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Review

Fifteen years of HIV Protease Inhibitors: raising the barrier to resistance

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ABSTRACT

HIV protease plays a crucial role in the viral life cycle and is essential for the generation of mature infectious virus particles. Detailed knowledge of the structure of HIV protease and its substrate has led to the design of specific HIV protease inhibitors. Unfortunately, resistance to all protease inhibitors (PIs) has been observed and the genetic basis of resistance has been well documented over the past 15 years.

The arrival of the early PIs was a pivotal moment in the development of antiretroviral therapy. They made possible the dual class triple combination therapy that became known as HAART. However, the clinical utility of the first generation of PIs was limited by low bioavailability and high pill burdens, which ultimately reduced adherence and limited long-term viral inhibition. When therapy failure occurred multiple protease resistance mutations were observed, often resulting in broad class resistance.

To combat PI-resistance development, second-generation approaches have been developed. The first advance was to increase the level of existing PIs in the plasma by boosting with ritonavir. The second was to develop novel PIs with high potency against the known PI-resistant HIV protease variants. Both approaches increased the number of protease mutations required for clinical resistance, thereby raising the genetic barrier.

This review provides an overview of the history of protease inhibitor therapy, its current status and future perspectives. It forms part of a special issue of *Antiviral Research* marking the 25th anniversary of antiretroviral drug discovery and development, vol. 85, issue 1, 2010.

Contents

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1. HIV protease: function and structure

HIV protease plays an essential role in the viral life cycle. It generates mature infectious virus particles through cleavage of the viral Gag and GagPol precursor proteins (Kohl et al., 1988). The Gag precursor protein codes for all the structural viral proteins, matrix (p17, MA), capsid (p24, CA) and nucleocapsid (p7, NC), the p6 protein and the two spacer proteins p2 (SP1) and p1 (SP2) (Fig. 1A). The GagPol polyprotein is generated through a −1 ribosomal frameshift event, occurring at a frequency of about 5–10% (Jacks et al., 1988). This polyprotein encodes MA, CA, p2, NC, the transframe protein (TFP) and the viral enzymes protease, reverse transcriptase and integrase (Fig. 1A).

HIV-1 protease recognizes the asymmetric shape of the peptide substrates, rather than a particular amino acid sequence (Prabu-Jeyabalan et al., 2002). So the amino acid sequence of all the cleavages sites is different, but the peptides have a superimposable secondary structure, yielding a substrate "envelope" which is designed to fit within the protease substrate-binding region. There are however, a few differences between the cleavage site substrates in which amino acid side chains protrude out of the "envelope". It is thought that these small differences in the structure of the peptides contribute to the highly ordered and regulated process in which all individual cleavages occur at different rates (Fig. 1B) (Erickson-Viitanen et al., 1989; Krausslich et al., 1989; Pettit et al., 1994; Wiegers et al., 1998). The first cleavage takes place at the C-terminal part of p2 (MA-CA-p2↓NC-p1-p6) and subsequent cleavages separate MA from CA-p2 (MA↓CA-p2) and NC-p1 from p6 (NC-p1↓p6). Separation of the small spacer proteins p2 (CA↓p2) and p1 (NC↓p1) are the final and rate limiting cleavages to occur. The ordered cleavage appears to be regulated by the amino acid sequence near the actual protease cleavage site. Although most studies have investigated the impact of the substrate sequence at p4-p3′ positions, which are in direct contact with the viral protease, it is demonstrated that the more distantly located p4′ and p5′ positions also affect the efficiency of cleavage (Nijhuis et al., 2007). The viral protease cleaves the two precursor proteins accumulating at the plasma membrane during or shortly after the assembled virus particles are released from the infected cells. So, HIV protease activity is not required for virus production and release per se, but is essential for viral maturation leading to infectious viral particles.

HIV protease is part of the family of aspartic proteases (with an aspartic acid in the active site at position 25) and is a symmetrically assembled homodimer consisting of two identical subunits of 99 amino acids (Navia et al., 1989; Wlodawer et al., 1989). The centre of the enzyme is formed by the substrate-binding cleft, which interacts with the different substrate cleavage site sequences in the Gag and GagPol proteins. The precise mechanism by which the viral protease, which is embedded in the GagPol protein, becomes activated is not fully understood; although it is recognized that dimerization of GagPol precursor proteins is required. Despite its critical function in viral maturation and infectivity, HIV protease has shown great plasticity and polymorphisms have been observed at one-third of the 99 amino acids (Stanford HIV Drug Resistance Database http://hivdb.stanford.edu/) (Rhee et al., 2003) (Fig. 2).

2. Inhibitors of the HIV protease

Detailed knowledge of the structure of HIV protease and its substrate has led to the development of specific protease inhibitors (PIs). They have been designed to bind the viral protease with high affinity but tend to occupy more space than the natural substrates. Currently, there are nine PIs approved for clinical use: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, tipranavir and darunavir (Fig. 3, Table 1). Most of them are prescribed with a concomitant low dose of ritonavir as boosting agent. All of them, with the exception of tipranavir, are competitive peptidomimetic inhibitors, mimicking the natural substrate of the viral protease. The peptidomimetic inhibitors contain a hydroxyethylene core, which prohibits cleavage of the protease inhibitor by the HIV-1 protease (Craig et al., 1991; Kempf et al., 1995; Koh et al., 2003; Partaledis et al., 1995; Patick et al., 1996; Robinson et al., 2000; Sham et al., 1998; Vacca et al., 1994) (Fig. 3). Instead of a peptidomimetic hydroxyethylene core, tipranavir contains a dihydropyrone ring as a central scaffold (Turner et al., 1998) (Fig. 3).

3. First generation protease inhibitor therapy

3.1. Saquinavir

The first protease inhibitor to be approved in 1995 by the FDA in the US under accelerated approval regulations for use in antiretroviral combination therapy was saquinavir (Fig. 3, Table 1). Saquinavir showed in vitro activity against HIV in human lymphoblastoid and monocytic cell lines and in peripheral blood mononuclear cells (PBMCs) (Plosker and Scott, 2003). The concentration of saquinavir required to inhibit 50% replication (IC_{50}) ranged from 20 to 500 nM in studies using cells that are either acutely or chronically infected with HIV (Perno et al., 1998). The drug was originally formulated as a hard-gel capsule (HGC) which was well tolerated but did not result in a sustained decline in plasma HIV-RNA load when used as monotherapy (Noble and Faulds, 1996; Kitchen et al., 1995). After a year of monotherapy approximately 50% of treated patients harboured viruses with reduced susceptibility to saquinavir, mainly characterized by mutations 48V and/or 90M in the protease coding gene (Fig. 2) (Jacobsen et al., 1996).

More clinical benefit was shown when the drug was used as part of a triple combination therapy. In the ACTG-229 trial combination of saquinavir with zidovudine and zalcitabine, two nucleoside transcriptase inhibitors (NRTIs), resulted in a greater reduction in plasma viral RNA level and modestly higher $CD4^+$ cell counts at 24 weeks compared to dual NRTI regimens. At 48 weeks the triple combination was still superior to the comparator arms, but efficacy markers tended to return to pre-treatment values (Collier et al., 1996). Reduced susceptibility to saquinavir was observed less frequently compared to mono and duo therapy but still occured in 22% of patients using a saquinavir-based triple combination regimen (Jacobsen et al., 1996).

As a result of limited absorption and extensive first-pass metabolism by the hepatic cytochrome P450 3A system, very low oral bioavailability of saquinavir was observed with the HGC formulation. In order to achieve more adequate absorption saquinavir had to be taken three times daily with meals with a high fat content. A pilot trial testing doses up to four times

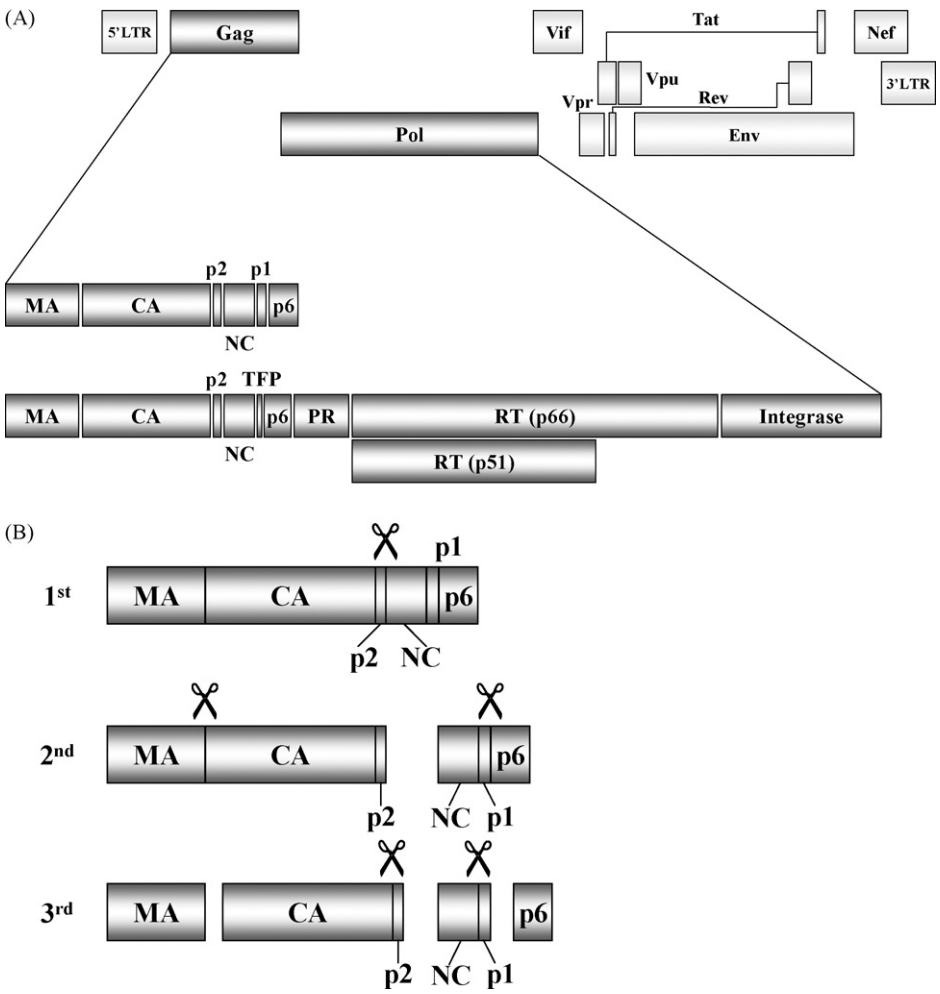

Fig. 1. (A) Schematic representation of the HIV-1 genome and organization of the Gag and GagPol precursor polyproteins. (B) Schematic overview of the sequential proteolytic processing of the HIV-1 Gag precursor protein. MA: matrix; CA: capsid; NC: nucleocapsid; TFP: transframe protein; PR: protease; RT: reverse transcriptase; IN: integrase.

higher than the registered dose showed much greater and more sustained HIV-RNA reductions, suggesting higher antiviral potential if suboptimal absorption could be overcome (Schapiro et al., 1996). To address this problem saquinavir was reformulated as a soft-gel capsule (SGC) which provided greater systemic exposure.

3.2. Ritonavir

Soon after approval of saquinavir two other protease inhibitors were licensed in the US for clinical use as monotherapy or as part of combination regimens. One of them was ritonavir which the first protease inhibitor approved for the treatment of HIV-infection in the European Union (Fig. 3, Table 1). The activity of ritonavir was assessed in vitro in acutely infected lymphoblastoid cell lines and in peripheral blood lymphocytes. The concentration of drug that inhibits 50% (EC50) of viral replication ranged from 3.8 to 153 nM depending upon the isolate and the cells employed (Ritonavir Package, 2008).

Its prolonged absorption phase and half-life permitted the use of a twice daily dosing schedule (Danner et al., 1995). When used as monotherapy, ritonavir resulted in impressive decreases in HIV-RNA plasma levels and increases in CD4 cell counts. Partial diminution of antiviral efficacy was seen after 12–16 weeks (Danner et al., 1995; Markowitz et al., 1995). Long-term use of ritonavir monotherapy was frequently associated with accumulation of ritonavir resistance characterized by signature mutations at positions 46I, 54V, 82 and 84 (Fig. 2) (Schmit et al., 1996; Molla et al., 1996).

Combination therapy consisting of ritonavir and two NRTIs resulted in impressive increase in CD4 cell counts and greater than 2 log declines in plasma HIV-RNA levels in observational studies (Notermans et al., 1998; Mathez et al., 1997). In patients with advanced disease addition of ritonavir to double nucleoside therapy reduced AIDS related complications and prolonged survival compared to placebo. Unfortunately, ritonavir was poorly tolerated due to numerous side effects including severe gastrointestinal symptoms, paresthesias, elevated hepatic aminotransferases and triglyceride levels forcing multiple patients to discontinue study medication (Notermans et al., 1998; Mathez et al., 1997; Cameron et al., 1998). Due to this extensive toxicity and the high pill burden, prescription of ritonavir as an antiretroviral in its own right was gradually rendered obsolete (Table 1).

3.3. Indinavir

The other protease inhibitor that became licensed in 1996 was indinavir (Fig. 3, Table 1). The concentration of indinavir required to reduce viral replication with 95% (IC_{95}) ranged from 25 to 100 nM in various in vitro studies (Emini et al., 1996; Plosker and Noble, 1999). Activity was assessed in human lymphoblast and monocyte cell lines as well as in PBL, and both laboratory-adapted strains of HIVand clinical isolates were investigated.

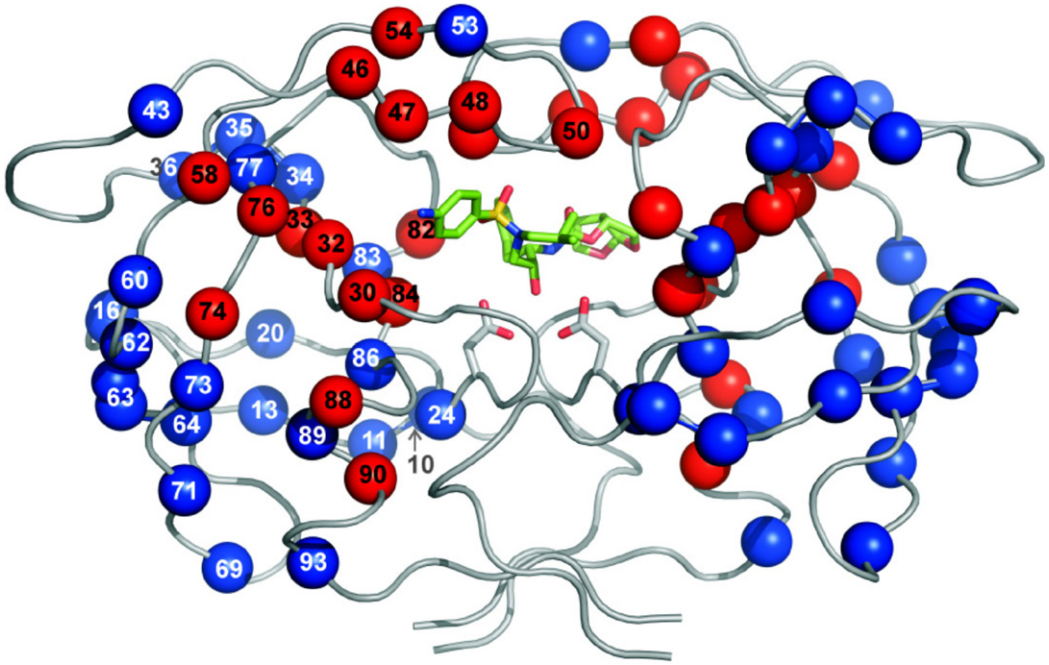

Fig. 2. Three-dimensional structure of HIV PR dimer depicting the primary (major) and secondary (minor) mutations associated with resistance to protease inhibitors (Johnson et al., 2008). Mutated residues are represented with their Cα atoms (spheres) and colored red and blue for major and minor mutations, respectively. Active site aspartates and darunavir bound to the active site are represented in sticks. The figure was generated using the structure of highly mutated patient derived HIV PR (Saskova et al., 2009) [PDB code 3GGU, doi:10.1128/JVI.00451-09] and program PyMol [DeLano Scientific LLC, San Carlos, CA, USA.; http://www.pymol.org].

Table 1
General characteristics and current indications of licensend antiretroviral drugs.

Protease inhibitor	Initial FDA approval	Boosting	Dose (mg) PI/ritonavir	Optimal formulation	Daily pill burden	Current indication
Saquinavir HGC	1995	Yes	1000/100 BID	Tabl 500 mg	2 × 3	Therapy naïve and experienced
Ritonavir	1996	–	–	Caps 100 mg	–	Pharmacokinetic enhancement
Indinavir	1996	No	800 TID	Caps 400 mg	3 × 2	Therapy experienced
		Yes	400/100 BID	Caps 400 mg	2 × 3	Therapy experienced
Nelfinavir	1997	No	1250 BID	Tabl 250 mg	2 × 5	Pregnancy only
Fosamprenavir[a]	1999	No	1400 BID	Tabl 700 mg	2 × 2	Therapy naïve
		Yes	1400/100 BID	Tabl 700 mg	2 × 3	Therapy naïve and experienced
		Yes	1400/100 QD	Tabl 700 mg	1 × 3	Therapy naïve
Lopinavir–ritonavir[b]	2000	Yes co-formulated	400/100 BID	Tabl 200/50 mg	2 × 2	Therapy naïve and experienced
		Yes co-formulated	800/200 QD	Tabl 200/50 mg	1 × 4	Therapy naïve
Atazanavir[c]	2003	No	400 QD	Caps 200 mg	1 × 2	Therapy naïve
		Yes	300/100 QD	Caps 300 mg	1 × 2	Therapy naïve and experienced
Tipranavir	2005	Yes	500/200 BID	Caps 250 mg	2 × 4	Therapy experienced
Darunavir	2006	Yes	600/100 BID	Tabl 600 mg	2 × 2	Therapy experienced
		Yes	800/100 QD	Tabl 400 mg	1 × 3	Therapy naïve

Tabl: tablet; Caps: capsule; QD: once daily; BID: twice daily; TID: three times a day.

[a] Amprenavir was approved in 1999. The prodrug Fosamprenavir was approved in 2003. Unboosted or once daily fosamprenavir is not approved by the European Medicines Agency (EMEA).

[b] Once daily lopinavir is not approved by the EMEA.

[c] Unboosted atazanavir is not approved by the EMEA.

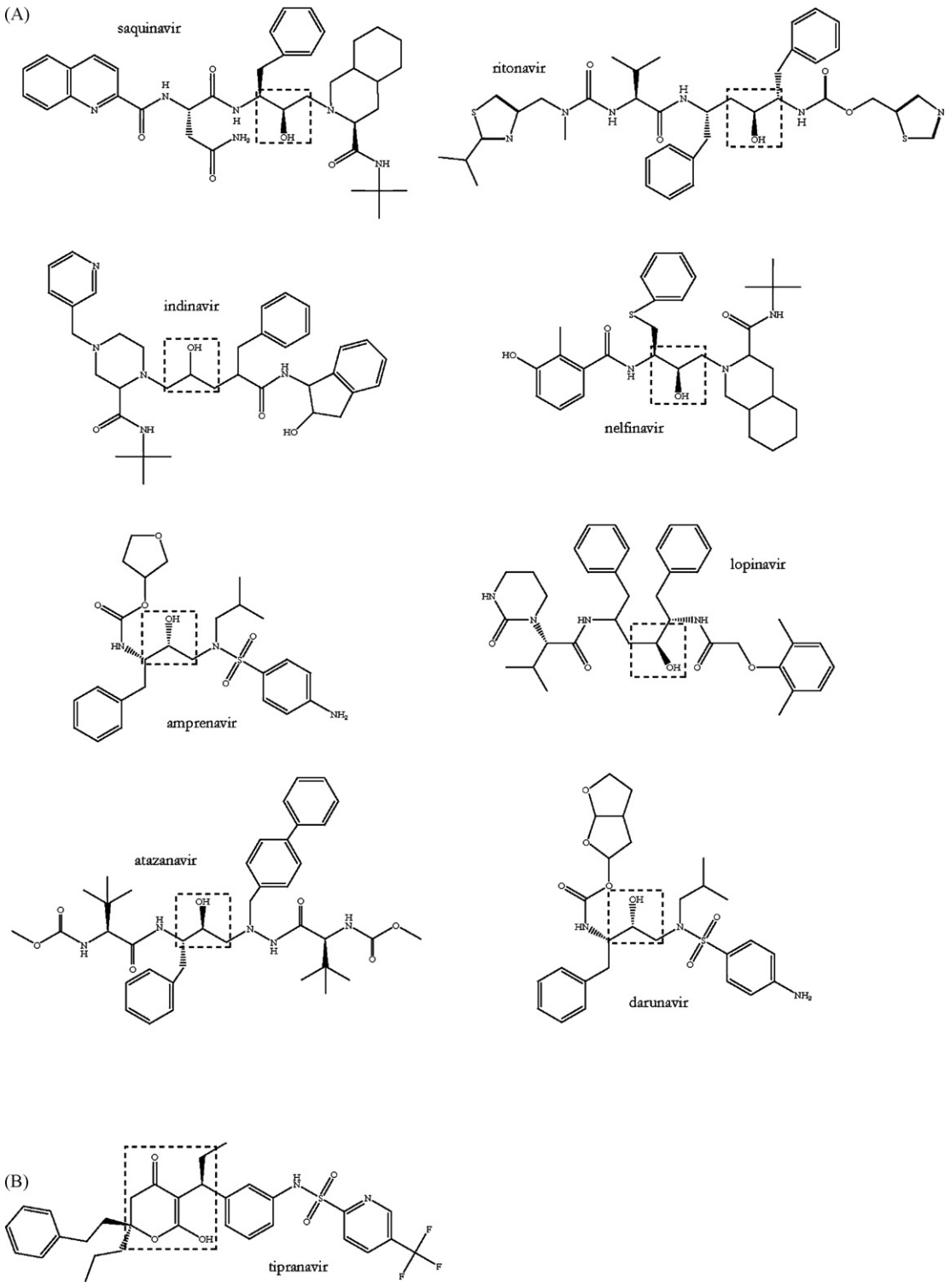

Fig. 3. Chemical structures of the nine HIV-1 protease inhibitors approved for clinical use. (A) Peptidomimetic protease inhibitors, characterized by a hydroxyethylene core, indicated with dashed-line boxes. (B) Non-peptidomimetic protease inhibitor characterized by a dihydropyrone rine, as indicated with a dashed-line box.

Indinavir, when used as monotherapy, also caused extensive declines in HIV-RNA plasma levels and increases in CD4 cell counts. But over time antiviral efficacy diminished and HIV-RNA almost returned to baseline values at 24 weeks (Stein et al., 1996). Multiple drug-resistance related mutations in the protease coding region were commonly detected during suboptimal monotherapy. Mutations at positions 46, 82 and 84 appeared to be most strongly correlated with decreased susceptibility to indinavir (Fig. 2). The observed mutational patterns also frequently induced cross-resistance to other PIs (Condra et al., 1996; Drusano et al., 1998).

A landmark breakthrough in the clinical management of HIV was observed when indinavir was combined with zidovudine and lamivudine in patients with prior zidovudine exposure. This triple combination not only dramatically reduced HIV-RNA plasma levels, to below the limit of quantification in the majority of patients (Gulick et al., 1997; Hammer et al., 1997), but also significantly slowed the progression of HIV disease and mortality compared

with the control arms of zidovudine and lamivudine or indinavir monotherapy (ACTG-320 trial) (Hammer et al., 1997).

Detectable plasma HIV-RNA levels appeared to be strongly correlated with a high risk for development of resistance (Drusano et al., 1998). At time of cross-over to open-label triple combination therapy, 64% of patients in the indinavir monotherapy arm had selected for virus with indinavir resistance mutations (Gulick et al., 1998). Prolonged suppression of HIV-RNA (<500 copies/mL) over 2 years was reached in only 30–45% of the patients in the cross-over group compared to 78% of patients who had received triple combination throughout (Gulick et al., 1998). As a result triple drug PI-based combination therapy became known as HAART and was implemented as standard of care for treatment of HIV-infection in resource rich areas all over the world.

On the back of these exciting results, indinavir swiftly became the most frequently prescribed PI, but long-term success was hampered due to strict intake recommendations and renal toxicity. The drug had to be dosed three times daily preferably on an empty stomach and a high intake of fluid was needed for prevention of indinavir-associated crystalluria and nephrolithiasis. Other adverse events included gastrointestinal symptoms, headache and fatigue (Plosker and Noble, 1999).

3.4. Nelfinavir

The antiviral efficacy of nelfinavir the fourth protease inhibitor that was registered by the FDA (Fig. 3, Table 1) was examined against a variety of laboratory and clinical strains in cell lines as well as macrophages and PBMCs (Patick et al., 1996). The IC_{50} ranged from 10 to 60 nM depending on the viral stain or cell types investigated. In vivo, only short-term monotherapy dose finding studies were performed with nelfinavir. A solid >2 log HIV-RNA decline was observed at day 28 using the initially registered three times daily dosing formula (Markowitz et al., 1998). In patients without previous exposure to antiretroviral therapy, nelfinavir-based triple combination therapy was found to be superior to a control consisting of dual NRTI regimens plus placebo in the multicenter AVANTI trial. At 28 weeks HIV-RNA was suppressed to below 400 copies/mL in 72% of patients in the nelfinavir arm compared with only 17% in the control arm (Gartland, 2001). In the Agouron trial similar suppression of HIV-RNA levels and durable CD4 cell count increases were observed after 48 weeks of nelfinavir-based HAART (Saag et al., 2001). Subsequently, equal HIV-RNA suppression was observed with a twice daily dosing regimen which was formally approved by the FDA and became standard of care in 1999 (Marzolini et al., 2001) (Table 1).

The most common adverse event associated with nelfinavir treatment was diarrhoea. Resistance was observed in viral isolates from patients experiencing incomplete or waning viral suppression during treatment. The nelfinavir-specific resistance mutation 30N, which does not lead to cross-resistance, was often found initially, followed by changes at position 88, which also affects susceptibility to future PIs (Fig. 2). Nelfinavir resistance may also occur as a result of the selection of other mutational pathways that result in more extensive PI class resistance (Pellegrin et al., 2002; Martinez-Picado et al., 1999; Atkinson et al., 2000). The latter was especially observed in patients who had been pre-treated with other PIs before experiencing failure on a nelfinavir containing regimen.

One of the major limitations of the first generation protease inhibitors was the low bioavailability of the drugs resulting in high pill burden and short half-life often necessitating multiple daily dosing. These factors reduced adherence and interfered with optimal viral inhibition. Despite the striking impact of protease inhibitor based HAART on plasma HIV-RNA, CD4 cell counts and clinical outcome, viral replication was not durably suppressed in a considerable number of patients (Bartlett et al., 2001). When therapy failure occurred, often multiple protease resistance mutations were detected, resulting in broad class resistance.

Two approaches were developed to prevent selection of PI resistance. The first approach was to increase the level of PI in the plasma of the patient by combining them with low-dose ritonavir, a cytochrome P450 3A4 enzyme inhibitor (Kempf et al., 1997). As a consequence of this, drug levels are higher and the virus needs to acquire more mutations in the protease sequence to achieve clinical resistance. The second approach is to develop novel HIV PIs, such as darunavir, with high potency against known PI-resistant HIV variants. Both approaches require a considerable increase in the number of protease mutations necessary to achieve clinical resistance, thereby increasing the genetic barrier.

4. Second-generation protease inhibitor therapy; boosting of protease inhibitors

As indicated, the next major advance in the use of protease inhibitors came when it was recognized that ritonavir reduces the metabolism of concomitantly administered PIs through hepatic and intestinal cytochrome P450 3A4 inhibition, leading to dramatically improved bioavailability and half-life of these PIs. The first combination used in clinical trials was saquinavir and ritonavir (400 mg each bid).

This combination with both PIs in therapeutic doses, delivered a powerful high antiviral efficacy punch leading to suppression of HIV-RNA below 200 copies/mL in 80% of treated individuals (Cameron et al., 1999). Although gastrointestinal symptoms improved, tolerability of a 400 mg dose of ritonavir remained, however, a concern. The approach really came to fruition when it was found that lower, non-therapeutic doses of ritonavir (100 mg) were sufficient to enhance the pharmacokinetics of co-administrated PIs allowing twice daily dosing schedules with greatly reduced toxicity (van Heeswijk et al., 2001).

Boosting of the former HGC formulation of saquinavir achieved similar or greater systemic exposure of saquinavir compared with boosted regimens using saquinavir SGC, resulting in reintroduction of the HGC formulation (Kurowski et al., 2003; Cardiello et al., 2003).

Unfortunately, boosting of indinavir induced high plasma peak (Cmax) levels leading to enhanced nephrotoxicity (Boyd et al., 2006; Voigt et al., 2002). As a consequence boosted indinavir was not implemented broadly. Boosting did not greatly enhance the bioavailability of nelfinavir. This would eventually limit the efficacy of nelfinavir compared to newly approved PIs, leaving the drug a limited role as a relatively safe PI for use in pregnancy.

4.1. Amprenavir/fosamprenavir

The next protease inhibitor to make its debut was amprenavir, the first protease inhibitor approved for twice daily dosing, in 1999 (Fig. 3, Table 1). Its antiviral efficacy was in vitro evaluated using laboratory strains and clinical isolates and appeared to be between 10 and 80 nM IC_{50} values (Arvieux and Tribut, 2005; St Clair et al., 1996). The combination of amprenavir with two NRTIs lamivudine and zidovudine was compared to the two NRTIs plus placebo in patients without prior use of PI or lamivudine. By 48 weeks, 89% of subjects in the amprenavir arm had plasma HIV-RNA <400 copies/mL compared to 60% of the comparator group (with amprenavir addition after week 12) in an as treated analysis. However, in the intention to treat analysis less than 30% of the individuals taking the amprenavir regimen had suppressed HIV-RNA (Haubrich et al., 1999). It appeared that a high percentage of patients prematurely discontinued amprenavir, probably because of difficulties swallowing eight very large capsules twice daily.

Furthermore the lowest drug (Cmin) concentrations in plasma achieved with the registered dose were very close to the EC_{90}. As a result the risk of suboptimal concentrations was high often resulting in weak activity of the PI and frequent selection of resistance (Sadler et al., 2001; Arvieux and Tribut, 2005). Resistance in the protease coding region appeared to be somewhat different from the earlier registered PIs and followed different pathways involving key mutations at positions I50V, I54L/M, V32I + I47V and less commonly I84V (Fig. 2) (Paulsen et al., 2003).

Introduction of the produg fosamprenavir led to improved plasma concentrations and lower pillburden and when combined with abacavir and lamivudine to higher efficacy compared to nelfinavir-based HAART in antiretroviral naïve individuals in the NEAT trial (Rodriguez-French et al., 2004). Subsequent boosting with ritonavir further improved the efficacy of fosamprenavir when combined with the nucleoside RTIs (NRTIs) abacavir and lamivudine resulting in suppression of HIV-RNA (<400 copies/mL) in 73% of antiretroviral naïve individuals at 48 weeks in the KLEAN-trial (Eron et al., 2006). Diarrhoea and elevation of fasting cholesterol and triglyceride levels were the most frequent observed adverse events with boosted fosamprenavir (Eron et al., 2006). There are only limited data available on the efficacy of boosted amprenavir in PI-experienced individuals, but in the CONTEXT trial viral suppression (<50 copies/mL) in about 50% of individuals was observed (Arvieux and Tribut, 2005; Quercia et al., 2005).

Just recently, the FDA approved once daily use of a high dose of boosted fosamprenavir (1400/100 mg) for combination therapy in therapy naïve individuals. The once daily regimen demonstrated high efficacy when combined with the NRTIs tenofovir and emtricitabine in a randomised trial although there was a non-significant trend to less successful HIV-RNA suppression in the intent-to-treat (ITT) analysis compared to the control, possibly related to more study discontinuations (Smith et al., 2008).

4.2. Lopinavir

Lopinavir was the first and thus far only PI co-formulated with a low dose ritonavir as Kaletra. The drug received approval in 2000 as a twice daily regimen of three capsules. Subsequently, the capsules were replaced by pills that were better tolerated and did not require dietary restrictions or refrigeration (Schrader et al., 2008) (Table 1). The in vitro activity of lopinavir was evaluated in a T cell line (MT4 cells) and showed a 6–17 nM IC_{50} range against various clinical and laboratory strains (Sham et al., 1998). The in vivo efficacy of lopinavir/ritonavir as initial therapy was compared in a randomised placebo controlled study with nelfinavir (three times a day) as comparator with an NRTI backbone of stavudine and lamivudine in both arms. At 48 weeks, 75% of individuals on the lopinavir-based regimen had suppressed plasma HIV-RNA (<400 copies/mL) compared to only 63% in the control arm while similar CD4 cell count increases of 200 cells/mm^3 was observed in both arms (Walmsley et al., 2002). Remarkably, no genotypic or phenotypic resistance to lopinavir was observed over 96 weeks in the small group of patients which experienced virological failure (Kempf et al., 2004). Although these results fuelled prescription of lopinavir, selection of resistance in first-line lopinavir-based HAART has remained extremely rare. Even today only isolated cases of the selection of major lopinavir resistance mutations have been reported involving the following mutations in protease 32I, 47A and 46I or L33F, I54V, and V82A or combinations among L76V, M46I, V82A in protease and A431V in gag (Friend et al., 2004; Conradie et al., 2004; Nijhuis et al., 2009).

In 2005, FDA approved the use of once daily lopinavir after reports of comparable efficacy with twice daily lopinavir-based HAART. Registration was limited, however, to antiretroviral naïve individuals because of lower Ctrough concentrations, which are the plasma drug levels just before a new dose is administered (Johnson et al., 2006b). Once daily dosing also resulted in a much higher incidence of diarrhoea, but this problem was curtailed through the use of lopinavir tablets (Gathe et al., 2009).

Lopinavir was the first boosted PI compared head-to-head with an NNRTI as initial therapy. In ACTG study 5142 48% of lopinavir–ritonavir recipients vs 61% of efavirenz recipients (both combined wit zidovudine/lamivudine) maintained plasma HIV-1 RNA at <50 copies/mL through week 96. Although efavirenz achieved superior viral suppression, more efavirenz failures than lopinavir failures resulted in double-class resistance. In addition, a significantly better CD4 response was obtained with lopinavir/ritonavir compared with efavirenz (median 285 cells versus 241 cells) (Riddler et al., 2008). Comparable efficacy of a third NRTI-sparing arm of lopinavir–ritonavir plus efavirenz (compared with efavirenz-NRTI) indicated that NRTIs are not absolutely essential for the treatment of HIV-1. More extensive elevation of triglyceride levels and a trend towards more NNRTI resistance upon therapy failure were observed in the NRTI-sparing arm (Riddler et al., 2008). More extensive selection of resistance was also recently reported with the use of NRTI-sparing regimens of either lopinavir or boosted indinavir with an NNRTI in the ANRS-121 trial (Soulié et al., 2009). The results from these two important trials clearly limit the role for future initiatives using NNRTI/PI combinations as NRTI-sparing regimens.

Investigations in previously PI-exposed patients experiencing failure on lopinavir-based HAART demonstrated that the high genetic barrier of lopinavir can be compromised by accumulation of mutations during prior PI-based regimens (Mo et al., 2005). Acquisition of mutations at codons 82, 54 and 46 and less commonly L33F, I50V and V32I + I47V/A were observed (Fig. 2).

In PI-experienced patients lopinavir-ritonavir-based combination therapy showed superior viralogical efficacy compared to several other investigator selected PI-based regimens or comparator arms in randomised clinical trials including non-boosted PIs, boosted indinavir or boosted saquinavir SGC, as nicely reviewed by Oldfield and Plosker (2006).

4.3. Atazanavir

The approval of atazanavir in 2003, the first PI that was immediately for once daily dosing, further simplified boosted PI-based combination therapy (Fig. 3, Table 1). The antiviral activity of atazanavir was evaluated using a variety of HIV-1 strains (clinical isolate and laboratory strains) and several host cell types (T cells lines and PBL). Atazanavir exhibited potent antiviral activity, with IC_{50}s between 2 and 5 nM (Robinson et al., 2000; Croom et al., 2009). Atazanavir raises plasma bilirubin levels in almost all treated individuals by inhibiting UDP glucuronyl transferase. The bilirubin elevation which is not seen during use of other PIs, except to some extent indinavir, does generally not result in clinically relevant symptoms. When used unboosted the drug has a favourable non-toxic profile with only limited effect on fasting cholesterol and triglyceride levels. Unboosted once daily atazanavir in the registered 400 mg dose showed equal immunogical (>200 cells increase in CD4 cell count) and superior virological efficacy when compared with nelfinavir twice daily when with the NRTIs stavudine and lamivudine in antiretroviral naïve patients (64% vs 53% HIV-RNA suppression <400 copies/mL) (Murphy et al., 2003). Disappointing virological results were obtained in a head-to-head comparison of efavirenz and atazanavir both combined with lamivudine and zidovudine in antiretroviral naïve patients. Although plasma HIV-RNA was suppressed below <400 copies/mL in 70% and 64% of the atazanavir and efavirenz arms, respectively, much lower rates of suppression (32% and 37%) were observed in both arms using the more stringent <50 copies/mL criterion (Squires et al., 2004).

These unexpected results were not in line with earlier reports of the efficacy of efavirenz. Soon after the trial it was reported that non-standard collection tubes might have influenced the results (Giordano et al., 2006).

Far more promising results were obtained in the Castle study in which once daily boosted atazanavir was demonstrated as being not inferior to twice daily lopinavir–ritonavir for the treatment of antiretroviral naïve patients in the background of the fixed-dose NRTI combination tenofovir/emtricitabine. In both arms high levels of suppression of plasma HIV-RNA were observed (78% vs 76% <50 copies/mL) together with a satisfying increase in CD4 cell counts of more than 200 cells/mm^3 (Molina et al., 2008).

Subsequently, it was recognised that a high genetic barrier to resistance was not limited to lopinavir–ritonavir but could be reached through boosting other PIs. Comparable efficacy and only rarely selection of resistance were demonstrated in several trials comparing lopinavir–ritonavir with either twice daily boosted fosamprenavir or twice daily saquinavir in antiretroviral naïve patients (Eron et al., 2006; Johnson et al., 2006a; Molina et al., 2008). Moreover, the use of boosted saquinavir or atazanavir also resulted in better lipid profiles and less diarrhoea compared with lopinavir–ritonavir (Johnson et al., 2006a; Walmsley et al., 2009; Molina et al., 2008).

In PI-experienced individuals, unboosted atazanavir proved to be virologically and immunologically inferior to lopinavir–ritonavir and, as a consequence, was not approved for use in this patient group (Cohen et al., 2005). However, when atazanavir was administered as a boosted PI, it was found to be not inferior to twice daily lopinavir–ritonavir in combination with two NRTIs in this patient group. Suppression of plasma HIV-RNA below 50 copies/mL was achieved in more than 50% of the patients in both treatment arms. The third comparator arm combined atazanavir with saquinavir plus two NRTIs and demonstrated less spectacular virological efficacy (Johnson et al., 2005).

The atazanavir resistance profiles detected during trials have been shown to be distinct from those of other PIs. Treatment-naïve patients experiencing therapy failure during unboosted atazanavir-based regimens developed a characteristic I50L mutation in protease which reduced susceptibility to atazanavir, but increases susceptibility to other PIs (Fig. 2) (Colonno et al., 2004). In contrast, in treatment-experienced patients and patients on boosted therapy other mutations conferring PI cross-resistance are generally selected including I84V, L90M, A71V/T, N88S/D, and M46I (Fig. 2) (Pellegrin et al., 2006).

4.4. Tipranavir

Tipranavir, was approved by the FDA in 2005 for the treatment of highly experienced patients with resistance to multiple PIs (Table 1). Tipranavir is a non-peptidomimetic protease inhibitor and as such less potent than the peptidomimetic PI as described above. Instead of a peptidomimetic hydroxyethylene core, tipranavir contains a dihydropyrone ring as a central scaffold (Fig. 3) (Turner et al., 1998). Tipranavir suppresses the in vitro replication of clinical isolates and laboratory strains in T cell lines and PBL with an average IC_{90} of 160 nM (Poppe et al., 1997; Croom and Keam, 2005). Tipranavir is a potent inducer of its own metabolism through induction of the cytochrome P450 expression, which results in a need for double dose ritonavir boosting. The registered dose of 500 mg twice daily needs therefore boosting with 200 mg of ritonavir, double the dose administered with other PIs.

Tipranavir showed superior efficacy in salvage relative to the comparator protease inhibitor (CPI), both with optimised background in heavily pretreated patients with extensive PI resistance in the RESIST trials. At 48 weeks, significantly more patients achieved and maintained treatment response in the tipranavir–ritonavir group than in the CPI-ritonavir group (plasma HIV-RNA <400 copies/mL: 33.6% vs 15.3% and <50 copies/mL: 22.8% vs 10.2%). However, tipranavir appeared not to be superior to lopinavir in lopinavir-naïve individuals. Notably, patients who were naïve for entry inhibitors and receceived enfuvirtide in their optimised background, had a significantly greater treatment response with tipranavir than with comparator PIs, underlining the efficacy of tipranavir. However, gastrointestinal system disorders, liver inflammation and raised transaminase levels, cholesterol, and triglycerides were more frequent in the tipranavir–ritonavir group than in the CPI-ritonavir group (Hicks et al., 2006; Cahn et al., 2006; Gathe et al., 2006).

In vitro TPV had selected for the following major PI-resistance mutations: V32I, I54V, V82L and I84V (Fig. 2) (Doyon et al., 2005). In vivo amino acid changes at positions V82A/T and I84 have been observed most frequently after failure of tipranavir-based regimens (Naeger and Struble, 2007). Multiple attempts have been made to optimise the prediction of tipranavir viral efficacy using lists of mutations in the protease coding region. Based on the results in the RESIST study a weighted genotypic susceptibility list has recently been reported including mutations T74P, I47V, V82L/T, Q58E, N83D as the strongested predictors of reduced efficacy and I54A/M/V, I84V, M36I, K43T, L10V and M46L as weaker predictors, whereas L24I, I50L/V, I54L and L76V were predictors of virological response (Fig. 2) (Scherer et al., 2009). Despite its clear virological benefits, tipranavir's narrow indication, lack of other reasonably tolerable agents to use in combination and significant risk of hepatotoxicty meant that the drug was not widely used.

Despite the availability of eight (boosted) protease inhibitors there was still a substantial need for new antiviral options in the clinical management of patients harbouring multi-drug-resistant HIV strains that would offer increased efficacy combined with a favourable toxicity profile.

4.5. Darunavir

Darunavir was approved as a ninth protease inhibitor in 2006 (Fig. 3, Table 1). In vitro studies against wild-type HIV-1 in acutely infected MT-2 and MT-4 cells, peripheral blood mononuclear cells and monocytes or macrophages, have shown that darunavir is a very potent PI with an IC_{50} ranging from 1 to 5 nM (De Meyer et al., 2005; Koh et al., 2003; McKeage et al., 2009). Although the drug was especially designed to inhibit drug-resistant strains, its powerful antiviral potency and limited adverse events profile may expand use of the drug into earlier lines of therapy. In the Artemis trial, efficacy in antiretroviral naïve patients was evaluated comparing once daily darunavir/ritonavir with once or twice daily lopinavir–ritonavir with a fixed-dose NRTI backbone of tenofovir/emtricitabine. At week 96, significantly more patients in the darunavir (79%) than the lopinavir arm (71%) had a plasma HIV-RNA less than 50 copies/mL. Median CD4 cell count increases from baseline were circa 180 cells/mm^3. Darunavir had a more favorable gastrointestinal and lipid profile compared to lopinavir (Mills et al., 2009).

Several randomized trials were performed in treatment-experienced patients. The POWER 1 and 2 studies were two dose finding trials comparing the efficacy and safety of boosted darunavir with that of currently approved protease inhibitors combined with an optimized background therapy. 24-Week efficacy and safety data of both trials confirmed the recommended darunavir/ritonavir 600/100 mg twice daily as the ideal option for pretreated individuals (Katlama et al., 2007). In a combined analysis of both trials 45% of patients receiving boosted darunavir-regimens had plasma HIV-RNA of less than 50 compared to 10% with comparator PI-based regimens (Clotet et al., 2007).

In the Titan study twice daily boosted darunavir plus optimized background (OB) was compared to twice daily lopinavir–ritonavir plus OB. At 48 weeks significantly more patients receiving darunavir achieved a plasma HIV-RNA load of <50 copies/mL. CD4 cell count increases were similar in both treatment groups (Madruga et al., 2007). Fewer patients with virologic failure in the darunavir arm than in the lopinavir arm developed primary protease inhibitor mutations or nucleoside reverse transcriptase inhibitor resistance-associated mutations (De Meyer et al., 2008a, 2009).

In vitro selection of darunavir-resistant HIV-1 appears to be slower and less frequent than with other PIs, probably reflecting the particularly strong binding of darunavir to the HIV protease resulting in a higher intrinsic genetic barrier than observed with the other boosted PIs (De Meyer et al., 2005; King et al., 2004). This makes it very difficult for the protease inhibitor naïve virus to escape via the traditional protease-based route, alternative gag substrate based resistance is being selected (De Meyer et al., 2006).

Pooled resistance data from the POWER trials which initiated boosted darunavir 600/100 mg in PI-experienced patients was used to determine a list of resistance mutations associated with a diminished response to darunavir (De Meyer et al., 2008b). Subsequently, the list was updated using data from more trials with therapy-experienced patients, including 11 mutations in the protease coding region: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V and L89V (Fig. 2). The presence of more than three darunavir listed mutations was associated with a median darunavir FC >10 and a diminished virological response (De Meyer et al., 2008a, 2009). Of interest, some of the darunavir mutations are associated with improved response to tipranavir, which might give additional options for future salvage therapy, if needed at all.

5. Double-boosting protease inhibitor based therapy

Preceding the recent approval of several new antiretroviral compounds physicians experienced difficulties building an effective regimen for a group of heavily therapy-experienced patients with extensive drug resistance. In this setting of limited therapeutic options, the use of double-boosted PI was explored to gain possible synergistic or added antiviral activity of both drugs and to increase the genetic barrier to PI resistance. Although no large randomised trials assessing the clinical efficacy of double-boosted PI-regimens were reported, several comparative and cohort studies suggested potential utility of combinations such as lopinavir/ritonavir with either saquinavir or atazanavir, or atazanavir plus low dose ritonavir with saquinavir all in combination with two NRTIs as a backbone (Stebbing et al., 2009; Petersen et al., 2007; von Hentig et al., 2007; Manosuthi et al., 2008; Ribera et al., 2006; Gilliam et al., 2006; Smith et al., 2005).

A high pill burden, increased risk for toxicity and lower efficacy compared to standard of care regimens containing either an NNRTI or a single PI with a dual NRTI backbone did not make the use of double-boosted PIs an appealing option beyond salvage therapy (van der Lugt et al., 2008; Ulbricht et al., 2009). The recent introduction of more successful regimens including novel agents, such as the integrase and CCR5-inhibitors and/or a second-generation PI (darunavir) and NNRTIs with high genetic barrier, have achieved impressive efficacy in heavily pretreated patients discarding the use of double-boosted PIs as salvage therapy in resource rich settings.

6. Ritonavir-boosted protease inhibitor-monotherapy

Although NRTIs had been the cornerstone of highly active antiretroviral therapy, the finding that the originally to PIs attributed lipo-atrophy was mainly induced by these NRTIs, fuelled a search for alternative regimens. Moreover, the profound efficacy of boosted PI-based HAART and the high genetic barrier to resistance questionned the paradigm of a three-drug regimen. Combined with the challence of life long adherence and the risk for selection of multidrug resistance these considerations led to re-evaluation of the concept of monotherapy in several trials.

A systematic review of these trials found an absolute risk difference of 10.3% of failure of boosted PI-monotherapy compared to standard PI-based HAART (Bierman et al., 2009). Low-level viremia and development of resistance was also more frequently observed during PI-monotherapy (Bierman et al., 2009). Of interest, no significant difference in efficacy was observed in individuals starting monotherapy after induction with full suppressive HAART (Bierman et al., 2009). Furthermore, among individuals who successfully controlled HIV after restarting nucleosides, failure rates did not significantly differ between monotherapy groups and standard-regimen groups (Bierman et al., 2009). In the future long-term follow-up of boosted PI-monotherapy strategies need to give further insight in the efficacy and safety of this NRTI-sparing approach.

A potential explanation for the increased risk of failure with monotherapy without induction phase may be insufficient viral suppression by boosted PIs in the central nervous system or other compartments. A lower genetic barrier to resistance for monotherapy than originally foreseen, may provide an alternative explanation for increased failure as recent reports revealed that just two mutations can be sufficient for viral breakthrough during (mono)therapy with lopinavir–ritonavir (Nijhuis et al., 2009; Delaugerre et al., 2009).

However, the reported combination of protease mutations M46I plus L76V is not frequently observed in cases of lopinavir-based therapy failure. This indicates that the genetic barrier to resistance is not simply a calculated sum of the two mutations, but also includes the selective advantage of these particular mutations in the viral quasispecies. More insight in the role of the genetic barrier of resistance may come from currently performed monotherapy trials using the recently approved protease inhibitor darunavir.

7. Evolution of resistance

Resistance to all the protease inhibitors has been observed and the genetic basis of resistance has been well documented. Within a chronically infected patient in the absence of effective treatment, continuous high level replication, lack of proofreading by the viral reverse transcriptase and recombination lead to the generation of massive numbers of genetically distinct viral variants, referred to as a viral quasispecies (Domingo et al., 1996). Within the viral quasispecies, wild-type is defined as the most fit and common individual sequence. However, it is important to realize that the total number of HIV variants is orders of magnitude higher than the number of wild-type viruses present in a patient population. It is therefore predicted that any single genome selected at random from the population is likely to have a mutation relative to wild-type, rendering it less fit.

The entire viral quasispecies is subject to selection and evolution and its genetic flexibility will allow the population to respond to different selection pressures. For example, a mutant that would be expected to be less fit may display an increased fitness under the selection pressure exerted by a protease inhibitor and would be considered as a drug-resistant variant.

Initially, it was assumed that the HIV-1 population size was infinite, evolution deterministic and antiretroviral resistance certain to occur (Coffin, 1995). However, it was demonstrated that the effective population size, defined as the average number of HIV variants that produces infectious progeny is relatively small (Leigh

Brown, 1997; Leigh Brown and Richman, 1997; Nijhuis et al., 1998). This can be explained by the fact that the majority of virus particles that are produced harbor deleterious mutations resulting in non-infectious virus. In addition, limited target cell availability and inactivation of potentially infectious viruses by the host immune system both reduce the effective population size.

These estimations indicate a relatively small effective population size in which solely single or double mutants as compared to wild-type are present. This estimation is supported by the observation that current Highly Active Anti-Retroviral Therapy (HAART) is capable of fully inhibiting viral replication, which would not have been possible if viral variants with more than two mutations would be present in the baseline viral population. The genetic barrier to resistance development is usually defined by the number of resistance-associated mutations necessary to confer virological failure. However, other factors also have to be taken into consideration, such as baseline variability (different subtypes) and the impact of the mutations on resistance development and viral replication capacity.

8. Mechanisms of HIV protease resistance

The development of protease inhibitor resistance is a stepwise process in which a substitution in the substrate-binding cleft of the viral protease is usually observed first. These resistance mutations in the viral protease result in an overall enlargement of the catalytic site of the enzyme. This leads to decreased binding to the inhibitor (causing a decrease in drug sensitivity) and, in parallel, to some decrease in binding to the natural substrate and thus to decreased viral replication (Croteau et al., 1997; Mammano et al., 2000; Nijhuis et al., 1999; Quinones-Mateu and Arts, 2001). These mutations, which are selected first and individually reduce the susceptibility to a protease inhibitor, are called primary or "major" resistance mutations (Fig. 2) (Johnson et al., 2008). Secondary or "minor" mutations generally emerge later and by themselves do not have a substantial effect on the resistance phenotype but improve replication of viruses containing major mutations (Fig. 2) (Mammano et al., 1998; Mammano et al., 2000; Nijhuis et al., 1999; Quinones-Mateu and Arts, 2001). Some minor mutations are present as common polymorphic changes in HIV-1 nonsubtype-B clades. The International AIDS Society-USA group reviews data on HIV-1 drug resistance that have been published or presented at recent scientific meetings to maintain a current list of mutations associated with PI resistance. At the moment major PI-resistance mutations at 15 protease codons and minor mutations at an additional 19 codons have been included in the update (Johnson et al., 2008).

Occasionally, amino acid insertions are selected during PI-based antiretroviral therapy. Insertions ranging from 1 to 6 amino acids have been detected at various sites in the viral PR sequence, e.g., in regions between codons 17 and 18, 22 and 25, 31 and 32, 35 and 38, 40 and 41, 70 and 71, and 95 and 96 (Kim et al., 2001; Winters and Merigan, 2005; Kozísek et al., 2008; Jordan et al., 2009). It is shown that protease insertions, particularly those between residues 32 and 42, have become more prevalent since 1999. Presence of these insertions is positively correlated with protease mutations associated with resistance to PIs whose usage has increased in recent years, including atazanavir, lopinavir, amprenavir and tipranavir (Kozísek et al., 2008). While the insertions lead to a decrease in PI susceptibility and modestly improve viral replication (Kim et al., 2001; Kozísek et al., 2008), they seem to contribute to PI resistance only in combination with other mutations either in the PR or in Gag (Kim et al., 2001).

Protease inhibitor associated mutatations have not only been observed in the viral protease itself, but also in the substrate of the viral protease, the Gag protein. They are commonly found in, or closely to, the protease cleavage sites and are thought to adapt the virus to the altered substrate-binding cleft of the mutant drug-resistant viral protease (Doyon et al., 1996; Maguire et al., 2002; Mammano et al., 1998; Zhang et al., 1997). In addition to traditional HIV protease-based resistance, alternative mechanisms of protease resistance have been postulated.

8.1. Gag substrate based protease inhibitor resistance

Several studies have identified an association between the selection of protease mutations and the appearance of mutations in the substrate of the viral protease, the gag protein (Doyon et al., 1996; Maguire et al., 2002; Mammano et al., 1998; Zhang et al., 1997). Changes at the p1/p6 cleavage site (L449F or P453L), which individually do not confer protease inhibitor resistance, were associated with reduced drug sensitivity in the background of the primary I50V in protease, proving that mutations in the viral protease and Gag protein can interact to increase PI resistance (Maguire et al., 2002; Prado et al., 2002). A few years ago, it was demonstrated that substitutions in the Gag NC/p1 cleavage site alone (A431V, K436E and/or I437V/T), without any alterations in the viral PR sequence were selected during in vitro protease inhibitor exposure (Nijhuis et al., 2007). When these changes were introduced into a reference strain they caused PI resistance, which could directly be related to increased gag processing. A detailed analysis of clinical isolates of patients on protease inhibitor therapy also indicated that mutations in the NC/p1 cleavage site strongly contribute to protease inhibitor resistance besides compensating for a loss in replication capacity (Dam et al., 2009). Taken together, this clearly demonstrates that selection of NC/p1 cleavage site substitutions allows the virus to overcome a PI induced reduction in protease activity and can also represent an alternative protease resistance pathway.

In vitro selection experiments with several protease inhibitors have shown to result not only in the selection of CS mutations in Gag but also in the selection of non-CS mutations in Gag (Callebaut et al., 2007; De Meyer et al., 2006; Gatanaga et al., 2002; Nijhuis et al., 2006). Although these non-CS mutations in Gag were often acompanied by resistance mutations in protease or in protease cleavage sites, they did contribute to protease inhibitor resistance. It is currently not known through which mechanism these non-CS mutations in Gag contribute to protease inhibitor resistance.

9. The influence of genetic diversity on protease inhibitor efficacy and selection of resistance

The vast majority of data regarding efficacy and selection of drug resistance to boosted PI have been generated for subtype B infections. Among different subtypes the variation in nucleotide sequence in the protease coding *pol* gene is approximately 10–15%, leading to distinct polymorphisms at amino acid level. These genetic differences have been reported to influence baseline susceptibility of PIs, the genetic barrier for selection of PI drug resistance and mutational pathways, but overall high efficacy of protease inhibitors has been observed in all HIV-1 subtypes (Frater et al., 2002).

In vitro decreased susceptibility to PIs was reported in a limited number of CRF02_AG isolates from therapy naïve individuals (Fleury et al., 2006; Kinomoto et al., 2005). However, conclusions have to be drawn carefully since deletion clones and phenotypic resistance cutt-offs applied are based on subtype B virus backbones. Other reports on small numbers of non-B HIV-1 isolates with decreased baseline susceptibility or hypersuscepbility for PIs in vitro have been published, but the majority of non-B subtype

isolates demonstrated similar suceptibility to that of subtype B isolates (Martinez-Cajas et al., 2008).

Wild-type sequences at several resistance related codons differ between non-B subtypes and subtype B (van de Vijver et al., 2006). In general these differences do not influence baseline susceptibility, but they may influence the genetic barrier or specific pathways to resistance. Examples are the minor mutations 10V and 36I, which are generally present in non-B viruses and which are included in the tipranavir resistance score (van de Vijver et al., 2006; Scherer et al., 2009). Diversity in nucleotide sequence may also lead to differential selection of PI-resistant variants on position 82 (van de Vijver et al., 2006; Abecasis et al., 2006).

In addition, the frequency of selection of specific mutational patterns may differ among subtypes. For instance, in subtype C and other non-B subtypes selection of resistance to nelfinavir preferably occurs via PI cross-resistance pathways including mutation L90M, and to a lesser extent via the, in subtype B most frequently observed, nelfinavir-specific pathway with mutation D30N (Cane et al., 2001; Grossman et al., 2004).

Finally, entirely alternative resistance pathways may occur among non-B subtypes. This is most comprehensively described for a PI-resistance pathway that includes positions 89 and 90 in non-B subtypes. In subtype C, F and G, M89 is the wild-type consensus amino acid, compared to L89 in subtype B. Acquisition of the mutations M89I and L90M results in decreased susceptibility to nelfinavir in these subtypes (Abecasis et al., 2005). Additional presence of mutation 71T or 74S, has been correlated with high levels of resistance to nelfinavir in subtype G viruses (Gonzalez et al., 2008).

10. The use of boosted PIs in resource-limited settings

HAART became available in resource-limited settings after adoption of the Doha-declaration, enabling developing countries to circumvent patent rights for better access to essential medicines. Two years later the World Health Organization (WHO) launched their "3by5" initiative aiming at provision of antiretroviral therapy to three million people by the end of 2005. Even though this goal was not met, the initiative led to an unprecedented increase in therapy roll out and 2 years later, by the end of 2007, nearly three million people were on HAART worldwide. An NNRTI-based regimen of nevirapine, stavudine and lamivudine is widely used as first-line therapy, and is recommended as such by the WHO for resource-limited settings. For second line therapy the WHO advises initiation of a PI-based regimen with lopinavir–ritonavir plus two NRTIs. This regimen has the important advantage above other PI-boosted regimens that no refrigeration of ritonavir capsules is required as ritonavir is co-formulated with lopinavir tablets. Furthermore a PI-based regimen is thought to demonstrate still viral efficacy even though the NRTI backbone may be compromised by first-line therapy. At present there are only limited data available regarding the efficacy of second line liponavir–ritonavir-based regimens in resource-limited settings. Several anecdotical reports exist of successful use of double-boosted PIs as an alternative for patients with second line therapy failure in these settings, where newer drugs are not currently available (personal communication: P. Schrooders, Ndlovu Medical Center, South Africa).

11. Novel boosting and PI strategies

Boosted PI containing regimens have proven to be a solid cornerstone of HIV-therapy. Still there are several concerns regarding use of PIs that require compelling attention, with respect to toxicity, and cross-resistance.

Recent in vitro experiments have demonstrated that several PIs show decreased insulin-mediated glucose disposal through an acute blockade of glucose transporter-4 (GLUT4) (Hruz, 2008). In addition, new onset diabetes mellitus, exacerbation of pre-existing diabetes mellitus and increased risk for cardiovascular events have been reported during post-marketing surveillance and cohort studies in many HIV-infected patients receiving protease inhibitor therapy (Bradbury and Samaras, 2008). Other adverse events, such as increased nephrotoxicity, hyperbilirubinemia and elevation of liver enzymes may depend more on the enhanced bioavailability of specific boosted PIs (Boyd et al., 2005; Rodriguez-Novoa et al., 2008; Gathe et al., 2007). Also ritonavir, although as boosting agent used in a much lower than therapeutic dose, has been strongly associated with toxicity such as lipid abnormalities and gastrointestinal side effects (Sension et al., 2009).

The development of new alternative boosting compounds posessing reduced metabolic toxicity offer important promises. One of these compounds is GS-9350, a non-PI potent cytochrome 3a inhibitor that lacks anti-HIV activity at concentations up to 90 μM. GS-9350 has reduced effects on adipocyte function in vitro compared to ritonavir, which may lead to reduced metabolic events, such as elevated lipid levels and insulin resistance (Ramnathan et al., 2009). In a phase 1 study, GS-9350 enhanced the pharmacokinetic profile of atazanavir equally to that obtained with co-administration of 100 mg ritonavir once daily. Moreover the physicochemical properties of GS-9350 will allow tablet co-formulations with other agents (Mathias et al., 2009). Another interesting compound in clinical development is SPI-452. SPI-452 is also a potent cytochrome 3a inhibitor with no activity against HIV. In a proof-of-concept trial involving 67 healthy HIV-negative individuals SPI-425 significantly increased minimum plasma darunavir and atzanavir levels. Although mild headache, nausea, diarrhoea were reported as side effects there were no significant changes in lipid profiles or liver-function tests in this short duration trial (Gulnik et al., 2009).

Even with the availability of seven boosted PIs as well as new drugs in other classes a small subset of heavily exposed individuals face limited therapeutic options due to extensive cross-resistance. Fortunately, new drugs in the development pipeline show interesting potential. One such compound is PPL-100/MK-8122, which is a novel PI that incorporates a lysine-based scaffold and binds the protease flap region. In vitro selection studies resulted in generation of novel active site mutations, T80I and P81S, that do not cause cross-resistance with other protease inhibitors, along with two well-known mutations that generally have a limited effect on PI susceptibility: K45R and M46I (Dandache et al., 2007). Other inhibitors such as GS-8374 and the Sequoia compounds bind to a similar target as most of the current available PI, the active site of protease. Still they seem to have excellent excellent potency against HIV strains with multiple drug PI-related mutations which may, at least in part, be explained by their high potency against wild-type enzyme (Gulnik and Eissenstat, 2008).

Future alternative drug development strategies may also include the design of protease inhibitors that closely fit within the substrate-binding region. Cocrystal structures of HIV-1 protease with its substrates have defined an overlapping substrate-binding region or substrate envelope. Novel HIV-1 protease inhibitors that have been designed to fit within this substrate envelope were found to retain high binding affinity and have a flat binding profile against a panel of drug-resistant HIV-1 proteases (Nalam and Schiffer, 2008).

In conclusion, recent progress in drug development has the potential to result in novel compounds with reduced toxicity and enhanced susceptibility to current resistant HIV variants.

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