. Molecular basis of action of HIV protease inhibitors and drug resistance

The HIV protease (PR) (HIV-1 and HIV-2) is a homodymeric aspartyl protease consisting of 99 amino acids per monomer. Three domains of the PR are frequently referred to in the scientific literature: the active site cavity, the dimerization domain, and the flaps (see Fig. 1). The main contribution of the HIV PR to the viral life cycle is in the maturation of the assembled viral particle. The PR recognizes a series of heptamers in the Gag (p55) and Gag-Pol (p160) polyproteins and cleaves them at 9 distinct sites releasing the constitutive components of the viral matrix (MA/p17) capsid (CA/p24) and nucleocapsid (NC/p7) as well as the functional enzymes reverse transcriptase (RT), PR and integrase (IN). At the core of the HIV PR, two aspartic acid residues (one in each monomer) stabilize the addition of water across the amide bond of a susceptible polypeptide to create a tetrahedral transition state intermediate. This intermediate form is then broken generating the C-terminal carboxylic acid and N-terminal amine, thereby resulting in cleavage of the substrate.

Protease inhibitors are competitive inhibitors of the HIV-1 PR. Inhibition of PR results in production of immature HIV particles that lack infectious ability. Saquinavir (SQV) was the first molecule of this type to be developed and approved for

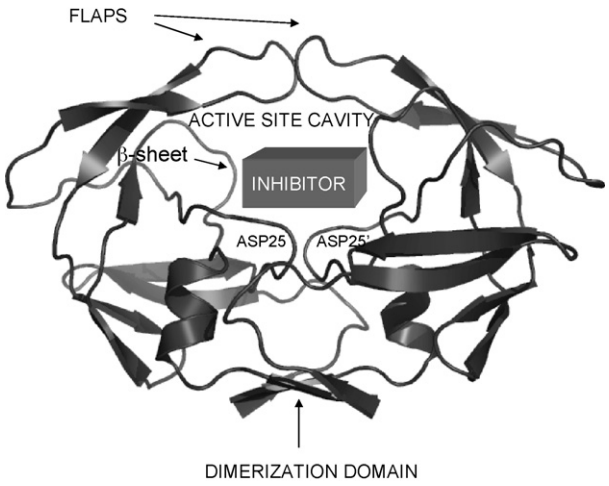

Fig. 1. The crystal structure of the wild-type HIV-1 protease. Inhibitors have been designed to fit inside the active site cavity. The flaps are flexible structures that open to permit entrance of the large gag-pol polyproteins. The aspartates (ASP) in the active site confer hydrolytic activity to the enzyme. *Note*: This cartoon was generated from the 3HVP structure in the RCSB PDB using software available from PyMol DeLano Scientific, Palo Alto, CA.

treatment of HIV infection. SQV, which works by simulating the PR transition state, was developed before HIV PR crystallographic data became available. Although it represented an important advance at the time, its low oral bioavailability proved to be a significant drawback, and resistance to this compound was rapidly recognized (Craig et al., 1991; Jacobsen et al., 1995; Noble and Faulds, 1996). In response to this drug's weaknesses, investigators focused on the discovery of agents with better oral bioavailability and improved potency. The design of later PIs largely benefited from the structural data provided by X-ray crystallography. The PIs subsequently developed had fewer peptidic properties, were based on structural designs, and were smaller in size. This second generation of PIs included indinavir (IDV), ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV) and amprenavir (APV) (Ogden and Flexner, 2001). Subsequent efforts were committed to the development of drugs with a lower impact on lipid metabolism and higher activity against drug resistant variants. These efforts lead to the discovery of atazanavir (ATV) (Piliero, 2002; Robinson et al., 2000), tipranavir (TPV) (Thaisrivongs and Strohbach, 1999) and darunavir (DRV) (Surleraux et al., 2005). APV was minimally modified to make fosamprenavir (FPV), a prodrug with better absorption after oral administration (Falcoz et al., 2002).

Overall, the affinity of these drugs for the HIV-1 PR principally relies on molecular interactions with atoms different from the catalytic residue Asp 25. Affinities of different PIs for wild-type HIV-1 PR have been measured. The dissociation constant (K_d), which is the concentration of drug at which 50% of the enzyme is bound to the drug, is inversely proportional to the drug affinity and is a good parameter of enzyme activity. K_ds fall into the nanomolar range for the earlier PIs, i.e. IDV (0.48 nM), SQV (0.40), and NFV (0.26 nM), and within the subnanomolar range for RTV (0.029 nM) and newer PIs APV (0.15 nM), LPV (0.0077 nM) (Velazquez-Campoy et al., 2002), TPV (0.008 nM) (Thaisrivongs and Strohbach, 1999) and DRV (0.0045 nM) (Surleraux et al., 2005).

Thermodynamic studies indicate that earlier inhibitors (IDV, SQV, NFV, RTV) utilize hydrophobic effects as a main driving force for binding (Luque and Freire, 2002, 1998). This confers to these molecules a dependence on shape for specificity that renders them poorly adaptable to mutations in the target enzyme. Consequently, the presence of only a few resistance mutations can cause significant loss of affinity and lead to resistance (Velazquez-Campoy et al., 2002). Newer PIs (APV, LPV, TPV, and probably DRV), which have very high affinities, are to some degree protected against changes in PR, since they can retain significant affinity despite the presence of several resistance mutations (Ohtaka et al., 2004). They also appear to better adapt to changes in the PR. The investigational drug TMC-126 combines high affinity and high adaptability to mutations, thanks to favorable enthalpy as shown by previous reports (Ohtaka et al., 2002; Yoshimura et al., 2002). These differences help to determine the resistance barrier of each PI.

Some PIs are especially susceptible to loss of activity when mutations are located on the flaps of PR (G48V for SQV, I50V for APV, I50L for ATV), while others are sensitive to mutations appearing inside the active site (D30N for NFV, V82L/T and I84V for TPV). Cross-resistance mutations that can substantially affect the binding affinity of most PIs in thermodynamic studies have been reported. They can lower the affinities of PIs with a different magnitude for each one. For instance, the mutations V82F/I84V lower the binding affinity of PR for SQV and NFV by a factor of ∼20, IDV by 70, RTV by 370, APV by 160 and LPV by 120 (Velazquez-Campoy et al., 2003). These two positions are located in the β-sheets of the active site cavity (see Fig. 1). Similarly, the IAS USA drug resistance mutation list includes V82 as a residue, that when mutated can affect all PIs in clinical use to date, except DRV (although the resistance profile of DRV is not yet completely determined). Similarly, the I84V mutation affects all eight PIs in clinical use and is a major mutation for five of them. Despite being outside the active site, the L90M mutation also compromises all PIs except DRV and, on its own, has less influence on susceptibility to TPV.

Crystallographic data suggest that resistant HIV PRs expand the active site cavity. For instance, a transition from a longer side chain to shorter chain amino acids is seen in resistant PRs (e.g., 82V → A, 84I → V) (Logsdon et al., 2004; Martin et al., 2005). Additionally, structural data from a highly resistant PR containing 10 resistance mutations revealed an expanded active site, as a result of separation of the flaps by as much as 10 Å, while this distance was only 4 Å in the case of wild-type PR. This "wide open expansion" appears to be the result of a domino effect led by the mutation M36V and the reshaping of the 23–32 amino acid segment by the L90M mutation (Logsdon et al., 2004; Martin et al., 2005). These active site cavity characteristics are preserved even when other major resistance mutations are introduced (Martinez et al., 2004). Interestingly, molecular dynamics simulations suggest that the L90M mutation can affect the triads 25D26T27G and cause subsequent important conformational changes at the flap region and the 79′P loop, despite being far from the active site cavity (Ode et al., 2005). This striking structural change is thought to make multi-drug resistant PRs targets for new drug development (Martin et al., 2005; Martinez et al., 2004).

Research aimed at developing PIs for treatment of highly PI-resistant HIV strains includes generation of molecules that should fit the expanded active site of the multi-drug resistant PRs (Logsdon et al., 2004). Such molecules should be expected to display high potency against multi PI-resistant viruses. This goal could be reached by designing molecules that fill empty pockets within the active site or "more adaptable" molecules that could accommodate themselves to fit into the expanded active site cavity.

3. The emergence of PI resistance

In vitro, resistance to PIs is attained by passage of the virus in tissue cultures (usually peripheral blood mononuclear cells) containing a gradually increasing level of drug. The results are expressed as IC_{50} or IC_{90} which are the concentrations of drug that inhibit 50% and 90% of viral growth, respectively. Resistance to PIs follows an ordered accumulation of mutations in the viral PR (Condra et al., 1996; Molla et al., 1996). These experiments have helped clarify the mutational pathways for specific PIs as well as the mutations that confer major impact in affin-

ity and mutations of lesser importance. Additionally, they have also permitted identification of the potential of various PIs to select for cross-resistance within this drug class. Major mutations are generally selected early in the process of resistance, are substantially inhibitor specific, and have a clearly evident effect on virus drug susceptibility (e.g., D30N, G48V, V82A, F, T, I84V, and L90M). Minor mutations accumulate in viral genomes already containing one or more primary mutations, have a less discernible effect on resistance, but may be selected because they improve viral fitness rather than decrease drug binding to target enzymes (Hirsch et al., 1998).

In vivo, the process is significantly more complex and cannot be directly monitored. The degree of suppression of viral replication depends on the relationship between exposure of the virus to the drug and the inherent susceptibility of the infecting virus to such drug. Drug exposure is importantly affected by drug bioavailability, metabolism, or degree of protein binding. Additionally, PR inhibition can only be accomplished if the PI can reach the intracellular environment. Evidently, *in vivo* drug resistance results from the interplay among viral, drug, and patient related factors. These factors are addressed below.

3.1. Viral factors affecting emergence of drug resistance

Three HIV characteristics are of principal importance: the high *in vivo* HIV replication rate (approximately 10^9 virions per day), the high mutation frequency of the HIV RT (one mutation per 10^4–10^5 viral particles), and the recombinogenic properties of the HIV genome (Hu and Temin, 1990; Preston et al., 1988). Every possible single point mutation occurs 10^4–10^5 times each day, including those that can reduce susceptibility to PIs (Coffin, 1995). Furthermore, drug-induced viral suppression exerted by PIs is not complete, and at least one study has reported that low-level viral replication in patients with VL <50 can suffice for selection of viral resistance (Elbeik et al., 2001).

The PR enzyme is highly adaptable. In general, several resistance mutations must accumulate in the PR gene in order to cause a significant loss of activity of PIs. However, the PR can tolerate as many as 15 amino acid substitutions and still sustain viral replication (Charpentier et al., 2003; Erickson et al., 1999; Gatanaga et al., 2002). In addition, resistance mutations in PR have been recognized in about 73% of amino acid positions (Torti et al., 2004). As a result of many resistance mutations, most PI-resistant viruses lose fitness (Quinones-Mateu and Arts, 2002) and probably pathogenicity, as suggested by continued immunologic restoration in patients with low plasma viral loads of resistant virus who continue to take PIs (Hunt et al., 2003; Kaufmann et al., 1998).

The genetic variability of HIV offers a wide spectrum of background polymorphisms that may affect the propensity of PR to become less susceptible to inhibitors, to preserve catalytic function while displaying low susceptibility to inhibitors, or to amplify the effect of drug resistance mutations. In one study, PRs from viruses of HIV-infected PI-naive patients had a mean number of polymorphisms of 4.2, and PRs with five or more polymorphisms, or that harbored the I93L or A71V/T polymorphisms, had a greater risk of developing virologic failure to the PIs IDV, RTV, or SQV (Servais et al., 2001). Others showed that the naturally occurring polymorphism 36I/71V, when combined with the NFV-selected active site mutation D30N, can lead to a greater level of inhibitor cross-resistance while retaining PR catalytic efficiency (Clemente et al., 2003). Additionally, in other HIV types and subtypes, genetic variability has been shown to affect drug affinity. One group reported that the presence of the double V82F/I84V resistance substitution within the C and A PR subtypes lowers the binding affinity of PIs by 40–3000 times more than when present in subtype B PR. In addition, the biochemical fitness of the C and A subtype drug-resistant mutants was up to 1000-fold higher than that of the wild-type B subtype PR in the presence of PIs. Therefore, naturally occurring PR polymorphisms of the C and A HIV-1 subtypes may be able to amplify the effects of common drug-resistance mutations (Velazquez-Campoy et al., 2002).

In contrast, Holguin et al. (2004) reported reduced susceptibility in only 2/58 non-B subtype viruses from drug-naive patients. Abecasis et al. (2006) recently explored the role of protease polymorphisms on the baseline susceptibility of subtypes G, CRF02_AG, F and C to the PIs APV, IDV, LPV, NFV, SQV, ATV, RTV and TPV. The influence of each polymorphic amino acid was individually assessed in a statistical model, and the results suggest that differences in baseline drug susceptibility to PIs depend on the differential prevalence of polymorphisms that affect drug susceptibility rather than the subtype per se (Abecasis et al., 2006). The implications of these findings on duration of virologic suppression and emergence of drug resistance will require further attention in prospective cohorts of patients infected with non-B subtypes who are taking PIs. Research in this area is important for ART programs in developing countries since ART regimens for massive use will need to be selected in the future based on resistance surveillance data. Finally, HIV-2, whose genome differs by 60% from that of HIV-1, shows decreased susceptibility to PIs, higher rates of failure with SQV, NFV, IDV and LPV, and more rapid emergence of drug resistance in viral culture (Ntemgwa et al., 2007; Rodes et al., 2006; Witvrouw et al., 2004).

Genetic variability also favors compensation of the otherwise reduced catalytic activity of PI-resistant PRs. The naturally occurring polymorphism L63P contributes to resistance in the presence of other substitutions that lower PIs affinities, as it helps to restore the fitness loss caused by the resistance mutations D30N and L90M (Martinez-Picado et al., 1999). In other studies, the naturally occurring polymorphisms L63P and V77I were found to partially restore the negative impact on PR function resulting from N88S, a resistance mutation selected for by NFV (Resch et al., 2002). Furthermore, natural substrates can also mutate and adapt to the mutated PR and regenerate the susceptibility of substrates to the proteolytic activity of drug-resistant PRs (Doyon et al., 1996).

Mutations in and in the vicinity of the Gag and Gag-Pol cleavage sites have been described (Gatanaga et al., 2002; Myint et al., 2004). Gag and gag-pol compensatory mutations generally appear after initial PI mutations have resulted in important loss of viral fitness (Carrillo et al., 1998; Zhang et al., 1997). Viruses containing Gag compensatory mutations partially regain

fitness and outcompete viruses lacking them (M.F. Maguire et al., 2002; Myint et al., 2004). It is likely that these compensatory mechanisms *in vivo* translate into higher plasma viral loads of PI-resistant viral strains and perhaps some gain in pathogenicity, but this has not yet been proven in clinical settings. It is not known how frequently substrate compensatory mutations are present as natural polymorphisms before resistance mutations develop or whether or not they are more or less likely to occur in non-B HIV subtypes.

3.2. Host factors affecting emergence of drug resistance

3.2.1. Adherence to therapy

Adherence to therapy greatly influences incidence of viral failure and emergence of drug resistance (Harrigan et al., 2005; Weiser et al., 2004; Yasuda et al., 2004). Drug adherence results from a multiplicity of factors, which can vary among different communities and geographic areas and include but are not limited to drug side effects and toxicity, cost of therapy and availability of medications (Ammassari et al., 2001; da Silveira et al., 2003; Knobel et al., 2005; Roge et al., 2004). While patient adherence to PIs has a clearly linear relationship with viral failure, there is a bell-shaped relation with regard to emergence of resistance (see Fig. 2). Regarding viral failure with PIs, Paterson reported that an adherence level to PIs <95% is significantly associated with viral failure in patients receiving non-boosted PIs as part of highly active antiretroviral therapy (HAART) (Paterson et al., 2000).

Suboptimal adherence has also been associated with the evolution of drug resistance. HIV drug resistance minimally occurs either at very high levels of adherence or at very low adherence levels (Friedland and Williams, 1999). In the former case, this is because maximal suppression of viral replication impedes emergence of resistant variants, and in the latter instance, exposure of virus to low levels of drugs does not suffice to select for resistance mutants. This leaves an intermediate scenario in which there is partial drug exposure that permits viral replication while favoring the replication of variants that are drug resistant. Poorly adherent patients miss doses, which can cause intermittent and/or suboptimal antiviral drug exposure ($C_{min} < IC_{50}$).

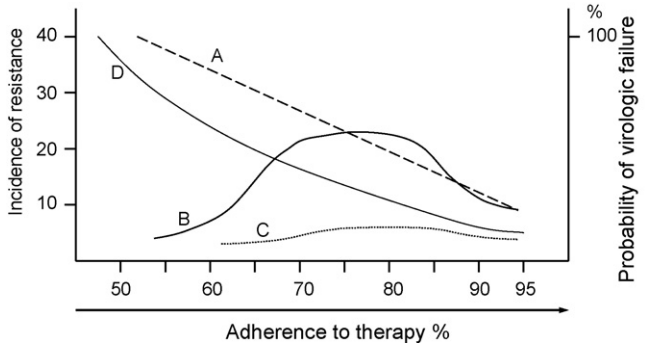

Fig. 2. Schematic representation of expected relationships between adherence to therapy and probability of viral failure (A), resistance incidence for non-boosted protease inhibitors (B), boosted protease inhibitors (C) and non-nucleoside transcriptase inhibitors (D). The figure does not present precise values, but attempts to outline probable relationships among variables.

Under such conditions, selection of drug-resistant (DR) variants is favored.

Bangsberg et al. (2003) examined the relationship between adherence, viral suppression, and HIV drug resistance among 148 participants who received at least 1 month of HAART. In this study, higher adherence was correlated with longer time on treatment and viral suppression (VL < 50 copies/mL). The investigators estimated that 23% of drug resistance mutations occur in patients with 92–100% adherence, 30% with 79–91% adherence, 15% with 58–78% adherence, 20% with 42–57% adherence, and 12% with 0–41% adherence. Additionally, in a prospective study of 195 patients who had maintained viral suppression while receiving ART, Sethi et al. (2003) reported an overall incidence of resistance of 14.5 per 100 person-years and of 9.6, 13.6, 36.6, 44.9, and 12.3 per 100 person-years for patients whose cumulative adherence was 100%, 90–99%, 80–89%, 70–79%, and <70%, respectively. Finally, the bell-shaped relationship was modeled by a group of investigators (Bangsberg et al., 2004b) and later validated by the results of a post hoc analysis of the M98–863 clinical trial (King et al., 2005). In this study by King et al., the relation between adherence and resistance was evaluated for a NFV-based compared to a LPV/r-based combination regimen. While the relation of adherence to NFV and incidence of NFV resistance was clearly bell-shaped, a flattened curve, representing a strikingly low incidence of resistance in the LPV/r arm was also found.

The relationship between adherence to drug regimens and drug resistance is still being studied in regard to boosted PIs. Early data suggested that RTV-boosted PIs can maintain high levels of efficacy at adherence rates lower than 95% (Gross et al., 2006). More recently, Shuter et al. (2006) reported high rates of virologic suppression in a prospectively followed cohort of patients taking LPV/r-based HAART. Virologic suppression (<400 c/mL) was achieved in 70% or more patients with adherence of 80% or higher. Interestingly, lower levels of suppression were found in patients at both very high and low levels of adherence (>90% and <70%), a finding that can be interpreted in a manner consistent with the pharmacokinetic advantages of RTV-boosted PIs. Additionally, comparisons of drug resistance between ATV and ATV/r have revealed differences in drug resistance patterns, in which neither I50L nor N88S emerged in patients taking ATV/r (Zolopa et al., 2006). Lower rates of virologic failure were also encountered with RTV-boosted SQV and APV (Ananworanich et al., 2005, 2006; Gathe et al., 2006). Further studies will be required to establish the reliability of RTV-boosting to maintain efficacy at adherence levels lower than 95%, including levels of 70 or 60%, which are common in clinical practice. Less adherence-dependent pharmacokinetics will likely prove to be advantageous for PI-based regimens compared to NNRTI-based combinations, where the genetic barrier for drug resistance is low.

3.2.2. Cytochrome P-450, sanctuary sites and efflux transporters

PIs are competitive inhibitors of cell transporters and are predominantly metabolized by the CYP3A4 pathway in hepatocytes and enterocytes. The extent of competitive inhibition

varies among PIs and ranges from weak (SQV) to modest (NFV, IDV) to potent (RTV) (Janneh et al., 2005). RTV produces a strong blockade of CYP3A4 that is used for pharmacologic enhancement of other PIs. Additionally, polymorphisms of some cytochrome P450 isoenzymes have been associated with variations in metabolism of NFV (2D6) and IDV (CYP3A5) (von Moltke et al., 1998). Polymorphisms of CYP 1A1 appear responsible for a risk of hyperbilirubinemia with ATV or IDV (Rotger et al., 2005). These variations can potentially affect the bioavailability of some PIs.

Interest has been placed on the role of drug transporter proteins in penetration of ARVs to so-called sanctuary sites. The P-glycoprotein (P-gp) is a transmembrane protein encoded by the multi drug resistance 1 (MDR1) gene, is found in many cell types, and mediates transport of certain molecules out of cells (efflux transporters), including ARVs (Gottesman and Pastan, 1993; Juliano and Ling, 1976; Krishna and Mayer, 2000; Sharom et al., 1993). The TT genotype of MDR1 has been linked to higher intracellular concentrations of NFV and immune response to it, as well as to higher plasmatic levels of ATV (Rodriguez Novoa et al., 2006). The T allele at this position is associated with better immune responses (Fellay et al., 2002), and the CC homozygote status has been linked to a trend toward virologic failure (Brumme et al., 2003). In a recent report, neither CYP3A4 variations nor MDR1 genetic variations influenced plasma or intracellular concentration of LPV/r (Winzer et al., 2003).

The evolution of primary and secondary drug resistance mutations to both RT and PR inhibitors in diverse regions of the brain during ART appears to be compartmentalized (Smit et al., 2004). Data are being accumulated that support the relevance of efflux transporters to decreased penetration of PIs into the CNS (Bachmeier et al., 2005; Ford et al., 2004; Park and Sinko, 2005; Thomas, 2004). For instance, blockade of P-gp by the inhibitor LY335979 in mice resulted in a near doubling of plasma NFV levels, an important increase in brain drug levels and an increase in brain-to-plasma drug ratio after IV NFV dosing (Choo et al., 2000). P-gp is also expressed in testes cells and similar results have been reported (Choo et al., 2000). RTV is only a moderate inhibitor of efflux transporters (Bachmeier et al., 2005), so the potential of more potent and selective inhibitors to improve PI activity within sanctuary sites is being investigated.

3.3. Drug factors affecting drug resistance

Pharmacokinetic concepts are relevant to understand how drug–virus interactions can affect clinical outcome. Briefly, the maximal or peak concentration of drug that is achieved after oral administration is known as maximal plasma concentration (C_{max}), and the minimal concentration (C_{min}) is known as the trough. The area under the concentration–time curve (AUC) expresses overall drug exposure. The elimination half-life ($t_{1/2}$) is the time necessary for plasma drug levels to decrease by 50%. The IC_{50} and IC_{90} refer to the concentration of drug required to inhibit 50% and 90% of viral replication, respectively. Inhibition of viral replication and reduction in viral load to an undetectable level requires maintaining maximally suppressive drug concentrations ($C_{min} > IC_{50}$ or IC_{90} or IC_{95}). A decrease in drug levels below IC_{50} can permit viral replication and the possible emergence of resistant virus (Molla et al., 1996). The C_{min}/IC_{50} ratio, also called the inhibitory quotient (IQ), has been shown to predict viral load response to PIs (Castagna et al., 2004; Condra et al., 2000; Winston et al., 2005).

On the other hand, the outcome of missing a dose depends on the drug C_{min}, $t_{1/2}$, intracellular drug concentrations, and the IC_{50} of a given HIV isolate. Even after a dose is missed, the greater the IQ and the longer the $t_{1/2}$ of the drug, the more likely the C_{min} would remain above the virus IC_{50}. In contrast, missing one dose of an ARV with a lower IQ and a shorter $t_{1/2}$ can cause suboptimal drug exposure of certain duration, thereby favoring the emergence of drug resistance. The duration of this suboptimal drug exposure becomes important and is discussed below.

3.3.1. Pharmacokinetic features of PIs

Characteristics of ARVs such as bioavailability and rate of drug metabolism importantly affect viral drug exposure, and, hence, the likelihood for viral failure and emergence of resistance. The main pharmacokinetic characteristics of non-boosted PIs and the effects of RTV-boosting are summarized in Table 1. Overall, the major disadvantage of PIs is their short elimination half-life ($t_{1/2}$), which results in rapid clearance of the drug and high likelihood of the C_{min} falling below the IC_{50} for wild-type virus when a dose is missed, hence rapidly losing antiviral activity. Also, important variability in pharmacokinetics exists among different PIs as well as among patients for each individual PI. Consequently, C_{min} and exposure levels for PIs can vary widely among patients and high chance of subtherapeutic exposure exists. Newer PIs offer more favorable pharmacokinetic properties that considerably improve drug absorption and simplify dosing.

Some of the previously mentioned disadvantages are overcome by RTV boosting. RTV is a particularly potent blocker of CYP3A4, an effect that can increase bioavailability and delay the elimination of several drugs including PIs. The RTV boosting of a PI refers to using a low dose of RTV (below the regular therapeutic antiviral range) in order to obtain increases in the pharmacokinetic parameters of another PI which is given within the therapeutic antiviral range. The results are variable augmentation of plasma C_{min}, plasma C_{max}, $t_{1/2}$, and the AUC (Kempf et al., 1997; Rathbun and Rossi, 2002). The increased drug exposure results in stronger viral suppression (Moyle and Back, 2001). The relative pharmacokinetic effects of RTV-boosting vary among PIs and are presented in Table 1. Nevertheless, toxicities can also be worsened by RTV boosting including impaired glucose tolerance (Petersen and McGuire, 2005; Tang et al., 2006), unfavorable changes in plasma lipids (Calza et al., 2004; Montes et al., 2005), fat redistribution (Ergun-Longmire et al., 2006; Martinez and Gatell, 1999) and probably promotion of atherosclerosis (Sudano et al., 2006).

3.3.2. Resistance barrier of PIs

A drug's genetic barrier to resistance generally refers to the number of mutations that the target enzyme must acquire so as

Table 1
Pharmacokinetic properties of HIV protease inhibitors

Properties of PI	SQV	IDV	NFV	LPV/r	APV	ATV	FPV	TPV/r	DNV/r
Bioavailabilty (%) (after oral administration)	4 (hard gel capsule)	60–65	20–80	NR	NR	60–68	NR	NR	37, 82 with RTV 100 mg
Food effect	Increase 6 to 7-fold in AUC	Decrease 77% in AUC	Increase 2 to 3-fold in AUC	Moderate fat meal increase AUC for capsules 48%and solution 80%	High fat meal decreases AUC in 21%	Light meal increases 70%, high fat meal increases 35%	No significant changes	High fat meal increase AUC 2-fold	Any food increase 30%
$t_{1/2}$ (h)	1.5–2	1.8	3.5–5.0	5–6	7.1–10.6	7.9	7.7	5.5–6.0	15.2 ± 6.76[a]
C_{max} (mg/mL)	0.2	7.7	3.0	9.6	7.7	5.4	4.82	51.94	4.628[b]
C_{min} (mg/mL)	0.16	0.15	1.4	5.5	0.32	0.22	0.35	22.05	3.8[c]
Protein binding (%)	97	60	>98	98–99	90	86	90	>99	95%
Pharmacokinetic effects of RTV-boosting	C_{max}, C_{min}, AUC	$t_{1/2}$, C_{min}, AUC	Complex and variable	Increase C_{max}, C_{min}, AUC	Increase $t_{1/2}$, C_{min}, AUC	Increase AUC, 7-fold increase in C_{min}	Increase AUC, minimal increase in C_{max}, 4–10 increase in C_{min}	Increase AUC 5 to 12-fold, and 10 to 25-fold increase in C_{min}	Increase 14-fold in AUC

Data obtained from manufacturers package information inserts and from references Acosta (2002), Acosta et al. (2004), Baldwin et al. (1998), Flexner (2000), McCallister et al. (2004), Moyle and Back (2001), Sekar et al. (2006) and Yu and Daar (2000).

[a] Mean ± 1S.D. in 8 individuals taking darunavir/r 600 mg/200 mg for 14 days.

[b] Mean C_{max} (calculated from information in package insert).

[c] Mean C_{min} (calculated from information in package insert).

not to be inhibited by such a drug at therapeutic levels. A higher resistance barrier requires more mutations to render an antiviral drug inactive. NNRTIs are more forgiving than PIs (Maggiolo et al., 2005), but boosted-PIs have a higher genetic barrier to resistance. A preliminary report of a recent head-to-head comparison between efavirenz (EFV) and LPV/r-based regimens showed that EFV usage was associated with a superior duration of viral suppression, but resulted in two class viral resistance more frequently that did a LPV/r-based regimen (Riddler et al., 2006). Other reports have also found less resistance for PIs (23%) than for NNRTIs (69%) at lower levels (0–48%) of patient adherence (Bangsberg et al., 2006).

The high genetic barrier of PIs to resistance is the major asset of this class and should be seriously considered when starting drug therapy. Extensive research has demonstrated that (1) PIs differ in the number of mutations necessary to confer high-level resistance, (2) PIs differ in the resistance mutations they select for, including the propensity to select cross-resistance mutations, and (3) PIs differ in their relative susceptibility to cross-resistance mutations. A schematic representation of the different PI resistance mutations in terms of susceptibility to resistance is found in Table 2.

On the other hand, the pharmacologic barrier of a drug refers to the likelihood that virologic failure might occur under conditions of suboptimal adherence. Drugs with a longer $t_{1/2}$ (e.g., NNRTIs and some NRTIs) can maintain virologic suppression with a lower adherence level, while those with a shorter $t_{1/2}$ require higher patient adherence to achieve viral suppression. This is doubtless a key reason for the success of the combination of TDF/FTC/EFV in clinical practice, in spite of the fact that each of the three drugs in the regimen has a very low genetic barrier for resistance. The other major reason is the high antiviral potency of each of the drugs in the regimen. However, a longer $t_{1/2}$ can also favor the emergence of resistance at very low adherence levels (Bangsberg et al., 2004a), in part, because it may facilitate viral exposure to only one drug in the context of combination therapy if all drugs are simultaneously discontinued. This is the result of faster clearance of the other drugs and the persistence of the drug with the longer $t_{1/2}$.

The plasma concentration curves of PIs may add one more protective factor against emergence of HIV drug resistance. In contrast to the pharmacokinetic properties of NNRTIs, whose very prolonged $t_{1/2}$ can result in a long decreasing plasma concentration slope, most PIs have a short $t_{1/2}$ and a sharp drop in plasma levels (King et al., 2004). This may limit the time of exposure to subtherapeutic drug concentrations ($<IC_{50}$ or $<IC_{90}$) that might otherwise exert selection pressure while allowing for some degree of viral replication. Three observations are of note: (1) in tissue culture, HIV drug selection pressure is usually initiated with drug concentrations close to the IC_{50}; (2) in the context of therapeutic drug monitoring, it is expected that the plasma C_{min} of an ARV drug, i.e. above the IC_{90} rather than IC_{50}, will result in more effective and prolonged viral suppression (despite increased chances of toxicity); (3) RTV-boosting substantially increases the C_{max} of most PIs, but prolongs $t_{1/2}$ to a much lesser extent. Hence, in the event of poor adherence, the exposure of virus to suboptimal levels of drug would be short (hours). In contrast, exposure time can be very prolonged for NNRTIs, since the $t_{1/2}$ of NVP and EFV are very long, 25–30 h and 40–55 h, respectively (Hoetelmans, 1999). Consequently, RTV-boosted PI usage can lead to two situations that are incompatible with selection of resistance: an initial exposure to high levels of drug

Table 2
HIV protease inhibitor resistance mutations

Protease inhibitor resistance mutations according to the IAS-US A panel for ARV resistance					PI resistance barrier
Protease inhibitor drug	Cross-resistance mutations		Unique mutations		Number of resistance mutations
	Major	Minor	Major	Minor	
Saquinavir/r	L90M, G48V	10IRV, 24I, 54VL, 62V, 71VT, 73S, 77I, 82AFTS, 84V			2 or more
Indinavir/r	46IL, 82AFT, 84V	10IRV, 20MR, 24I, 32I, 36I, 54V, 71VT, 73SA, 77I, 90M			3 or more
Nelfinavir	90M	10FIRV, L24I, M36I, M46IL, A71VT, G73S, V77I, V82AFTS, I84V, N88DS	30N		2 or more D30N and other substitution
Fosamprenavir/r	I50V	L10FIRV, V32I, M46IL, I47V, I54LVM, G73S, V82AFST, L90M			3 or more
Lopinavir/r	V32I, I47VA, V82AFTS	L10FIRV, K20MR, L24I, L33F, M46IL, I50V, F53L, I54VLAMTS, A71VT, G73S, I84V, I90M		L63P	6 or more
Atazanavir/r	I84V, N88S	L10IFVC, K20RMITV, L24I, V32I, L33IFV, M36ILV, M46IL, G48V, F53LY, I54, LVMTA, I62V, A71VITL, G73CSTA, V82ATFI, L90M	I50L	G16E, E34Q, D60E, I64LMV, I93LM	3 or more
Tipranavir/r	L33F, V82LT, I84V	L10V, K20MR, E35G, M36I, K43T, M46L, I47V, I54AMV, L90M		I13V, Q58E, H69K, T74P, N83D	7 or more
Darunavir/r	I50V, I54ML, I84V	V11I, V32I, L33F, I47V, G73S	L76V	V11I, L89V	3 or more

Data obtained from Baxter et al. (2006), Condra et al. (1996), De Meyer et al. (2006), J.A. Johnson et al. (2006), V.A. Johnson et al. (2006), Kempf et al. (2001), Parkin et al. (2003), Partaledis et al. (1995), Patick et al. (1998), Pazhanisamy et al. (1996), Schapiro et al. (1998), Schapiro et al. (1999), Vora et al. (2006).

(with strong viral suppression) and a rapid fall of drug levels (as compared to what occurs with NNRTIs) from above the IC_{90} to IC_{50} levels or lower that results in minimal selection pressure for resistance. In clinical practice, excellent patient adherence to PI dosage and timing leaves little opportunity for viral replication to occur. In non-adherent patients, rapid drug elimination may reduce the likelihood for selection of drug resistance.

4. Protease inhibitor resistance in clinical studies

In general, most HIV mutations found *in vitro* have been confirmed *in vivo* but frequencies of occurrence in patients can vary. In vitro studies have described the selection of drug resistance mutations for each of the PIs currently used in clinical practice. Detailed description of PI resistance selection experiments can be found elsewhere. Resistance mutations emerging during clinical studies are addressed below.

4.1. Early generation protease inhibitors

4.1.1. Saquinavir/r

In the ACTG229 trials, 144 treatment experienced patients received non-RTV boosted SQV (1800 mg/day) as part of combination regimens in two of the three arms of the study. The L90M substitution appeared in 39.6% (57/144) of patients, G48V in 0.7% (1/144) of patients and both mutations in 6.3% (9/144) of patients (Schapiro et al., 1999). A genotypic analysis of the pooled plasma samples from the NV14256 and SV14604 phase III clinical trials demonstrated an incidence at week 48 of 39–45% for L90M, 5–8% for G48V + L90M and less than 5% for G48V alone (Race et al., 1998). Another study that used higher doses of SQV (3600 mg/day and 7200 mg/day) reported a switch in the relative frequency of these mutations. At a dose of 3600 mg/day, the frequencies were 2/20 for G48V and 7/20 for L90M, while a frequency of 2/20 for each mutation occurred at 7200 mg/day. In this latter study, both mutations together did not occur (Schapiro et al., 1996). Finally, one study in patients taking monotherapy with SQV found other mutations and natural polymorphisms (10I, L63P/Q/T, 71V/T, 36I and, I84V), besides the typical mutations at codon 90 in viral isolates with reduced susceptibility to SQV (Ives et al., 1997).

4.1.2. Nelfinavir

Clinical trials of NFV monotherapy have reported the predominant role of the D30N substitution in patients taking NFV and experiencing viral failure. Concurrent or sequential emergence of other mutations (at residues 35, 36, 46, 71, 77, and 88) were occasionally seen. Mutations associated with resistance to other PIs were not observed except for L90M which was rarely seen (Patick et al., 1998). A systematic review of genotypes from four clinical trials and two observational studies of patients failing NFV-containing combination ARV regimens as initial therapy revealed that only 38% of isolates had phenotypic resistance to NFV. The resistance mutations in these isolates were D30N in 81% of case, L90M in 13% and one sample had both mutations (Clotet et al., 2002). A recent Brazilian study in patients failing NFV-based ARV combination therapy as a first regimen showed similar results with the D30N mutation being found in 57%, L90M in 18% and the wild-type virus in 25% of subjects (Tupinambas et al., 2005). The strong predominance of D30N over L90M and the rarity of selection for cross-resistance mutations is intriguing. Others reported a lower frequency of D30N than L90M in subtype C HIV-1 (Grossman et al., 2004), suggesting that non-B subtypes may have important differences in resistance pathways for NFV.

4.1.3. Indinavir/r

Studies of samples from patients enrolled in phase I and II clinical trials demonstrated no preferred pathway for resistance and a correlation was shown between phenotypic resistance and mutations at positions L10, K20, L24, M46, I54, L63, I64, A71, V82, I84, and L90. Mutations at position 82 (V82A, T or F) were most commonly associated with IDV monotherapy and caused a 4 to 8-fold reduction in susceptibility to IDV (Condra et al., 1995). Some initial clinical trials of IDV-based combination therapy failed to demonstrate resistance mutations in viruses of patients experiencing virologic failure (Descamps et al., 2000; Havlir et al., 2000). This may have been due to several factors including patient non-adherence to therapy. Of interest, Dykes et al. (2004) performed subspecies analyses of plasma samples from patients failing an IDV-based combination regimen and found the V82A resistance mutations in 1/6 samples that had not revealed any mutations by bulk sequencing.

4.2. Newer protease inhibitors and their role in rescue therapy

4.2.1. Amprenavir/r and fosamprenavir/r

Susceptibility data provided by the PROAB3006 study showed that an APV/NRTI combination regimen in NRTI experienced patients can result in the selection of one of four alternative HIV PR genotypes, i.e. I50V, I54L or I54M, V32I + I47V, or rarely, I84V. Viruses with >4-fold decreased susceptibility harbored I54L, I54M, V32I + I47V, or I50V. The role of these mutations was confirmed with both clinical isolates and site-directed mutant phenotypic assays. The clinical isolates resistant to APV infrequently exhibited reduced susceptibility to the other PIs employed in this study (IDV, NFV, SQV, RTV, LPV) (M. Maguire et al., 2002). Another study reported that six or more mutations were predictive of a reduced response to APV/r in PI-experienced patients (Marcelin et al., 2003).

The NEAT study revealed an APV-like pattern of mutations in drug-naive patients who received unboosted FPV; most frequently L10I, V31I, L33F, M46I, I47V, I50V, I54L (Macmanus et al., 2003). Follow-up of the SOLO study, a clinical trial of drug-naive patients on therapy with FPV/r, reported no detected resistance mutations in patients undergoing treatment for up to 160 weeks. At week 160, however, virus from one patient failing this regimen possessed a resistance genotype associated with APV failure, i.e. M46I + I50V, and a cleavage site mutation at position L449F. This variant also harbored numerous polymorphisms as compared to the reference HXB2 strain (T12P K14R, G16E, L19I, M36I, S37N, R41K, I62V, L63A, V82I, and I93L) (Sax et al., 2005). A second report of another patient in the same

trial, who underwent viral rebound after a prolonged period with low viral load (<1000 copies/mL), showed the mutations V32I, I47V, M46I, A71V. Clonal analysis of the virus before therapy was started demonstrated the presence of small proportions of the resistance mutations V32I, I47V, and V82A (Schurmann et al., 2006).

4.2.2. Lopinavir/r

Abundant information has been generated on LPV resistance from PI-experienced patients (LPV/r is the only form available). Kempf et al. (2001) obtained viruses from PI-experienced patients and performed genotypic and phenotypic analyses. The following mutations were associated with reduced susceptibility: L10F/I/R/V, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V, and L90M. The mutations 82, 54, 10, 63, 71, and 84 were associated with modestly (4- and 10-fold) decreased LPV susceptibility, while the K20M/R and F53L substitutions, when accompanied by multiple other mutations, were related to >20- and >40-fold-decreased susceptibility, respectively. The number of mutations was associated with greater loss of susceptibility and 6–7 resistance mutations resulted in an increased IC_{50} (13.5 times higher). Since LPV/r results in a very high plasma C_{min} of LPV (>75-fold IC_{50}), it was thought possible to use this approach to suppress some viruses with 6–8 resistance mutations.

When used in PI-naive patients, LPV/r has demonstrated a very low propensity to select resistance mutations. Through 7 years (360 weeks) of follow-up, ARV-naive subjects receiving LPV/r-based therapy exhibited sustained virologic responses, with 59% demonstrating HIV-1 RNA <50 copies/mL by intention-to-treat (NC = F) analysis. The corresponding on-treatment response rates were 95–98%. Two cases of resistance developing on LPV/r as initial therapy have been reported so far. The first involved the V32I, M46M/I, and I47A mutations (Friend et al., 2004) and the other substitutions at M36I, I54V, L63P, V82A, I93l and L33F (Conradie et al., 2004).

Several studies have addressed the predictive value of mutations emerging in viruses exposed to other PIs in regard to probability for LPV/r failure. The first study examined the impact of various PR mutations on viral load response to LPV in 792 extensively PI-treated patients. This investigators determined the importance of mutations at positions 10, 20, 33, 47, 48, 50, 54, and 82 for LPV resistance (Marcelin et al., 2005). Another study reported the emergence of mutations at positions 10, 20, 33, 46, 47, 50, 54, and 82 following a salvage regimen with LPV/r (Masquelier et al., 2002). A third study found that mutations at positions 33, 47, 48, 50, 58 and 73 emerged following virologic failure in 21 patients receiving salvage therapy with LPV (Mo et al., 2005).

Recently, Mo et al. (2005) also examined new mutations appearing in virus variants from patients who experienced viral rebound while taking a LPV/r-containing salvage regimen. The probability of viral rebound increased as more baseline resistance mutations were present in the PR gene. Mutations at positions 82, 54, and 46 were seen frequently and were highly prevalent after viral failure. In contrast, emerging mutations at positions 84, 90, and 71 were not observed at rebound, despite having been frequently present at baseline. In addition, the substitutions L33F and I50V were seen in seven subjects and were associated with resistance to the PI class. A different study revealed that the association of mutations I47A, I32V and M46I, and several polymorphisms, although rare, resulted in greater phenotypic resistance than predicted (Parkin et al., 2003). de Mendoza et al. (2006) reported a prevalence of 0.6% of mutation I47A among samples obtained from PI-experienced patients, corresponding to four patients who failed LPV/r. Phenotypic testing revealed that I47A caused high-level LPV resistance (>100-fold), cross-resistance to APV, but caused hypersusceptibility to SQV. Rodes et al. (2006) described high levels of resistance in HIV-2 harboring I47A alongside hypersusceptibility to SQV. The I47A substitution was seen at a high frequency in HIV-2 (8.6% of PI-experienced patients).

4.2.3. Atazanavir and atazanavir/r

Studies of treatment-naive patients with virologic rebound who receive an atazanavir regimen reveal that I50L is the signature drug resistance mutation for this drug (Colonno et al., 2004). In all 23 isolates with genotypic testing, the mutation I50L was found. The I50L substitution does not confer cross-resistance to APV. Other PI-associated mutations were also identified: A71V (52%), K45R/Q/N (30%), G735 (26%), and M46I/L (13%). The mean time to detection of ATV resistance mutations in these studies was 62 weeks (Colonno et al., 2004).

The I50L substitution found in ATV-resistant isolates can result in hypersusceptibility to other PIs. In phenotypic assays, a fold change of ≤0.4 was noted for LPV (100% of isolates), RTV (81%), IDV (46%), SQV (67%), APV (43%), and NFV (29%) (Colonno et al., 2004). In treatment-experienced patients, the I50L mutation does not emerge and ATV resistance is due to accumulation of other substitutions that frequently cause PI cross-resistance: I84V, L90M, A 71V/T, N88S/D, and M46I. In treatment-experienced patients with high-level resistance to other PIs, susceptibility to ATV is often reduced (Colonno et al., 2003; Schnell et al., 2003). The mutation pattern seen in viral isolates from patients on ATV/r differs from that described in patients taking non-boosted ATV and from in vitro data. For instance, I50L has not been seen in patients taking ATV/r, but N88S (along with K20T, M36I/V, L63P, A71T,) and G73S (along with L10I, L63P, V77I, I93L) have been reported in two separate cases exposed to this regimen (Coakley et al., 2005; McGrath et al., 2006; Salama and Caplivski, 2005). However, patients who may have developed I50L while on ATV may be subsequently non-responsive to ATV/r.

4.2.4. Tipranavir/r

TPV is active against multiple clinical isolates that harbor mutations (I15V, E35D, N37D, R41K, D60E, A71T) that cause resistance to IDV, RTV and NFV (Poppe et al., 1997; Rusconi et al., 2000). Larder et al. (2000b) found that 90% of 105 HIV clinical isolates from heavily PI pretreated patients (having received SQV, RTV, NFV, IDV) were phenotypically susceptible to TPV.

Data from phase II and III clinical trials in which TPV/r was used as part of salvage therapy in patients failing other PIs revealed a broader range of mutations associated with decreased

susceptibility (10V, 13V, 20M/R/V, 33F, 35G, 36I, 43T, 46L, 47V, 54A/M/V, 58E, 69K, 74P, 82L/T, 83D, and 84V). Importantly several major PR mutations, associated with resistance to other PIs (30N, 48V, 50V/L, 82A/F, and 90M), did not contribute to the TPV resistance score and some others (30N, 50V) were associated with increased susceptibility to TPV. The results of this study suggest a "cut-off" of ≥8 TPV score mutations as predictive of full resistance and lack of clinical response with use of TPV. However, ≥5 TPV mutations were associated with diminished 24 week virologic responses (Baxter et al., 2006).

A recent analysis of 9860 HIV isolates assessed the extent of resistance to current PIs conferred by key mutations at positions 33, 82, 84, and 90, plus L33I and L33F. In this study, the presence of three or more key mutations resulted in 70% of isolates being resistant to LPV, 79% resistant to ATV, 82% to SQV, and 85% resistant to APV. In contrast, 17% of isolates with two key mutations and 38% with three key mutations displayed full resistance to TPV. Additionally, V82A did not confer resistance to TPV, and rarely did so when associated with one or two other key mutations. In vitro, TPV selects for V82L or V82T, not V82A. Importantly, L90M plus one other key mutation resulted in 33–75% of isolates becoming resistant to LPV, APV, ATV, and SQV, but less than 20% of isolates showing resistance to TPV (Piliero et al., 2006).

The RESIST I and II trials demonstrated the superiority of RTV-boosted TPV over an alternative comparator PI (both in combination with an optimized background regimen), in terms of viral suppression and immunologic response, in highly ARV-experienced patients who had VL of ≥1000 copies/mL. The fact that the optimized background PI regimen (the comparator arm) consisted of the best available approved drugs at the time adds clinical relevance to the findings. Co-usage of TPV and enfuvirtide (ENF) resulted in a large proportion of patients achieving effective treatment responses for up to 48 weeks (Hicks et al., 2006).

The Food and Drug Administration analysis of TPV resistance reported reduced virologic response rates in patients receiving TPV/r when substitutions I13V/A/L/S, V32I/L, M36I/A/V/L/N, I47V/A, Q58E, D60E/K/A/N, and I84V/A were present at baseline (Naeger and Struble, 2007). Mutations V82S/F/I/L but not V82A/T/C were also associated with lower virologic response rate. When the number of baseline PI resistance mutations were five or more, a reduced virologic response and a loss of virologic response after either week 4 or 8 were seen (Naeger and Struble, 2007). Additionally, an analysis of pooled data from RESIST 1 and 2 in regard to TPV phenotype showed an association with response rate as follows: with an IC_{50} FC of 0–3, 45% of patients responded to TPV/r versus 77% if ENF was present; with a IC_{50} FC >3–10, 21% responded to TPV/r versus 43% if ENF was present; and with an IC_{50} FC > 10, no-one responded who was not given ENF versus 53% if ENF was used (Naeger and Struble, 2007).

Marcelin et al. (2007) reported a 55% virological response in 143 PI-experienced patients who were treated with TPV. Mutations at six codons, i.e. E35D/G/K/N, M36I/L/V, Q58E, Q61D/E/G/H/N/R, H69I/K/N/Q/R/Y, L89I/M/R/T/V were linked to a lower rate of response to TPV/r. One mutation F53L/W/Y was associated with an augmented virological response to TPV/r, indicating that these substitutions may cause hypersensitization to TPV.

Similarly, Parkin and Chappey (2006) studied a data set of 1411 phenotyped samples, containing at least one PI resistance mutation with a cut-off for resistance to TPV of 2-fold. By introducing several modifications to previous algorithms, they showed that each of I47V, I54A, V82T, I84V, V11L increased resistance by a factor of two while each of V32I, A71L, G73T, L89V did so by a factor of one, and L10I, M46I, and L90M each did this by a factor of 0.5. In contrast, each of L10V, I13V, K20R, and M46L and L24I, D30N, I50L/V, I54L, L76V, V82I resulted in diminished levels of resistance (Parkin and Chappey, 2006). A genotypic and phenotypic analysis may be essential in order to predict which PI-experienced patients may best be able to benefit from the use of TPV/r as an active drug.

4.2.5. Darunavir/r

The POWER 1, 2 and 3 trials led to the FDA approval of darunavir (DRV) in June of 2006. The POWER 3 study was, an open label safety study, and showed that 65% of patients who received DRV/r 600/100 bid had a >1 log drop in HIV RNA by week 24, with 40% reaching <50 copies/mL and a mean VL reduction of −1.65 log. Importantly, an advantage was provided by the concomitant use of enfuvirtide as part of the salvage regimen (Vangeneugden et al., 2006). The presence of 10 or more PI resistance mutations from the IAS-USA panel correlated with a 10-fold change in phenotypic susceptibility. Eleven mutations were specifically associated with a diminished virologic response in the POWER studies. In order of importance, I50V (4-fold decrease in susceptibility) was the most relevant followed by L76V, I84V, V32I, L33F, I 47V, V11I, I54L/M, G73S, and L89V (De Meyer et al., 2006). The package insert of DRV (Prezista™) states that amino acid substitution V32I developed in greater than 30% and a substitution at amino acid I54 developed in greater than 20% of DRV/r virologic failure isolates from treatment-experienced patients.

5. Clinical value of HIV drug resistance testing in selecting PI-based therapies

Genotype, real phenotype, and virtual phenotype-based ARV resistance tests can now be used to diagnose HIV drug resistance and help select future therapy. Most studies have found a therapeutic advantage in terms of likelihood of viral suppression and mean virologic RNA reduction for resistance-test-guided treatments when compared to treatments based only on clinical judgment (De Luca et al., 2003; DeGruttola et al., 2000; Durant et al., 1999; Gianotti et al., 2006; Ormaasen et al., 2004; Tural et al., 2002). Genotype-based resistance testing is currently the most widely used resistance test, given its low cost and technical simplicity. A major limitation of genotyping is the interpretation of results, and most HIV resistance non-expert clinicians rely on genotyping resistance algorithms to obtain an interpretation.

Large databases of paired genotype–phenotype assays have been used to build "virtual phenotypes", i.e. calculations that provide a quantitative estimation of HIV-1 resistance to ARV

drugs based on a statistical prediction of the most likely phenotype for a given genetic sequence. Although a good correlation of virtual phenotypes with "real phenotypes" has been reported (Graham et al., 2001; Larder et al., 2000a), there is no consensus among experts on the degree of correlation between virtual and real phenotype. Virtual phenotype also has limitations of probability analysis, but higher correlation is expected as the number of matched genotype–phenotype samples increases.

Although concern has been raised regarding the long-term benefits of resistance testing on maintaining viral suppression (Panidou et al., 2004), a recent well-designed study which followed patients for 48 weeks found benefit in likelihood of viral suppression for both a rule-based interpretation algorithm and a virtual phenotype interpretation method (Gianotti et al., 2006). In addition, data from the latter suggest that resistance testing could even provide benefit in clinical settings in which a resistance expert is not available.

In phenotype-based resistance testing, assays directly measure viral replication under drug pressure. In these assays, a genetic segment of interest from a clinical isolate is spliced into a DNA vector. Appropriate cells are then transfected with the assembled clone and the effect measured with respect to drug susceptibility. The virus is grown under a range of drug concentrations, and the IC_{50}, IC_{90} or IC_{95} are obtained. The ratio IC_{50} (IC_{90} or IC_{95}) of the mutated virus/IC_{50} (IC_{90} or IC_{95}) of the wild-type virus can be determined, and the results are expressed in terms of fold change (FC) in susceptibility. Cut-off points have been established for each drug, above which resistance is considered to be present, in order to facilitate interpretation (resistance present or not). This strategy, can potentially predict resistance for viruses that *in vivo* might still be suppressed by a drug. The use of the inhibitory quotient (IQ) can improve the application of this data. The IQ and a normalized IQ (NIQ) have been found to predict virologic response to several PIs in patients who experienced drug failure due to resistance (Casado et al., 2003; Castagna et al., 2004; Gonzalez de Requena et al., 2004; Marcelin et al., 2003; Shulman et al., 2002; Winston et al., 2005). Higher IQs predict better responses. The IC_{50} FC for each drug, along with confidence intervals, can provide information on the potential contribution that each single PI might offer as part of the next drug regimen being considered. It is important to emphasize that phenotyping in clinical practice offers the greatest advantage in cases involving viruses with complex and/or rare mutational combinations.

All resistance tests currently used in the standard clinical setting are incapable of detecting viruses that may have been archived in viral reservoirs. Nor can they detect minority viral quasispecies (less than 20% of the total viral mixture). Current methods appear to have similar performance at detecting ARV resistance (Antinori et al., 2006; Ferrer et al., 2003; Mazzotta et al., 2003; Saracino et al., 2004), but genotyping is more sensitive than phenotyping for minority populations. Of interest, the presence of minority variants was found to predict subsequent treatment failure in a study conducted by the US Centers for Disease Control and Prevention (V.A. Johnson et al., 2006). Pre-existing resistant viral subspecies were recently reported as a cause of viral failure in a PI-naive patient experiencing virologic rebound while on RTV-boosted FPV, that had not been detected by bulk population sequencing (Schurmann et al., 2006). More sensitive genotyping techniques are now able to detect as few as 0.1–2.0% of minority viral sequences in plasma and may be further improved (Flys et al., 2006; Hare et al., 2006; J.A. Johnson et al., 2006; Palmer et al., 2006). Finally, several studies have clearly demonstrated that expert advice adds benefit to results from resistance testing (Badri et al., 2003; Bossi et al., 2004; Clevenbergh et al., 2003; Saracino et al., 2004; Torre and Tambini, 2002; Tural et al., 2002).

6. Transmission of PI resistance

The prevalence of PI resistance mutations among drug-naive individuals has been reported in several areas of the world and demonstrates the transmission of HIV drug resistance. In New York, this prevalence was 27% for all ARVs and 8% for PIs in the period 2003–2004, in Europe 13.5% for all ARVs and 3.4% for PIs in the period 1996–2002, in the United Kingdom this was 19.2% for all ARVs and 6.6% for PIs in the period 2002–2003, in Canada 12.2% for all ARV and 2.3% for PIs. In a cohort from ten cities of the United States, the prevalence of resistance to all ARVs was 8.9% and 1.9% for PIs in the period 1997–2001. Primary HIV resistance may lead to reduced treatment options, treatment failure and clinical progression (Harzic et al., 2002; Little et al., 2002; Markowitz et al., 2005). These facts led HIV-treatment guidelines-developing agencies in the United States and Europe to recommend routine genotype resistance testing for all treatment-naive patients (Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, 2006; Vandamme et al., 2004).

Resistance mutations are most likely transmitted from patients failing a PI containing regimen and who practice high transmission risk behaviors. MDR HIV is capable of dissemination and establishment of a cellular reservoir at the time of primary HIV infection; the same transmitted resistant strain can persist for years (24–48 months) (Barbour et al., 2004; Brenner et al., 2004; Ghosn et al., 2006). An additional concern is PI access in countries with massive numbers of infected people, since second and third line ARV regimens are not yet widely available. It is possible that more PI resistance mutations will accumulate in countries in which early non-boosted PIs are still used instead of newer PIs. Encouraging a rapid switch to newer PIs in these countries as first line regimens will reduce rates of resistance in such settings.

7. Summary

Structure-based design of PIs has resulted in development of increasingly PIs active drugs that have improved options for successful treatment of HIV infection in both ARV-naive and experienced patients. RTV-boosting has helped simplify PI dosing, and has improved and prolonged the antiviral suppression exerted by many PIs, despite some worsening of toxicities. In addition, despite the better pharmacokinetics of the RTV-boosted PIs, high levels of adherence still appear necessary

to maintain low likelihood of viral failure. Virologic failure in patients taking newer PIs is seldom accompanied by resistance mutations. The underlying reason for this fact is still not well understood. Comparisons with the NNRTI efavirenz indicate that there is room for pharmacokinetic improvement of PIs whose high genetic barrier for resistance continues to be their principal advantage. The study of efflux transporters and their role in PI penetration into and distribution within so-called sanctuary sites may lead to ways of making HIV in these compartments more susceptible to ARVs. Rational use of genotyping and phenotyping resistance testing is necessary to optimize formulation of active ARV regimens, given high levels of PI resistance transmission and the complex and not always predictable presence of cross-resistance. Resistance transmission will warrant close monitoring in settings in which ARV use is being expanded.

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