

Resistance of hepatitis C virus to NS3-4A protease inhibitors: mechanisms of drug resistance induced by R155Q, A156T, D168A and D168V mutations

Jérôme Courcambeck^{1*}, Mourad Bouzidi¹, Régis Perbost¹, Besma Jouirou¹, Nolwenn Amrani¹, Patrice Cacoub², Gérard Pèpe³, Jean-Marc Sabatier⁴ and Philippe Halfon¹

¹Genoscience, Marseille, France

²Department of Internal Medicine Hopital La Pitié-Salpêtrière, Paris, France

³Laboratoire GCOM2, CNRS-UMR 6114, Faculté des Sciences de Luminy – Marseille, France

⁴Laboratoire de Biochimie – Ingénierie des protéines, IFR Jean Roche, Faculté de Médecine Nord, Marseille, France

*Corresponding author: Tel: +33 491 82 94 06; Fax: +33 491 82 94 07; E-mail: courcambeck@3dgenoscience.com

Part of this work was presented at the Annual Congress of the American Association for the Study of Liver Diseases, October 29–2 November 2004, Boston, USA. Abstract 513.

Background/aims: One of the main issues in the development of antiviral therapy is the emergence of drug-resistant viruses. In the case of hepatitis C virus (HCV), selection of drug-resistant mutants was evidenced by *in vitro* studies on protease inhibitors (PIs); for example, BILN-2061, VX-950 and SCH-6. Four mutations in the HCV protease (R155Q, A156T, D168A and D168V) have been identified *in vitro* in the HCV replicon system that confer resistance to BILN-2061 (a reference inhibitor). However, the molecular mechanism of drug resistance is still unknown. The aim of this study is to unravel, using an molecular modelling strategy, the structural basis of such molecular mechanism of HCV resistance to PIs. We focused on protease mutations conferring HCV resistance to BILN-2061 and described for the first time such mechanism at a molecular level. **Methods:** The structures of drug-resistant NS3 proteases were obtained by mutation of selected residues (R155Q,

A156T, D168A and D168V) and the ternary complexes formed between NS3-4A and BILN-2061 were optimized using GenMol software (www.3dgenoscience.com; Genoscience, Marseille, France).

Results: Two mechanisms were evidenced for viral resistance to BILN-2061. A 'direct' resistance mechanism is based on contacts between the mutated R155Q and A156T protease residues and its inhibitor. In the 'indirect' resistance mechanism, the mutated D168A/V residue is not in close contact with the drug itself but interacts with other residues connected to the drug.

Conclusions: These data provide new insights in the understanding of the mechanisms of HCV drug escape, and may allow predicting potential cross-resistance phenomenon with other PIs. This approach can be used as a basis for future rational PI drug design candidates.

Introduction

The overall prevalence of hepatitis C virus (HCV) chronically infected people is 3–5%, corresponding to more than 175 million people worldwide. The standard of care for chronic HCV infection is based on a 48-week therapeutic combination of pegylated interferon- α 2 (peg-IFN- α 2) and ribavirin. This peg-IFN- α 2 and ribavirin therapy fails in approximately 50% of patients infected with the HCV-1 genotype, which corresponds to the majority of HCV-infected patients in developed countries [1–3]. Furthermore, this therapeutic approach is limited by the frequent side effects of both agents in long-term

therapy [4–7]. There is therefore an urgent need for the development of potent and more specific HCV antiviral drugs associated with a shorter effective therapy. Major research efforts have focused on the identification of agents that inhibit specific steps of the HCV life cycle as NS3-4A serine protease, which was firstly described to be involved in the late HCV polyprotein maturation step [8,9]. NS3-4A is a multifunctional HCV protein with a serine protease function at the amino-terminal domain and with a helicase function at the carboxy-terminal domain. The later

function supports HCV replication by unwinding the viral dsRNA [10]. More recently, HCV NS3-4A protein was described to take part in host immune evasion [11–13]. Indeed, NS3-4A proteolytic activity is incriminated in the immune host evasion by blocking the activation of interferon regulatory factor 3 (IRF-3) [11,14]. Thus, a potent HCV NS3-4A protease inhibitor (PI) can have a synergic impact on HCV replication. Indeed, the HCV NS3-4A PI acts at one of the end steps of the HCV replication cycle (the maturation step), and at a more early step during the triggering of the host response to HCV infection, as NS3-4A interferes in the IRF-3 signalling pathway. Therefore, a potent HCV NS3-4A PI can prevent the maturation of HCV polyprotein and can restore an innate immune response. This finding may explain the exceptional antiviral activity found *in vitro* and *in vivo*, with potent HCV NS3-4A PIs.

BILN-2061 was the first proof-of-concept in the application of a NS3-4A PI for HCV therapy. BILN-2061 was found to induce, within 48 h, a 2–3 log₁₀ reduction of viral load in HCV-infected patients [15]. However, BILN-2061 was stopped in a Phase II clinical trial (in 2005) because of drug-induced cardiac toxicity. More recently, other PIs have emerged, such as VX-950 [16,17] and SCH-6, [18]. However, BILN-2061 is still a reference compound for HCV-related PIs. Emergence of drug-resistant viruses limits the long-term efficacy of these antiviral products. Key factors of appearance of drug-resistant viruses are specific to viral enzymes such as reverse transcriptase for retrovirus like HIV [19–21] or HBV [22–24], DNA polymerase for DNA virus (for example, HSV) [25–27], or RNA-dependent RNA polymerase for RNA virus (for example, HCV) [28]. It is noteworthy that actual resistance to antiviral drugs appears during the course of a long-term treatment of patients with specific drug(s). The resistance phenomenon is therefore one of the main focus in the development of a candidate chemotherapeutic agent [29]. Mutations in HCV protease seem to be key to the emergence of drug-resistant viruses. Unravelling the molecular mechanisms of drug resistance or sensitivity is crucial for the design and production of future effective anti-HCV drugs.

In HCV subgenomic replicon cells, the capacity of HCV to select mutation(s) conferring viral resistance *in vitro* was described in 2003 by Trozzi *et al.* for PIs [30], Migliaccio *et al.* for nucleoside analogues [31] and Nguyen *et al.* for non-nucleoside analogue NS5B polymerase inhibitors [32]. The capacity of HCV to select mutation(s) *in vitro* under PI drug pressure was confirmed in 2004 by Lu *et al.* [33] with the potent BILN-2061. The results were also strengthened by Lin and colleagues [16,17], who carried out *in vitro* resistance studies with BILN-2061 and VX-950. Four

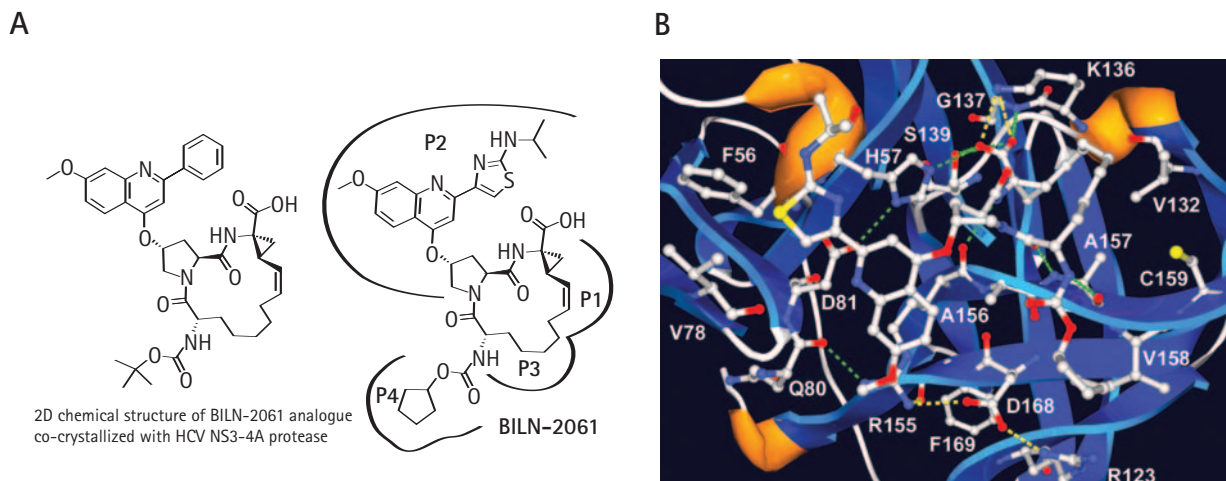
mutations that confer viral resistance to BILN-2061 in the HCV NS3-4A protease are currently known: R155Q, A156T, D168A and D168V [16,17,33].

The aim of this study is to describe, using a molecular modelling strategy, the structural basis of such molecular mechanism of HCV resistance to PIs. We focused on protease mutations conferring HCV resistance to BILN-2061 and described for the first time such mechanisms at a molecular level [34]. Basically, although the former hypothesis that HCV can select drug-resistant mutants is validated by several *in vitro* studies on BILN-2061 [33], VX-950 [16,17] and SCH-6 [18], the precise molecular mechanism of drug resistance is still unknown and should be carefully examined. We highlighted two mechanisms for viral resistance to BILN-2061: a ‘direct’ resistance mechanism based on contacts between the mutated R155Q and A156T protease residues and the inhibitor, and an ‘indirect’ resistance mechanism in which the mutated D168A/V residue is not in close contact with the drug itself, but interacts with other residues connected to the drug. This distinction is helpful to understand and to discern the mechanisms of HCV-resistant virus selection, and to predict the potential for cross-resistance with other characterized PIs.

Materials and methods

The crystal structure of the ternary complex between NS3-4A genotype 1b and BILN-2061 is not available. To generate this complex, we used a ternary complex in which NS3-4A contained a small peptidomimetic inhibitor (PDB ID: 1DY9) [35]. The selection of this template is based on the high sequence homology with genotype 1b (>97%) [36] and the presence of a small molecule in the NS3-4A active site (it shares the same binding site with BILN-2061). The presence of this compound can induce a local and/or global rearrangement that refines the NS3-4A complex to optimize the resulting ternary complex. This is also called the ‘induced-fit’ mechanism. The crystallographic water molecules close to the protein were kept. For the construction of the NS3-4A genotype 1 structural model, the mutations D30E, L36V, Y56F, P86Q and V150A were introduced using GenMol software (www.3dgenoscience.com; Genoscience, Marseille, France) [37–40]. The Arg-155 side chain was modelled in an extended conformation (see Results). This conformation can be found in one crystal structure of the binary complex NS3-4A, with the pdb ID:1JXP in chain B [41]. To validate our structural model, construction of a ternary complex between BILN-2061 and NS3-4A was preceded by the construction of the published crystal structure of a close BILN-2061 analogue (Figure 1A), but the corresponding crystal

Figure 1. Chemical structure of HCV NS3-4A protease inhibitors BILN-2061 and one analogue co-crystallized with HCV NS3-4A protease (A) [42]; model of ternary complex between wild-type HCV NS3-4A protease and BILN-2061 (B)



(A) Two-dimensional (2D) chemical structure of one BILN-2061 co-crystallized with HCV NS3-4A protease [42], and 2D chemical structure of BILN-2061 with the schematic representation of its P1 to P4 binding domain. (B) The wild-type NS3-4A protease (genotype 1b) is shown in a ribbon representation, based on its secondary structures. The active-site NS3-4A protease residues and BILN-2061 are shown in ball and stick with atom type colours. Hydrogen bonds are highlighted in green dotted line. The two salt bridges between Asp¹⁶⁸-Arg¹²³ and Asp¹⁶⁸-Arg¹⁵⁵, as well as electrostatic interactions between Phe¹⁶⁹ and Arg¹⁵⁵ are shown in yellow dotted line. All pictures were created with Swiss PDB Viewer 3.7 [49] and rendered with PovRay 3.6 (www.povray.org).

structure coordinates were unavailable [42]. In this first model, the structural characteristics of the BILN-2061 analogue model were found to be similar to the crystallographic data reported by Tsantrizos *et al.* [42]. The distances between the BILN-2061 analogue and NS3-4A protease are reportedly in the same range (<0.2 Å). All structural models were checked with Procheck software [43].

As a final step, this validated model was used to construct the ternary complex between BILN-2061 and NS3-4A. The three-dimensional structures of mutated NS3-4A proteases were obtained by substituting selected residues (R155Q, A156T, D168A and D168V) using GenMol software. The resulting ternary complexes between BILN-2061 and NS3-4A were then optimized using the GenMol all atoms force field and its sigmoid distance-dependent dielectric model [37–40].

Results

Selected HCV mutants showed high resistance level to BILN-2061

In studies of BILN-2061 resistance *in vitro*, two patterns of residue mutations conferring drug resistance (with distinct resistance profile) were selected and characterized with two common mutations: D168V and A156T (Table 1). According to Lin *et al.* mutation D168V in HCV NS3-4A protease (FR 1273) was more drug-resistant than A156T (FR 273, Table 1) [16,17].

By contrast, Lu *et al.* reported D168V (FR 144) was found to be less resistant than A156T (FR 357, Table 1) [33]. The fold resistance for each mutation was markedly different. These differences were attributed to non-normalized HCV phenotype assays. Apart from A156T and D168V mutations, two additional mutations (one in each assay) were evidenced, corresponding to D168A (FR 465; Lin *et al.* [16,17]) and R155Q (FR 24; Lu *et al.* [33]).

Structural basis of interactions between BILN-2061 and HCV NS3-4A

Four residue mutations – R155Q, A156T, D168A and D168V – were located in the HCV NS3-4A protease domain. These mutations were selected *in vitro* during HCV PI resistant cell-based assays [16,17,33]. The structural analysis of each ternary complex between HCV mutated NS3-4A and BILN-2061 showed 2 types of resistance mechanisms. The first involves a ‘direct’ resistance mechanism that relies on a close contact between BILN-2061 and NS3-4A mutated residues (R155Q and A156T). The second mechanism, referred to an ‘indirect’ resistance mechanism, does not involve any close contact between BILN-2061 and the mutated D168A/V residue. However, the latter residue interacts with two residues (Arg¹²³ and Arg¹⁵⁵) that are connected to BILN-2061 (Figure 1B). The distances between mutated residues and BILN-2061 were <4 Å or 4–6 Å for the ‘direct’ or ‘indirect’ resistance mechanism, respectively.

Table 1. Resistance profile of BILN-2061 in HCV replicon cell-based assay

Protease mutations	BILN-2061 susceptibility					References
	WT	R155Q	D168A	D168V	A156T	
IC ₅₀ , μ M	0.004	ND*	1.86	5.09	1.09 [†]	16,17
FR [‡]	–	ND*	465	1,273	273 [†]	
IC ₅₀ , μ M	0.003	72	ND*	0.432	1,072	33
FR [‡]	–	24	ND*	144	357	

*ND, not determined: the corresponding HCV NS3-4A protease mutation was not found in this *in vitro* resistance selection [16,17,33]. [†]Data from [16]. [‡]FR, fold resistance in drug sensitivity compared with the parental wild-type replicon cell-based assay (genotype 1b).

BILN-2061 is a peptidomimetic inhibitor interacting with the S1–S4 binding pocket of NS3-4A through hydrophobic, electrostatic and hydrogen bond interactions (Figure 1A and 1B). P1 and P3 of BILN-2061 are connected together by an alkyl macrocycle that contracts hydrophobic interactions with the S1 and S3 binding pocket. S1 is defined by Val¹³², Leu¹³⁵, Lys¹³⁶ and Phe¹⁵⁴ residues (Ala¹⁵⁷ is at the junction of S1-S3) and S3 is defined by Cys¹⁵⁹, Ala¹⁵⁷ and Ala¹⁶⁴ residues, as well as Val¹³², which connects S1 and S3. P3 forms two hydrogen bonds with the Ala¹⁵⁷ backbone (Figure 1B, green dotted line). Of note, the C-terminal carboxylic acid extremity of BILN-2061 makes electrostatic interactions, as well as strong hydrogen bonds, with the NS3-4A His⁵⁷, Ser¹³⁹ catalytic residues, and with Lys¹³⁶ (Figure 1B). His⁵⁷ possesses a formal positive charge with its protonated N- π . Thus, one oxygen atom of the C-terminal carboxylate extremity of BILN-2061 can contract a strong hydrogen bond and synergizing electrostatic interactions with the His⁵⁷ residue. Anchoring of this carboxylate is strengthened by a bidentate hydrogen bond with the hydroxyl group of the catalytic Ser¹³⁹ residue (O- γ). Additionally, the C-terminal carboxylate makes one hydrogen bond with N-H of Gly¹³⁷, which corresponds to the NS3-4A oxanion hole. Noting equally a hydrogen bond between the C=O of Arg¹⁵⁵ backbone and the N-H of P1.

The large aromatic P2 of BILN-2061 constituted by a 7-methoxy-2-thiazol-4-quinolinol substituting a *trans*-hydroxyproline makes mainly hydrophobic interactions with the large solvent-exposed S2 binding pocket of NS3-4A. This S2 pocket includes Phe⁵⁶, Val⁷⁸, Asp⁷⁹, Gln⁸⁰, Arg¹⁵⁵ and Ala¹⁵⁶ residues, and catalytic His⁵⁷ and Asp⁸¹ residues. P2 entity represents \approx 57% of the total BILN-2061 accessible surface area. This compound takes its functional potency mainly through P2–S2 interactions. The large P2 part of BILN-2061 allows a strong stabilization of the ternary complex between BILN-2061 and NS3-4A.

P4 of BILN-2061 makes only weak hydrophobic interactions with the corresponding S4 binding pocket

through its hydrophobic cyclopentyl moiety. The S4 solvent-exposed binding pocket is mainly defined by Arg¹²³ and Val¹⁵⁸ residues. Arg¹²³ participates in the orientation of hydrophobic cyclopentyl toward hydrophobic side chain of Val¹⁵⁸. This orientation is also favored by one hydrogen bond between the N-terminal extremity (N-H) of BILN-2061 and the C=O of Ala¹⁵⁷ backbone (Figure 1B, green dotted line). It is worth noting that Asp¹⁶⁸ connects S2 and S4 through two salt bridges with Arg¹²³ and Arg¹⁵⁵, participating to their conformational stabilization (Figure 1B, yellow dotted line).

Before detailing the molecular mechanisms of BILN-2061 drug resistance, it is crucial to understand, in the wild-type complex, the key role of each of the mutated residues (Arg¹⁵⁵, Ala¹⁵⁶ and Asp¹⁶⁸) in their interactions with BILN-2061. Arg¹⁵⁵ is located on the E2 strand; it exhibits both hydrophobic (alkyl side chain) and polar characteristics (guanidinium side chain extremity) and contracts with BILN-2061 these two types of interaction. Under the pressure of the large aromatic P2 part, Arg¹⁵⁵ adopts an extended conformation to improve the planarity of the large S2 binding pocket and increases the interactions with BILN-2061 through its large planar P2. The extended conformation of Arg¹⁵⁵ was not clearly described in the reported crystal structures of HCV NS3-4A protease in complex with a small molecule or peptide. This conformational peculiarity of Arg¹⁵⁵ improves P2-S2 interactions. BILN-2061 makes some electrostatic contacts with the guanidinium polar extremity of Arg¹⁵⁵ through the methoxy group (Me-O-) of the P2 quinoline (distance between Arg^{155-CZ} and oxygen of Me-O- is 3.5 Å), and some additional hydrophobic interactions with the aromatic quinoline ring (distance between Arg^{155-C γ -C δ} and quinoline ranged from 4 to 4.6 Å). To complete P2-S2 interactions, Arg¹⁵⁵ makes additional π - π interactions (like π staking) between methoxyquinoline extremity (P2 of BILN-2061) and the guanidinium moiety of Arg¹⁵⁵ (both π systems). Ala¹⁵⁶ is on the E2 strand and in closed contact with both *trans*-hydroxyproline and quinoline P2 part of BILN-2061. The nature of the

interactions between BILN-2061 and Ala¹⁵⁶ is hydrophobic. The distances between Ala^{156-C β} and P2 ranged from 3.7 to 4.0 Å (for quinoline and hydroxyproline ring). Asp¹⁶⁸ is on the F2 strand and connects S2-S4 binding pockets by two salt bridges with first, S2-Arg¹⁵⁵ and second, S4-Arg¹²³. It provides a conformational stabilization of these two residues. We assumed that Asp¹⁶⁸ behaves as a chain link connecting S2 and S4, two normally independent binding pockets in chymotrypsin-like proteases. Thus, Asp¹⁶⁸ does not directly participate in the anchoring of BILN-2061 but is indirectly involved in the stabilization of the ternary complex between BILN-2061 and NS3-4A.

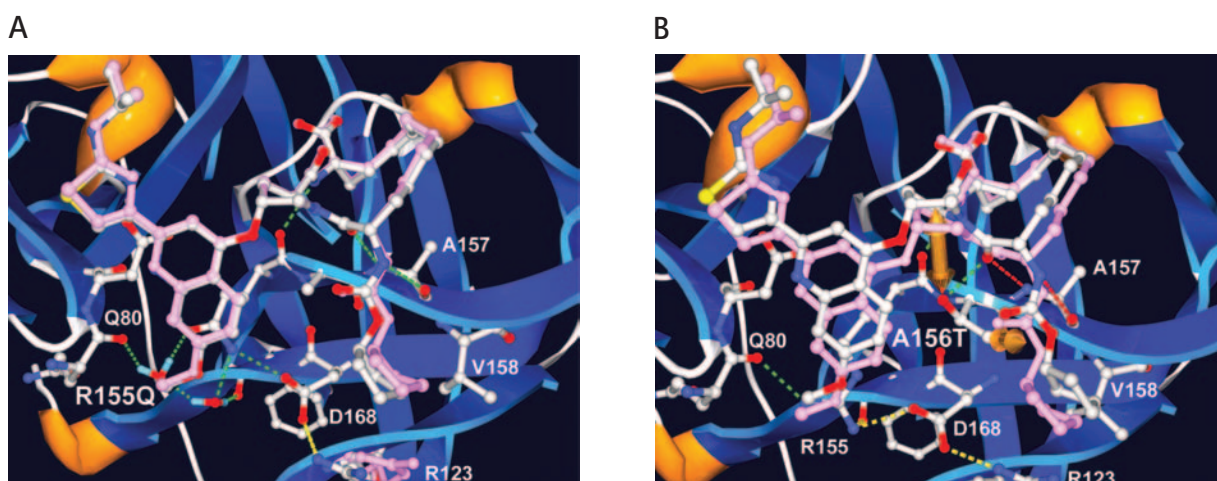
Molecular mechanisms of BILN-2061 drug resistance: 'direct' resistance mechanism

Here we highlight two types of molecular mechanisms of BILN-2061 drug resistance. The first mechanism – the 'direct' resistance mechanism – relies on a direct contact between mutated NS3-4A protease residue and BILN-2061 inhibitor. R155Q shows a low-fold resistance as compared to other mutations (Table 1), which showed up to 24-fold change [33]. This R155Q mutation modifies the hydrophobic and electrostatic environments of the S2 binding pocket. Gln¹⁵⁵ is uncharged, whereas Arg¹⁵⁵ is positively-charged on its guanidinium moiety. The electrostatic interactions between Arg¹⁵⁵ and -O-Me of P2 quinoline are thus lost. Moreover, in the 1DY9 crystal structure, [35] we found two water molecules that are located in the same position of extended Arg¹⁵⁵ terminal nitrogen atoms

(Figure 2A). These water molecules were taken into account during molecular modelling of R155Q mutated protease, and formed a new hydrogen bond network in the co-complex that stabilizes the bottom part of S2 (Figure 2A, green dotted line). The lower part of S2 binding pocket evolved from electronic deficiency to electronic enrichment (increasing the electronic density). Therefore, the lower part of S2 changed from a local positive charge to a neutral one (Arg to Gln). The P2-S2 electrostatic interactions follow this reversion, and -O-Me has finally unfavourable electrostatic interactions with S2. Methoxy group of BILN-2061 is opposed to oxygen atoms of water molecules, with a distance of 4.1 Å. Moreover, the new hydrogen bond network slightly modifies the Asp¹⁶⁸ conformation. Gln¹⁵⁵ is shorter than Arg¹⁵⁵, and makes one hydrogen bond with the Asp¹⁶⁸ side chain through its -NH₂ side chain (Figure 2A, green dotted line), thereby reorienting Asp¹⁶⁸ upward (carboxylate movement of Asp¹⁶⁸ is approximately of 1.3 Å, side-chain rotation of 18°). However, Asp¹⁶⁸ usually forms a salt bridge with Arg¹²³. Consequently, Arg¹²³ follows the Asp¹⁶⁸ motion (movement of Arg¹²³ guanidinium is *ca.* 1.8 Å). Thus, the R155Q protease mutation slightly impacts on P4-S4 interactions (see Figure 2A). R155Q is still related to a 'direct' resistance mechanism, because its clear impact in P2-S2 interactions.

A156T is the second mutation involving a 'direct' resistance mechanism. This resistance mutation was first described by Lu *et al.* [33] and, thereafter, confirmed by Lin *et al.* [16,17]. A156T is a highly

Figure 2. Model of ternary complex between R155Q mutated NS3-4A protease and BILN-2061 (A) and between A156T mutated NS3-4A protease and BILN-2061 (B)



(A) The ternary complex (shown in atom type) NS3-4A^{R155Q}-BILN-2061 is superimposed with the wild-type complex (pink) NS3-4A-BILN-2061. Hydrogen bond network and salt bridge between Asp¹⁶⁸ and Arg¹²³ are highlighted in green and yellow dotted lines, respectively. (B) The ternary complex (shown in atom type) NS3-4A^{A156T}-BILN-2061 is superimposed with the wild-type complex (pink) NS3-4A-BILN-2061. Hydrogen bonds and salt bridges Asp¹⁶⁸-Arg¹⁵⁵ and Asp¹⁶⁸-Arg¹²³ are highlighted in green and yellow dotted lines, respectively. Steric hindrance between Thr¹⁵⁶ mutated residue and BILN-2061 is highlighted by two double arrows (in gold colour). This steric hindrance induces the loss of two hydrogen bonds (with Ala¹⁵⁷), which are shown in red dotted lines.

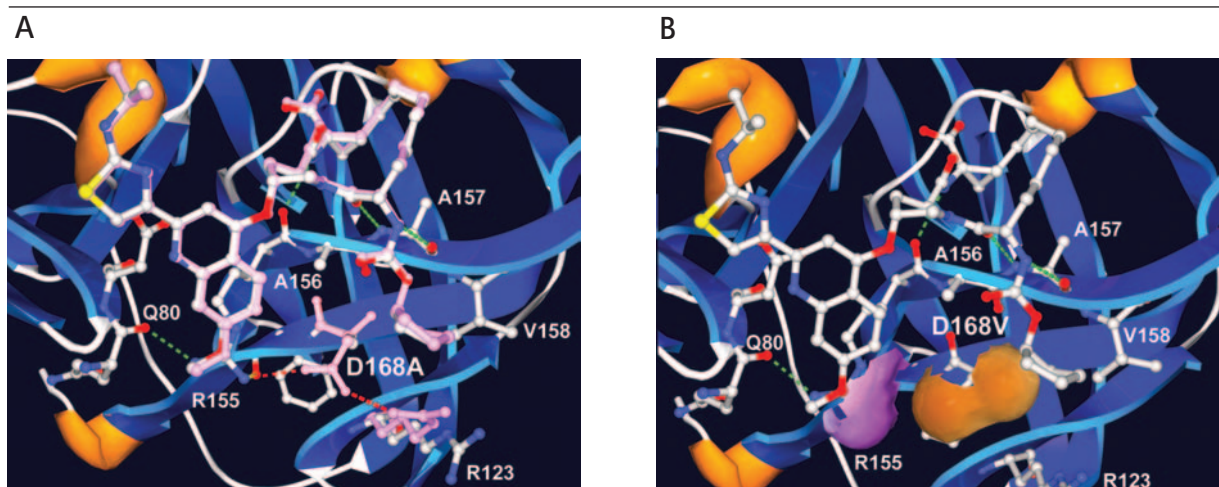
discriminating protease mutation with regard to BILN-2061 (357- and 273-fold resistances according to Lu *et al.* [33] and Lin *et al.* [16,17], respectively, Table 1). A156T mutated protease can adopt three main conformations. None of these are compatible with BILN-2061 from the steric viewpoint, and more precisely, with its P2 or P4 moiety, or both. One of these conformations supports the strong resistance profile of BILN-2061 (see Table 1). This particular conformation produces strong steric constraints regarding hinge P2, P3 and P4 parts of BILN-2061 (see Figure 2B, double arrows). In the case of A156T mutation, the molecular mechanism of viral resistance to BILN-2061 relies on a steric conflict. The hydroxyl group of Thr^{156-O γ} is in steric conflict with the P2 hydroxyproline motif of BILN-2061. The steric requirement of the hydroxyl Thr¹⁵⁶ (a β -branched residue) induces a BILN-2061 motion. As compared with the wild-type ternary complex, the P2, P3 and P4 parts of BILN-2061 moved ≈ 1 Å, inducing the loss of two hydrogen bonds (red dotted line, Figure 2B) and van der Waals (vdW) interactions. The impact of this steric clash is reflected on the entire molecule and markedly affects the affinity of BILN-2061 for the NS3-4A A156T mutated strain (Table 1). Moreover, another steric conflict occurs with the urethane function linking P3 and P4. The methyl group of Thr^{156-C γ} provokes a steric hindrance with the P4 part of BILN-2061, which is characterized by a widening out (see Figure 2B). This motion is characterized by 1 Å and 1.3 Å movements for N-H terminal

and CH cyclopentyl, respectively. Thus, A156T mutation can further alter P4-S4 interactions. In summary, A156T mutation of NS3-4A protease impacts directly on all BILN-2061 interactions.

Molecular mechanisms of resistance to BILN-2061: the 'indirect' resistance mechanism

The second molecular mechanism of resistance to BILN-2061 is 'indirect'. In this case, the mutated residue does not directly interact with the inhibitor. This type of mechanism is observed with BILN-2061 and the D168A or D168V protease mutations. The D168A mutation results in a strong 465-fold resistance (Lin *et al.* Table 1) [16,17]. This mutation prevents the formation of two salt bridges between Asp¹⁶⁸ and Arg¹²³ (S4 pocket), as well as Asp¹⁶⁸ and Arg¹⁵⁵ (S2 pocket; Figures 1B and 3A). Asp¹⁶⁸ does not significantly interact with BILN-2061, but stabilizes the conformations of both Arg¹²³ and Arg¹⁵⁵ residues. Asp¹⁶⁸ connects S2 and S4 through Arg¹⁵⁵ and Arg¹²³, respectively. Arg¹⁵⁵ no longer forms a salt bridge with Asp¹⁶⁸ and loses its S2 anchoring property towards the P2 quinolin moiety of BILN-2061 (Figure 3A). Likewise, Asp¹⁶⁸ stabilized the 'upward' structure of Arg¹²³ side chain, thereby acting on the orientation of the P4 cyclopentyl toward Val¹⁵⁸, which favours P4-S4 vdW interactions. Arg¹²³ from S4 pocket is exposed to the solvent and exhibits an extended conformation in the absence of stabilizing Asp¹⁶⁸ salt bridge. Thus, being located at the S2-S4 junction, D168A indirectly affects P4-S4 and main P2-S2

Figure 3. Model of ternary complex between D168A mutated NS3-4A protease and BILN-2061 (A) and between D168V mutated NS3-4A protease and BILN-2061 (B)



(A) The ternary complex (shown in atom type) NS3-4A^{D168A}-BILN-2061 is superimposed with the wild-type complex (pink) NS3-4A-BILN-2061. Hydrogen bonds and wild-type lost salt bridges Asp¹⁶⁸-Arg¹²³ and Asp¹⁶⁸-Arg¹⁵⁵ are highlighted in green and red dotted lines, respectively. With the D168A protease mutation, motion of Arg¹²³ side chain guanidinium are 3.8 Å (N-H1) and 1.7 Å (N-H2). (B) The ternary complex NS3-4A^{D168V}-BILN-2061 is shown in atom type. Hydrogen bonds are highlighted in green dotted lines. With the D168V protease mutation, Arg¹²³ side chain motion was similar to the one observed for D168A mutation (see A). To distinguish D168V from D168A protease mutation, as well as to highlight the additional van der Waals unfavourable interactions between the hydrophobic Val¹⁶⁸ and polar hydrophilic Arg¹⁵⁵, molecular surfaces are shown in brown and purple, respectively.

interactions in the ternary complex between BILN-2061 and NS3-4A protease.

Similar to D168A, D168V mutation abrogates the two salt bridges with Arg¹²³ and Arg¹⁵⁵ residues (Figure 3). The D168V mutation shares the same molecular mechanism that D168A mutation even though with a higher 1,273-fold resistance (Lin *et al.*; Table 1). The D168V mutation does not have any direct impact on BILN-2061 and does not provoke any steric hindrance or unfavourable non-bonded interactions with BILN-2061. Basically, D168V directly alters Arg¹²³ and Arg¹⁵⁵ side-chain conformations, similar to D168A. Therefore, this higher resistance of D168V versus D168A should be inferred to the intrinsic properties of the mutation; that is, Ala or Val. The D168V mutation provides some additional unfavourable vdW interactions between hydrophobic/bulky Val¹⁶⁸ side chain and hydrophilic Arg¹²³ and Arg¹⁵⁵ guanidinium (Figure 3B). Based on the lost of Asp¹⁶⁸-Arg¹²³ salt bridge, together with some additional unfavourable non-bonded interactions, Arg¹²³ adopts an extended conformation in the solvent-exposed domain, and loose its P4 cyclopentyl-related orientating effect toward Val¹⁵⁸. Thus, D168V mutation indirectly decreases vdW interactions of P4-S4.

The D168V mutation also induced some additional unfavorable vdW interactions with Arg¹⁵⁵, as compared to the D168A mutation (Figures 3). D168V directly impacts on the conformational stabilization of Arg¹⁵⁵. When the wild-type HCV NS3-4A is complexed or not with a small inhibitor harbouring a small P2 moiety (like VX-950), Arg¹⁵⁵ does not display any extended conformation but a more compact one. During the formation of the ternary complex between BILN-2061 and NS3-4A, Arg¹⁵⁵ adopts an extended conformation that locates its guanidinium at the bottom of the S2 pocket. Arg¹⁵⁵ is stabilized in this extended conformation by, first, one hydrogen bond with the C=O of Gln⁸⁰ backbone and, second, a potent salt bridge with Asp¹⁶⁸, as well as an electrostatic interaction with the C=O of Phe¹⁶⁹ backbone. The lack of this salt bridge and the additional unfavorable interactions associated with the D168V mutation might be crucial to the stabilization of Arg¹⁵⁵ extended conformation during the formation of a ternary complex between BILN-2061 and NS3-4A. In such a wild type ternary complex, Asp¹⁶⁸ induces stacking of Arg¹⁵⁵ to the large P2 quinoline of BILN-2061. With D168V mutation, Val¹⁶⁸ has, at the opposite, a repulsive effect on Arg¹⁵⁵ (in comparison with D168A mutation) thereby decreasing BILN-2061 affinity, in an indirect manner. Thus, D168V mutation indirectly alters P4-S4 and main potent P2-S2 interactions in the ternary complex between BILN-2061 and NS3-4A.

Discussion

The present study highlights the molecular mechanism of HCV NS3-4A resistance to PIs such as BILN-2061, a HCV PI reference compound. Four mutations located in the HCV NS3-4A protease domain in or close to the BILN-2061 binding site have been recently selected *in vitro* that conferred viral resistance to BILN-2061: R155Q, A156T, D168A and D168V. These mutations either slightly or markedly alter protease sensitivity to BILN-2061 (from 24- to 1273-fold difference in sensitivity, Table 1) [16,17,33]. In the present study, two types of molecular mechanisms of viral resistance to drug are described for the first time, referred to 'direct' and 'indirect' mechanisms.

The 'direct' mechanism of viral resistance is associated with a physical interaction between BILN-2061 and the mutated residues of NS3-4A protease (for example, R155Q and A156T). First, with R155Q mutation, the resistance mechanism corresponds to a lost of protease to drug affinity by decreasing the non-bonded interactions (electrostatic and vdW) between P2 of BILN-2061 and R155Q mutated protease (Figures 1B, 2A). The R155Q protease mutation results in the lower resistance profile (24-fold), as compared with other three protease mutations that conferred higher viral resistance (Table 1) [33]. One can anticipate that such resistance profile could take place with a PI having a BILN-2061 P2-like motif which stacks to Arg¹⁵⁵.

The 'direct' mechanism of viral resistance is found with A156T protease mutation. As shown in Figure 2B, this mechanism relies on a steric clash between BILN-2061 P2 and the bulky β -branched Thr¹⁵⁶ (vs Ala¹⁵⁶). The latter mechanism is responsible for a marked difference (273- to 357-fold) in protease to drug sensitivity (Table 1), similar to the paradigm of 3TC (lamivudine) with regard to its HIV-RT M184V signature mutation [44,45]. The A156T mutation induces roughly a 1 Å motion of BILN-2061 (P1 to P4 domains) toward the S1 to S4 binding pockets. This mutation was predicted to be the molecular basis of some cross resistance phenomena with proline-based inhibitors such as VX-950 (it contains a bicyclic proline-like motif as P2) [46]. This structural prediction was confirmed by Lin *et al.* [16]. Such a resistance profile would be difficult to circumvent with P2 proline-based inhibitors. Therefore, A156T behaves as one of the signature mutations for viral resistance to PIs having a P2-like proline-based motif. This assumption has been recently validated by the resistance profile of SCH-6, which can select the A156T/V mutation *in vitro* [18]. Remarkably, the SCH-6 PI is also based on a P2 proline-based motif.

The second molecular mechanism of viral resistance to BILN-2061 is referred to as an 'indirect' mechanism. It relies on the D168A/V protease mutations that do not favour major contacts between NS3-4A and BILN-2061 (Figure 3). A resistance mutation (for example, Asp¹⁶⁸), without any essential contact with the inhibitor, can induce a strong viral resistance through an 'indirect' mechanism. D168A/V might behave as one of the signature mutations for viral resistance to HCV PIs that possess a large P2 planar motif. This hypothesis is supported by the *in vitro* resistance study of Trozzi *et al.* [30], who used a PI harbouring a large planar P2 (proline-substituted quinoline derivative) similar to BILN-2061, and showed that HCV can select *in vitro* (in a HCV replicon system) the D168A/Y/V mutations.

Another important factor affecting the outgrowth of the resistant population is the fitness of the mutant viruses. The replicative fitness of resistant viruses is also a crucial parameter of viral resistance and it has a prognostic value with regard to achieving sustained virological response [38,47]. Kinetic studies correlate the proteolysis efficiency of mutated HCV NS3-4A to the replication capacity and the viral fitness [48]. Mutated HCV NS3-4A is less active and the virus has a weak replication capacity. For example, cells bearing the A156T replicon were less fit than wild-type replicon cells, and the mutant cells were relatively stable in the absence of inhibitor or competition from wild-type cells [48].

This innovative molecular modelling strategy was previously used to describe the molecular mechanism of the resistance or sensitivity of tenofovir to the K65R, L74V and M184V HIV-RT mutants [20,38,47]. Futures research lead to serve as a model to understand the molecular mechanisms of others HCV NS3 protease variants selected to the PI SCH 503034 recently reported: T54A, V170A, A156S and A156T [48].

In conclusion, the structural basis of the molecular mechanism of HCV resistance to PIs is described for the first time at a molecular level. These data provide new insights in the understanding of the mechanisms of HCV drug escape, and may allow us to predict potential cross-resistance phenomena with other PIs. This approach can be used as a basis for future rational PI drug design candidates.

References

1. Manns MP, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**:958–965.
2. Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**:975–982.
3. Hadziyannis SJ, Sette H, Jr, Morgan TR, *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**:346–355.
4. Ascione A, De Luca M, Di Costanzo GG, *et al.* Incidence of side effects during therapy with different types of alpha interferon: a randomised controlled trial comparing recombinant alpha 2b versus leukocyte interferon in the therapy of naive patients with chronic hepatitis C. *Curr Pharm Des* 2002; **8**:977–980.
5. Schaefer M, Schmidt F, Folwaczny C, *et al.* Adherence and mental side effects during hepatitis C treatment with interferon alfa and ribavirin in psychiatric risk groups. *Hepatology* 2003; **37**:443–451.
6. Toniutto P, Fabris C, Fumo E, *et al.* Pegylated versus standard interferon-alpha in antiviral regimens for post-transplant recurrent hepatitis C: Comparison of tolerability and efficacy. *J Gastroenterol Hepatol* 2005; **20**:577–582.
7. Lebray P, Nalpas B, Vallet-Pichard A, *et al.* The impact of haematopoietic growth factors on the management and efficacy of antiviral treatment in patients with hepatitis C virus. *Antivir Ther* 2005; **10**:769–776.
8. Bartenschlager R, Lohmann V, Wilkinson T, Koch JO. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. *J Virol* 1995; **69**:7519–7528.
9. Yao N, Reichert P, Taremi SS, Prosis WW, Weber PC. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. *Structure* 1999; **7**:1353–1363.
10. De Francesco R, Steinkuhler C. Structure and function of the hepatitis C virus NS3-NS4A serine proteinase. *Curr Top Microbiol Immunol* 2000; **242**:149–169.
11. Gale M, Jr, Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005; **436**:939–945.
12. Li K, Foy E, Ferreon JC, *et al.* Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 2005; **102**:2992–2997.
13. Meylan E, Curran J, Hofmann K, *et al.* Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**:1167–1172.
14. Foy E, Li K, Wang C, *et al.* Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003; **300**:1145–1148.
15. Lamarre D, Anderson PC, Bailey M, *et al.* An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 2003; **426**:186–189.
16. Lin C, Gates CA, Rao BG, *et al.* *In vitro* studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem* 2005; **280**:36784–36791.
17. Lin C, Lin K, Luong YP, *et al.* *In vitro* resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem* 2004; **279**:17508–17514.
18. Yi M, Tong X, Skelton A, *et al.* Mutations conferring resistance to SCH6, a novel hepatitis C virus NS3/4A protease inhibitor: Reduced RNA replication fitness and partial rescue by second-site mutations. *J Biol Chem* 2006 **281**:8205–8215.
19. Daar ES, Richman DD. Confronting the emergence of drug-resistant HIV type 1: impact of antiretroviral therapy on individual and population resistance. *AIDS Res Hum Retroviruses* 2005; **21**:343–357.
20. Deval J, Courcambek J, Selmi B, Boretto J, Canard B. Structural determinants and molecular mechanisms for the resistance of HIV-1 RT to nucleoside analogues. *Curr Drug Metab* 2004; **5**:305–316.
21. Turner D, Schapiro JM, Brenner BG, Wainberg MA. The influence of protease inhibitor resistance profiles on selection of HIV therapy in treatment-naive patients. *Antivir Ther* 2004; **9**:301–314.

22. Bartholomeusz A, Tehan BG, Chalmers DK. Comparisons of the HBV and HIV polymerase, and antiviral resistance mutations. *Antivir Ther* 2004; **9**:149–160.
23. Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J Hepatol* 2006; **44**:422–431.
24. Yang H, Qi X, Sabogal A, *et al.* Cross-resistance testing of next-generation nucleoside and nucleotide analogues against lamivudine-resistant HBV. *Antivir Ther* 2005; **10**:625–633.
25. Morfin F, Thouvenot D. Herpes simplex virus resistance to antiviral drugs. *J Clin Virol* 2003; **26**:29–37.
26. Stranska R, van Loon AM, Bredius RG, *et al.* Sequential switching of DNA polymerase and thymidine kinase-mediated HSV-1 drug resistance in an immunocompromised child. *Antivir Ther* 2004; **9**:97–104.
27. Thomsen DR, Oien NL, Hopkins TA, *et al.* Amino acid changes within conserved region III of the herpes simplex virus and human cytomegalovirus DNA polymerases confer resistance to 4-oxo-dihydroquinolines, a novel class of herpesvirus antiviral agents. *J Virol* 2003; **77**:1868–1876.
28. De Francesco R, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. *Nature* 2005; **436**:953–960.
29. Dalmau D, Klimkait T, Telenti A. Opinion paper. Resistance to new anti-HIV agents: problems in the pathway of drug registration. *Antivir Ther* 2005; **10**:867–872.
30. Trozzi C, Bartholomew L, Ceccacci A, *et al.* *In vitro* selection and characterization of hepatitis C virus serine protease variants resistant to an active-site peptide inhibitor. *J Virol* 2003; **77**:3669–3679.
31. Migliaccio G, Tomassini JE, Carroll SS, *et al.* Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication *in vitro*. *J Biol Chem* 2003; **278**:49164–49170.
32. Nguyen TT, Gates AT, Gutshall LL, *et al.* Resistance profile of a hepatitis C virus RNA-dependent RNA polymerase benzothiadiazine inhibitor. *Antimicrob Agents Chemother* 2003; **47**:3525–3530.
33. Lu L, Pilot-Matias TJ, Stewart KD, *et al.* Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor *in vitro*. *Antimicrob Agents Chemother* 2004; **48**:2260–2266.
34. Courcambek J, Perbost R, Chabaud P, *et al.* Molecular mechanism susceptibility profile of BILN-2061 to various hepatitis C virus genotypes and NS3–NS4A protease mutations: D168A and D168V. *55th Annual Meeting of the American Association for the Study of Liver Diseases*. 29 October–2 November 2004. Abstract 513.
35. Di Marco S, Rizzi M, Volpari C, *et al.* Inhibition of the hepatitis C virus NS3/4A protease. The crystal structures of two protease-inhibitor complexes. *J Biol Chem* 2000; **275**:7152–7157.
36. Thibeault D, Bousquet C, Gingras R, *et al.* Sensitivity of NS3 serine proteases from hepatitis C virus genotypes 2 and 3 to the inhibitor BILN 2061. *J Virol* 2004; **78**:7352–7359.
37. Pepe G, Guiliani G, Loustalet S, Halfon P. Hydration free energy a fragmental model and drug design. *Eur J Med Chem* 2002; **37**:865–872.
38. Deval J, White KL, Miller MD, *et al.* Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations. *J Biol Chem* 2004; **279**:509–516.
39. Pepe G, Seres B, Laporte D, Del Re C. Surface electrostatic potentials on macromolecules in a monopole approximation: A computer program and an application to cytochromes. *J Theor Biol* 1985; **115**:571–593.
40. Pepe G, Siri D. GENMOL. A fast program for molecular modeling. Application to the determination of the psychotonic or sedative effect of tricyclic antidepressant drugs. *Stud Phys Theor Chem* 1990; **71**:93–101.
41. Yan Y, Li Y, Munshi S, *et al.* Complex of NS3 protease and NS4A peptide of BK strain hepatitis C virus: a 2.2 Å resolution structure in a hexagonal crystal form. *Protein Sci* 1998; **7**:837–847.
42. Tsantrizos YS, Bolger G, Bonneau P, *et al.* Macrocyclic inhibitors of the NS3 protease as potential therapeutic agents of hepatitis C virus infection. *Angew Chem Int Ed Engl* 2003; **42**:1356–1360.
43. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Cryst* 1993; **26**:283–291.
44. Gao HQ, Boyer PL, Sarafianos SG, Arnold E, Hughes SH. The role of steric hindrance in 3TC resistance of human immunodeficiency virus type-1 reverse transcriptase. *J Mol Biol* 2000; **300**:403–418.
45. Sarafianos SG, Das K, Clark AD, Jr, *et al.* Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proc Natl Acad Sci USA* 1999; **96**:10027–10032.
46. Courcambek J, Perbost R, Chabaud P, *et al.* Molecular susceptibility profile of BILN-2061 for common HCV genotypes and NS3–NS4A protease resistance mutations: D168A and D168V. *Gastroenterol Clin Biol* 2004; **28**:768.
47. Deval J, Navarro JM, Selmi B, *et al.* A loss of viral replicative capacity correlates with altered DNA polymerization kinetics by the human immunodeficiency virus reverse transcriptase bearing the K65R and L74V dideoxynucleoside resistance substitutions. *J Biol Chem* 2004; **279**:25489–25496.
48. Tong X, Chase R, Skelton A, *et al.* Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral Res* 2006.
49. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; **18**:2714–2723.

Accepted for publication 15 June 2006