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Short communication

Potent inhibition of drug-resistant HIV protease variants by monoclonal antibodies

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Abstract

The monoclonal antibodies 1696 and F11.2.32 strongly inhibit the activity of wild-type HIV-1 protease (PR) by binding to epitopes at the enzyme N-terminus (residues 1–6) and flap residues 36–46, respectively. Here we demonstrate that these antibodies are also potent inhibitors of PR variants resistant to active-site inhibitors used as anti-AIDS drugs. Our *in vitro* experiments revealed that the inhibitory potency of single-chain fragments (scFv) of these antibodies is not significantly affected by the presence of mutations in PR; inhibition constants for drug-resistant protease variants are 5–11 nM and 13–169 nM for scFv1696 and for scFvF11.2.32, respectively. Tethered dimer of HIV-1 PR variant proved to be a model protease variant resistant to dissociative inhibition by 1696, and, strikingly, it also displayed resistance to inhibition by F11.2.32 suggesting that dimer dissociation also plays a role in the inhibitory action of F11.2.32.

Keywords: HIV protease; Drug resistance; Inhibiting antibody; Dissociative inhibition

Since the demonstration that a specific aspartic protease (PR) is essential for the human immunodeficiency virus (HIV) replication, this enzyme became one of the primary targets for antiviral drug design leading to development of hundreds of inhibitory compounds, nine of which are currently approved for clinical use. The major problem that limits the therapeutic efficiency of protease inhibitors (PIs) is drug resistance caused by extensive mutations in PR (Condra et al., 1995; Schinazi et al., 1997). Development of new inhibitors acting by an alternative mode of inhibition is thus essential for successful treatment of HIV-positive patients.

Design of alternative inhibitors, acting outside of the PR active site, is hindered by the absence of naturally occurring templates that could be used as a lead. With the aim of aiding the alternative inhibitor design, we have identified two monoclonal antibodies that inhibit the PR catalytic activity with inhibition constants in the nanomolar range (Rezacova et al., 2002).

Antibody 1696 recognizes an epitope at the N-terminus of PR (residues 1–6, Fig. 1) and inhibits PR by perturbing the enzyme dimer interface structure (Rezacova et al., 2001). Antibody F11.2.32 inhibits PR through distortion of the flap, a flexible loop that closes over the active PR site (residues 36–46, Fig. 1; Lescar et al., 1997). Since both epitopes belong to well conserved parts of PR, these antibodies could serve as useful leads for development of alternative inhibitors effective against PI-resistant PR variants.

We tested the ability of antibodies to inhibit drug-resistant PRs on four variants with mutations typically selected upon exposure to various PIs in use as anti-AIDS drugs (Fig. 1). Variants PR^{SAQ}, PR^{IND} and PR^{RIT}, containing mutations associated with resistance to saquinavir (G48V/L90M), indinavir (M46I/A71V/V82T/I84V) and ritonavir (V82A), respectively, were prepared by site-directed mutagenesis (Weber et al., 2002). PR^{K4} variant, containing nine muta-

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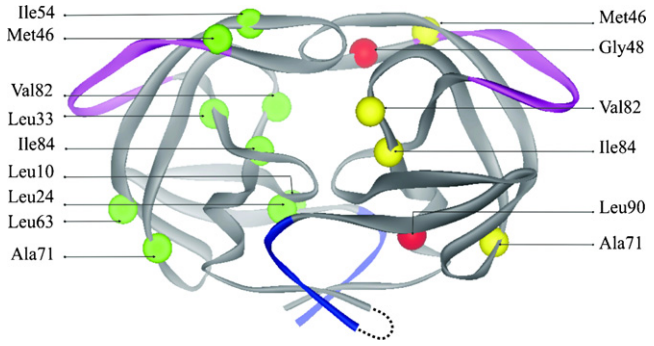

Fig. 1. Position of the mutations in PR variants used in this work. Mutations in PR^{SAQ}, PR^{IND} and PR^{K4} are shown in red, yellow and green, respectively. Mutation V82A of PR^{RIT} is not specifically colored in the figure. Dotted line represents linker connecting two monomers in PR^{SC}. The epitopes recognized by 1696 and F11.2.32 are colored blue and magenta, respectively.

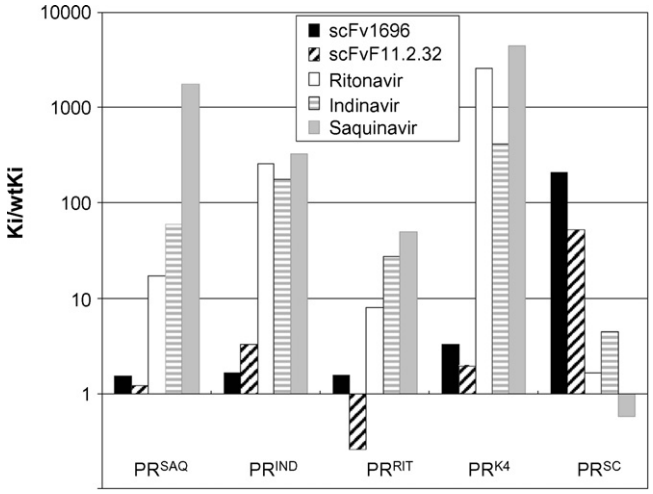

Fig. 2. Relative increase in K_i for resistant PR variants in relation to PR^{WT}.

tions (L10I/L24I/L33P/M46L/I54V/L63P/A71V/V82A/I84V), was amplified from proviral DNA originating from an HIV-positive patient under long-term HAART therapy and represents a PR variant with high cross-resistance to several PIs. All PR variants were expressed in *E. coli* and purified as described previously (Kožíšek et al., 2004; Weber et al., 2002). We also tested tethered dimer of PR (PR^{SC}) for inhibition by antibodies, as this variant was expected to be resistant to dissociative inhibition by 1696. PR^{SC} has two monomers joined by Gly–Gly linker (DiIanni et al., 1990) and is more stable than the natural PR dimer (Cheng et al., 1990).

Instead of the whole immunoglobulin molecules, single-chain fragments (scFv), the smallest functional antibody fragments, were used in this study. Our recombinant scFv contained 112 N-terminal light chain residues and 120 heavy chain N-terminal residues linked by a flexible linker (Johnson and Bird, 1991). Both scFv were expressed in *E. coli* using a T7 promoter driven expression vector and were isolated from inclusion bodies (Kurucz et al., 1995; Bayly et al., 2002).

Inhibition effect of antibody fragments on catalytic activity of resistant PR variants was evaluated at pH 7.2, i.e. under conditions optimal for antibody–antigen binding. Inhibition of PR activity *in vitro* was measured using chromogenic substrate (Kožíšek et al., 2004). The inhibition constant values (K_i) were compared to values of three well established PIs (Table 1) determined at pH 4.5. Although experimental conditions of the two assays differ, relative inhibition values (mutant to wild-type K_i value ratio) for uncharged inhibitors remain constant for various pH values (Kožíšek et al., unpublished results) and can thus be used for comparison.

Antibody fragment scFv1696 proved to be a potent inhibitor of wild-type PR and also exhibits a strong inhibitory effect on all tested PR variants with K_i values in low nanomolar range. Antibody fragment scFvF11.2.32 inhibits the wild-type enzyme 15 times less effectively than scFv1696, and the K_i values for tested protease mutants vary from 13 to 168 nM. Although the inhibitory effect of scFv on PR^{WT} is much weaker compared to clinically used inhibitors, in several cases the antibody has actually a greater inhibitory effect on drug-resistant PR variants. To assess the impact of mutations on the inhibitory potency of individual inhibitors, the relative inhibition values (mutant to wild-type K_i value ratio) were calculated (Fig. 2).

For all PR mutants, the inhibition potency of scFv is affected far less compared to clinically used inhibitors. The difference is most evident for patient-derived PR^{K4}, where the relative inhibition values for PIs vary from ~400 to 4500, while the ratio for scFv1696 and scFvF11.2.32 is 3.1 and 1.9, respectively.

Even though the mutation at position M46 is the only one within the epitope recognized by the antibodies and which could directly influence F11.2.32 binding, our results indicate that also other mutations in PR variants alter antibody binding and inhibitory effect indirectly as well, probably by changing PR structure and dynamic properties. The presence of mutations I84V, V82T and L90M destabilizes PR dimer (Xie et al., 1999) and this could influence the 1696 inhibition dissociative mechanism. Similarly, F11.2.32 inhibition mediated through the distortion of PR flap region is likely to be affected by the presence of mutations influencing the flap flexibility: mutation M46I reduces the flap flexibility (Collins et al., 1995; Piana et al., 2002), mutations L90M and G48V accelerate the flap openings and decrease the flap closing rates (Maschera et al., 1996). Why scFvF11.2.32 inhibits V82A single mutant PR^{RIT} better than the PR^{WT} remains to be answered.

The K_i values obtained for tethered dimer PR^{SC}, revealed a 200-fold increase for scFv1696 compared to PR^{WT} inhibition. Thus, the tethered PR dimer can be considered as a model variant resistant to 1696 inhibition. Strikingly, a 50-fold increase in K_i value was observed for scFvF11.2.32 as well. This indicates that the inhibitory effect of antibody F11.2.32 is not only mediated through PR flap distortion but might also be coupled to dimer dissociation. Results of molecular dynamics simulation suggested that local conformational changes in PR structure induced by F11.2.32 binding might produce long-range effects leading to easier dimer dissociation (Lescar et al., 1997). This hypothesis needs to be supported by experimental evidence.

In conclusion, recombinant fragments of 1696 and F11.2.32 antibodies are potent inhibitors of PR variants resistant to inhibitors used as anti-AIDS drugs. In contrast to clinical inhibitors, the inhibitory potency of antibody fragments is not

Table 1

	K_i values (nM)					
	PR^{WT}	PR^{SAQ}	PR^{IND}	PR^{RIT}	PR^{K4}	PR^{SC}
scFv1696	3.33 ± 0.78	5.1 ± 1.2	5.5 ± 1.3	5.17 ± 1.03	11.0 ± 1.8	684 ± 83
scFvF11.2.32	51.1 ± 3.4	62.9 ± 5.9	168.8 ± 7.4	13.22 ± 1.78	100 ± 12	2679 ± 83
Ritonavir	0.015 ± 0.003[a]	0.26 ± 0.04[a]	3.9 ± 2.0	0.12 ± 0.02[a]	39.1 ± 3.2	0.025 ± 0.004
Indinavir	0.12 ± 0.02[a]	7.1 ± 0.6[a]	21.1 ± 4.0	3.30 ± 0.04[a]	49.2 ± 2.6	0.54 ± 0.15
Saquinavir	0.04 ± 0.01[a]	70.7 ± 4.1[a]	13.1 ± 2.0	2.00 ± 0.14[a]	180.1 ± 13.1	0.023 ± 0.006

[a] As determined in (Rinnova et al., 2000).

substantially affected by the presence of mutations in tested PR variants. A more stable tethered dimer PR^{SC} proved to be a model variant resistant to dissociative inhibition by mAb1696, and it also showed resistance to inhibition by scFvF11.2.32 indicating that dimer dissociation might play a role in the mAbF11.2.32 inhibition mechanism. Strong inhibition potency toward wild-type and resistant PR variants together with available structural information (Lescar et al., 1996, 1997, 1999; Rezacova et al., 2001, 2005) makes antibodies 1696 and F11.2.32 promising leads for structure-based inhibitor design or good candidates for studies aimed at controlling the viral infection with intracellularly expressed antibodies (Rondon and Marasco, 1997).

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