Bovine leukemia virus protease: comparison with human T-lymphotropic virus and human immunodeficiency virus proteases

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Bovine leukemia virus (BLV) is a valuable model system for understanding human T-lymphotropic virus 1 (HTLV-1); the availability of an infectious BLV clone, together with animal-model systems, will help to explore anti-HTLV-1 strategies. Nevertheless, the specificity and inhibitor sensitivity of the BLV protease (PR) have not been characterized in detail. To facilitate such studies, a molecular model for the enzyme was built. The specificity of the BLV PR was studied with a set of oligopeptides representing naturally occurring cleavage sites in various retroviruses. Unlike HTLV-1 PR, but similar to the human immunodeficiency virus 1 (HIV-1) enzyme, BLV PR was able to hydrolyze the majority of the peptides, mostly at the same position as did their respective host PRs, indicating a broad specificity. When amino acid residues of the BLV PR substrate-binding sites were replaced by equivalent ones of the HIV-1 PR, many substitutions resulted in inactive protein, indicating a great sensitivity to mutations, as observed previously for the HTLV-1 PR. The specificity of the enzyme was studied further by using a series of peptides containing amino acid substitutions in a sequence representing a naturally occurring HTLV-1 PR cleavage site. Also, inhibitors of HIV-1 PR, HTLV-1 PR and other retroviral proteases were tested on the BLV PR. Interestingly, the BLV PR was more susceptible than the HTLV-1 PR to the inhibitors tested. Therefore, despite the specificity differences, in terms of mutation intolerance and inhibitor susceptibility of the PR, BLV and the corresponding animal-model systems may provide good models for testing of PR inhibitors that target HTLV-1.

INTRODUCTION

All replication-competent retroviruses, including bovine leukemia virus (BLV), encode an aspartic protease [PR; nomenclature of viral proteins is according to Leis et al. (1988)]. The function of the mature PR is critical for virion replication (reviewed by Tózsér & Oroszlan, 2003). The PR of human immunodeficiency virus (HIV) proved to be a good target of antiretroviral therapy; various PR inhibitors are now in clinical use (De Clercq, 2004). However, as in the case of reverse transcriptase inhibitors, resistance develops rapidly both in vitro and in vivo (Prejdiva et al., 2004). Most HIV PR inhibitors, including those in clinical use, were designed specifically against the wild-type HIV-1 PR, which appears to have unique specificity determinants among retroviral PRs (Tózsér, 1997; Bagossi et al., 2005). The types of amino acid residue that are mutated in drug-resistant HIV-1 PR can frequently be found in structurally equivalent positions of other wild-type retroviral PRs, as indicated for BLV in Fig. 1. Hence, comparative studies of various retroviral PRs can reveal the common features of their specificity. Consequently, the design and use of antiviral inhibitors that are efficient against several different retroviral PRs may reduce the possibility of selection for viable HIV-1 mutants when utilized in AIDS therapy.

BLV is considered to be an important model system for understanding and inhibiting human T-lymphotropic virus 1 (HTLV-1) (Willems et al., 2000). BLV infection of rabbits can be used as a model system to evaluate vaccination strategies against lymphotropic retroviruses (Altanerova et al., 2004). HTLV-1 PR, like HIV-1 PR, is a target for chemotherapy, especially in the case of tropical spastic...
paraparesis where active replication is thought to be involved in pathogenesis (Sheremata et al., 1993). The availability of an infectious molecular clone of BLV, together with cattle and sheep models (Willems et al., 1997, 2000), provides an excellent model system for the development of anti-HTLV-1 therapies. Therefore, it is important to determine to what extent BLV can serve as a model for in vivo studies using PR inhibitors. Furthermore, although it has been generally accepted that BLV cannot infect, replicate or induce cancer in humans, recent findings with state-of-the-art immunological techniques have shown that antibodies reactive against BLV are frequently present in humans, emphasizing the importance of further studies (Buehring et al., 2003).

The three-dimensional structures of several different retroviral PRs [from HIV-1, HIV-2, simian immunodeficiency virus, Rous sarcoma virus (RSV), feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV)] have been determined (Wlodawer & Gustchina, 2000), with the latest addition of the HTLV-1 PR structure (Li et al., 2005). The primary and secondary structures of all retroviral PRs resemble a single domain of the bilobal cellular aspartic PRs. Comparison of modelled structures of HIV-1, FIV and EIAV PRs with subsequently determined crystal structures revealed that the models were essentially correct in prediction of the structure of the substrate-binding sites (Weber, 1991; Wlodawer et al., 1995; Gustchina et al., 1996). Therefore, molecular modelling of retroviral PRs can serve as an important tool in the absence of crystal structures.

Retroviral PR cleavage sites are currently classified into two groups (Pettit et al., 1991; Griffiths et al., 1992; Tözser et al., 1992). Type 1 cleavage sites have an aromatic residue and Pro at P1 and P1′, whereas type 2 sites have hydrophobic residues (excluding Pro) at the site of cleavage. The P2 and P2′ positions have been shown to be critical in determining the specificity (Griffiths et al., 1992; Tözser et al., 1992). In type 1 cleavage sites of primate lentiviruses, such as HIV-1, there is a preference for Asn at P2 and a β-branched hydrophobic residue (Val or Ile) at P2′, whereas in type 2 cleavage sites, the P2 position typically has a β-branched side chain. Here, we describe a molecular model of the BLV PR, as well as a detailed specificity study of the enzyme in comparison with HTLV-1 and HIV-1 PRs, using a set of oligopeptides representing naturally occurring cleavage sites of various retroviruses, a type 2 cleavage site substrate series and various inhibitors.

**METHODS**

**Molecular modelling.** The sequence of the BLV PR was aligned with those of other PRs based on the known crystal structures for the HTLV-1 and HIV-1 PRs, as shown in Fig. 1. The crystal structure of the HTLV-1 PR with a substrate-based statine inhibitor (Li et al., 2005) was the basis for the model for BLV PR. The amino acid residues of the HTLV-1 PR were replaced by those of the BLV PR. The BLV PR dimer was modelled with the peptide substrates TKVLQVVQP and SQNYQPIVQ by using the program AMMP (Harrison, 1993), as described previously for the HTLV-1 PR (Tözser et al., 2000). The C-terminal residues 118–126 were not included in the model. A conserved water molecule was included between the flaps and the peptide, and a proton was used to stabilize the charged oxygen atoms of the two catalytic Asp residues. The positions for all new atoms were generated with the sp4 potential set using 15 cycles of the Kohonen algorithm (Harrison, 2000), followed by conjugate-gradient minimization of the non-bonded and geometrical terms. Finally, the entire PR–substrate complex was minimized by using 400 steps of conjugate gradients. Structural models were examined by using the molecular graphic programs Sybyl (Tripos Inc.) or RasMol (Sayle & Milner-White, 1995), run on Silicon Graphics workstations or Linux PCs. The models have been deposited at http://vir.sgmjournals.org.
in the Protein Database (PDB) with codes 2IM6 for the BLV PR in complex with TKVL↓VVQPK, and 2IM7 for the BLV PR with SQNY↓PIVQ.

**Enzyme assay with oligopeptide substrates.** The BLV PR was cloned into the pMal-c2 vector. The PR processed itself out from the maltose-binding protein (MBP) fusion protein and was purified to homogeneity from inclusion bodies as described previously (Zahuczky et al., 2000). The concentrations for wild-type BLV PR preparations were determined by active-site titration using IB-268, a reduced peptide analogue of an HTLV-1 cleavage site (KTKVLR↓VVQPK, where r represents a reduced peptide bond) by using the HPLC-based method (K, 3.1 mM), as described for the HTLV-1 PR (Kadas et al., 2004). Comparison of the protein content determined by Bradford assay (Bio-Rad) with the active-site values suggested that the folding of the PR was completely efficient. The PR assays were initiated by the mixing of 5 μl (12 mM to 7.8 μM) purified BLV PR with 10 μl 2× incubation buffer (0.5 M potassium phosphate buffer (pH 5.6) containing 10% glycerol, 2 mM EDTA, 10 mM dithiothreitol (DTT), 4 M NaCl) and 5 μl 0.03–2.90 mM substrate. The substrate-concentration range was selected depending on the approximate Km values. For some peptides, the kcat/Km values were determined from the linear part of the rate versus concentration profile or by using competition assays (Fersht, 1985). The reaction mixture was incubated at 37°C for 1 h and the reaction was stopped by the addition of 180 μl 1% trifluoroacetic acid (TFA), and an aliquot was injected onto a Nova-Pak C18 reversed-phase chromatography column (3.9 × 150 mm; Waters Associates Inc.) using an automatic injector. Substrates and cleavage products were separated by using an increasing water–acetonitrile gradient (0–100%) in the presence of 0.05% TFA. Amino acid analysis of the collected peaks was used to confirm the site of cleavage with at least one PR (typically with the PR of the same retrovirus) and to quantify the amount of substrate cleaved; for other PRs, the site of cleavage was assumed to be identical if cleavage products eluted with the same retention time and gave the same relative integration values as those identified by analysis. In case of ambiguity, cleavage sites were also verified by mass-spectrometric analysis. Kinetic parameters were determined by fitting the data obtained at <20% substrate hydrolysis to the Michaelis–Menten equation (or by linear fitting for kcat/Km values determined under pseudo first-order conditions) by using the Fig.P program (Fig.P Software Corporation). The standard errors of the kinetic parameters were <20%. Kinetic parameters for the same set of HTLV-1 cleavage site-based substrates were published previously for the HIV-1 and HTLV-1 PRs (Toszés et al., 2000). For the inhibitor assays, a microtitre plate-reader assay was used with a fluorescent Dabcyl/Edans-tagged analogue of the capsid ↓ nucleocapsid (CA/NC) substrate of HTLV-1 PR [RE(Edans)TKVL↓VVQPK(Dabcyl)k, where the arrow represents the cleavable bond]. The method was described in detail elsewhere (Bagossi et al., 2004). Briefly, enzyme, substrate and inhibitor were incubated in 250 mM phosphate buffer (pH 5.6) containing 5% glycerol, 1 mM EDTA, 5 mM DTT, 500 mM NaCl, 1% DMSO, in 96-well microtitre plates. The increase of fluorescence was detected at 460 nm, using an excitation wavelength of 355 nm in a Wallac 1420 Victor^2 fluorimeter–luminometer (Wallac Oy). The inner filter-effect correction and KI values were calculated with the KiDet program (Bagossi et al., 2004).

**Mutagenesis of the BLV PR and studies of its self-processing and folding ability.** The pMAL-BLVPR clone, encoding the BLV PR with an N-terminal but without a C-terminal flanking region (Zahuczky et al., 2000), was used as a template for the mutagenesis. Mutants were generated by the Quik-Change mutagenesis protocol (Stratagene) with the appropriate oligonucleotide pairs. Mutations were verified by DNA sequencing performed with an ABI Prism Dye Terminator cycle sequencing kit and a model 373A sequencer (both from Applied Biosystems). Protein expression was induced by the addition of 1 mM IPTG for 3.5 h to *Escherichia coli* DH5α cells harbouring the plasmid encoding the wild-type or mutant MBP–BLV PR fusion proteins in 5 ml cultures. After expression, cells were collected in 50 mM Tris/HCl (pH 8.2) containing 1 mM EDTA, 1 mM DTT, 1% Triton X-100, and disrupted with sonication. Protein samples were separated by SDS-PAGE and transferred to a PVDF membrane. Immunoblots were developed by using an antiserum of a rabbit immunized with chemically synthesized BLV PR (Zahuczky et al., 2000) and a peroxidase-conjugated anti-rabbit antibody, using an ECL detection kit (Pierce). Mutant PRs that showed substantial self-processing ability were purified after large-scale expression (500 ml) by ion-exchange chromatography as described for the wild-type enzyme (Zahuczky et al., 2000), whereas the other mutants were purified in the fusion form by using amylase-affinity chromatography, as described previously for human foamy virus PR (Fenyősfalvi et al., 1999). All of the mutants were >95% pure based on Coomassie staining of SDS–polyacrylamide gels (data not shown).

**RESULTS**

**Modellled structure of the BLV PR and its comparison with the structures of HTLV-1 and HIV-1 PRs**

In the absence of a crystal structure for the BLV PR, a model was built with two peptide substrates in order to understand the molecular basis for the specificity. The amino acid sequence of the BLV PR was aligned with the sequences of other retroviral PRs of known structure to determine the best starting structure for building the model. The structural alignment of the sequences of HIV-1, HTLV-1 and BLV PRs is shown in Fig. 1. Although numerous crystal structures are available for the HIV-1 PR, the HTLV-1 PR structure was determined only recently (Li et al., 2005). The BLV, HTLV-1 and HIV-1 PRs have different lengths. The HIV-1 PR is the shortest with 99 aa, followed by the HTLV-1 PR (125 aa), whilst the BLV PR has 126 aa. Despite the differences in length, the crystal structures of the HIV-1 and HTLV-1 PRs share a conserved core region that includes the substrate-binding site and the dimer interface. Therefore, the molecular model of the BLV PR was predicted to share the conserved core region of the HIV-1 and HTLV-1 PRs. The extra amino acids of the BLV and HTLV-1 PRs form longer loop structures on the surface of the molecules. In addition, the BLV and HTLV PRs have C termini that are extended by an additional 10 aa compared with the HIV-1 PR. The function of the C-terminal extension (aa 116–125) is controversial. Based on some previous studies, these residues were not required for activity of the HTLV-1 PR (Precigoux et al., 1993; Herger et al., 2004), whilst five of the C-terminal residues (aa 116–120) appeared to be important in another study (Hayakawa et al., 1992). The final model of the BLV PR dimer with substrate had a root mean square (rms) difference of 0.79 Å (0.079 nm) for 223 pairs of Cx atoms compared with the crystal structure of the HTLV-1 PR, and 1.72 Å for 175 pairs of Cz atoms compared with the crystal structure of the HIV-1 PR (with PDB code 2AOD). These values are comparable to the rms difference of 1.25 Å observed for 194
Table 1. Residues predicted to form the subsites of the BLV, HTLV and HIV PRs

<table>
<thead>
<tr>
<th>Subsite</th>
<th>BLV/HTLV-1/HIV-1 residues*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4</td>
<td>Glu32/Asp30/Asp28, Asn39/Met37/Asp36, Ala56/Ser55/Met56, Val37/Val35/Ile37, Leu55/Leu53/Glu46, Tyr80/Phc57/Val56, Trp90/Leu92/Gln90, Leu92/Leu90/Ile92</td>
</tr>
<tr>
<td>S3</td>
<td>Arg30/Arg28/Arg26, Leu31/Leu30/Leu32, Glu32/Asp30/Asp28, Leu56/Leu54/Glu46, −/−/Thr80†, −/−/Pro89†, Trp90/Trp89/Val90</td>
</tr>
<tr>
<td>S2</td>
<td>(Gly32/Gly34/Gly37), Ala37/Ala35/Ala28, (Glu32/Asp30/Asp28), Asn39/Met37/Asp36, Val37/Val35/Val32, Val37/Val35/Ile37, Leu55/Leu53/Glu46, Gly46, Gly59/Gly58/Gly49, Ala56/Ala55/Ile56, Tyr80/Phc57/Val56, Leu92/Leu90/Ile92, Ile100/Ile98/Ile94, Ala56/Ala55/Ile56, −/−/Thr80†, −/−/Pro89†, Trp90/Trp89/Val90</td>
</tr>
<tr>
<td>S1</td>
<td>Arg30/Arg28/Arg26, Leu31/Leu30/Leu32, (Asp34/Asp32/Asp37), (Asp34/Asp32/Asp37), (Gly32/Gly34/Gly37), (Gly59/Gly58/Gly49), Ala56/Ala55/Ile56, −/−/Thr80†, −/−/Pro89†, Trp90/Trp89/Val90, Ile101/Ile100/Ile100/Ile94</td>
</tr>
</tbody>
</table>

*Residues that are different in all three PRs are indicated in bold, whilst residues differing in two PRs are indicated in italics. Amino acid residues in the second subunit are indicated by a prime. Only the residues forming the S4–S1 subsites are given. Primed binding sites (such as S1′) have the same composition as the non-primed ones, but they are built from residues of the other subunit. Residues involved only in interactions with the main-chain atoms of the peptide substrate are shown in parentheses.

†Whilst Thr 80’ and Pro 81’ of the HIV-1 PR participate in the formation of binding sites, the corresponding residues of the BLV and HTLV-1 PRs do not interact with ligand; their position is very different due to an upstream loop insertion.

pairs of Cα atoms in the dimers of the RSV S9 PR and HIV-1 PR with inhibitor (Wu et al., 1998). Residues forming the substrate-binding sites of BLV, HTLV-1 and HIV-1 PRs are listed in Table 1, and a schematic representation of the substrate-binding sites is given in Fig. 2.

Studies on the specificity of the BLV PR using oligopeptides representing naturally occurring cleavage sites in different retroviruses

To compare the specificity of the BLV PR with that of the HTLV-1 and HIV-1 PRs, a large set of oligopeptides representing naturally occurring cleavage sites in HIV-1, HIV-2, EIAV, RSV, mouse mammary tumor virus (MMTV), Moloney murine leukemia virus (MMLV), HTLV-1 and BLV was used. These peptides have been characterized previously. They were found to be hydrolysed by the respective PR coded within the same virus and have been used to compare the specificity of the HTLV-1 and HIV-1 PRs (Kadas et al., 2004). The measured specificity constants are shown in Table 2. The majority of the peptides were hydrolysed by both the BLV (40 of 50) and HIV-1 (40 of 50) PRs. However, only two peptides were not substrates for either enzyme and 16 were substrates for only one of them (Table 2). By selecting 1 mM−1 s−1 as an arbitrary threshold to separate efficient substrates from inefficient ones, the majority of the substrates can be considered as efficient in both cases. However, only 20 of the 50 peptides were substrates for the HTLV-1 PR and only half of them were hydrolysed efficiently (Kadas et al.,

![Schematic representation of the HTLV-1 CA/NC substrate in the S4–S3′ subsites of the BLV, HTLV-1 and HIV-1 PRs. The relative size of each subunit is indicated approximately by the area enclosed by the curved line around each substrate side chain. PR residues important in forming the subsites are shown only for those that differ between at least two PRs as BLV/HTLV-1/HIV-1 residues. Residues of the BLV PR that were mutated in this study are underlined.](http://vir.sgmjournals.org)
Table 2. Comparison of the specificity of the BLV and HIV-1 PRs with peptides representing naturally occurring cleavage sites in various retroviruses

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Virus</th>
<th>Site*</th>
<th>Substrate sequence</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BLV PR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HIV-1 PR†</td>
</tr>
<tr>
<td>1</td>
<td>HIV-1</td>
<td>MA/CA</td>
<td>VSQNY ↓ PIVQ</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>HIV-1</td>
<td>CA/p2</td>
<td>KARVL ↓ AEAMS</td>
<td>5.7</td>
</tr>
<tr>
<td>3</td>
<td>HIV-1</td>
<td>p2/NC</td>
<td>TATIM ↓ MQRGN</td>
<td>32.5‡</td>
</tr>
<tr>
<td>4</td>
<td>HIV-1</td>
<td>NC/p1</td>
<td>ERQAN ↓ FLGKI</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>HIV-1</td>
<td>p1/p6</td>
<td>RPGNF ↓ LQSRP</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>HIV-1</td>
<td>IN/p6</td>
<td>DKELY ↓ PLTLS</td>
<td>12.5</td>
</tr>
<tr>
<td>7</td>
<td>HIV-1</td>
<td>TF/PR</td>
<td>VSNF ↓ PQITL</td>
<td>0</td>
</tr>
<tr>
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<td>HIV-1</td>
<td>PR/RT</td>
<td>CTLNF ↓ PISP</td>
<td>2.5</td>
</tr>
<tr>
<td>9</td>
<td>HIV-1</td>
<td>RT/RI</td>
<td>AETF ↓ YVDGAA</td>
<td>0.4§</td>
</tr>
<tr>
<td>10</td>
<td>HIV-1</td>
<td>RT/IN</td>
<td>IRKIL ↓ FLDG</td>
<td>71.3</td>
</tr>
<tr>
<td>11</td>
<td>HIV-2</td>
<td>MA/CA</td>
<td>KGGNY ↓ PVQHV</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
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<td>CA/p2</td>
<td>KARLM ↓ AEALK</td>
<td>0.05§</td>
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<td>13</td>
<td>HIV-2</td>
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<td>IPFAA ↓ AQQRK</td>
<td>0</td>
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<td>14</td>
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<td>KPRNF ↓ PVAQV</td>
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<td>15</td>
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<td>TF/PR</td>
<td>RGLAA ↓ PQFSL</td>
<td>0.002§</td>
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<td>16</td>
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<td>HIV-2</td>
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<td>IQRQL ↓ FLEKI</td>
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<tr>
<td>18</td>
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<td>PSEEE ↓ PIMID</td>
<td>4.8Il</td>
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<td>QKMM ↓ LAKAL</td>
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<tr>
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<td>NC/p9</td>
<td>QKQTG ↓ PIQQK</td>
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<tr>
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<td>AKLVL ↓ AQLSK</td>
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<td>RT/UT</td>
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<tr>
<td>23</td>
<td>RSV</td>
<td>MA/p2A</td>
<td>GTSCY ↓ CHGTA</td>
<td>0</td>
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<tr>
<td>24</td>
<td>RSV</td>
<td>p2B/p10</td>
<td>PPYYG ↓ SGLYP</td>
<td>0</td>
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<tr>
<td>25</td>
<td>RSV</td>
<td>p10/CA</td>
<td>PVMAM ↓ PVPVK</td>
<td>15.1ll</td>
</tr>
<tr>
<td>26</td>
<td>RSV</td>
<td>CA/p3</td>
<td>IAAAM ↓ SSAIQ</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>RSV</td>
<td>p3/NC</td>
<td>IQPLIM ↓ AVVNR</td>
<td>137ll</td>
</tr>
<tr>
<td>28</td>
<td>RSV</td>
<td>NC/PR</td>
<td>PPAVS ↓ LAMTM</td>
<td>1.2‡</td>
</tr>
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<td>RSV</td>
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<td>RATVL ↓ TVALH</td>
<td>173ll</td>
</tr>
<tr>
<td>30</td>
<td>RSV</td>
<td>RT/IN</td>
<td>TFQAY ↓ PLREA</td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>MMTV</td>
<td>MA/pp21</td>
<td>SDLVL ↓ LSAEARR</td>
<td>(11.4)ll,#</td>
</tr>
<tr>
<td>32</td>
<td>MMTV</td>
<td>pp21/p3</td>
<td>DSKAF ↓ LADTW</td>
<td>0.03</td>
</tr>
<tr>
<td>33</td>
<td>MMTV</td>
<td>p3/p8</td>
<td>DELIL ↓ PVKRR</td>
<td>2.6†</td>
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<tr>
<td>34</td>
<td>MMTV</td>
<td>p8/n</td>
<td>PVGFAG ↓ AMA</td>
<td>0 &lt;0.01</td>
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<tr>
<td>35</td>
<td>MMTV</td>
<td>n/CA</td>
<td>LTTFF ↓ PVVFMRRE</td>
<td>1.3‡</td>
</tr>
<tr>
<td>36</td>
<td>MPMV</td>
<td>p12/CA</td>
<td>PKDIF ↓ PVTTET</td>
<td>0.45</td>
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<tr>
<td>37</td>
<td>MMLV</td>
<td>MA/p12</td>
<td>PRSSL ↓ PALTP</td>
<td>0.2</td>
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<tr>
<td>38</td>
<td>MMLV</td>
<td>p12/CA</td>
<td>TSQAF ↓ PLRAG</td>
<td>0.1</td>
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<tr>
<td>39</td>
<td>MMLV</td>
<td>CA/NC</td>
<td>MSKLL ↓ ATVVS</td>
<td>7.9ll</td>
</tr>
<tr>
<td>40</td>
<td>MMLV</td>
<td>NC/PR</td>
<td>QTSSL ↓ TLDDQ</td>
<td>3.5</td>
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<tr>
<td>41</td>
<td>MMLV</td>
<td>PR/RT</td>
<td>PLQVL ↓ TLIERR</td>
<td>43.9ll</td>
</tr>
<tr>
<td>42</td>
<td>MMLV</td>
<td>RT/IN</td>
<td>TSTLL ↓ IENSS</td>
<td>(7.5)ll,**</td>
</tr>
<tr>
<td>43</td>
<td>HTLV-1</td>
<td>MA/CA</td>
<td>APQV ↓ PVMHP</td>
<td>186ll,††</td>
</tr>
<tr>
<td>44</td>
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<td>45</td>
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<td>DPASIL ↓ PVIP</td>
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<td>PR/pX</td>
<td>KGPPVL ↓ PIQAP</td>
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<td>MA/CA</td>
<td>PPAIL ↓ PISE</td>
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<td>ELEC ↓ LSIPL</td>
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<td>PR/p13</td>
<td>PPMVG ↓ VLDAI</td>
<td>0.4††</td>
</tr>
</tbody>
</table>

*Nomenclature is according to Leis et al. (1988). The HIV-1 p2 and p1 are frequently called SP1 and SP2, respectively.
Substrate specificity of BLV protease

†The corresponding specificity constants were published previously, together with the values for the HTLV-1 PR (Kádas et al., 2004).
‡This peptide was assayed as a competitive substrate with DKELY ↓ PLTSL to determine the specificity constant.
§In the studied concentration range, the [S] versus ν curve was linear and was used to determine the $k_{cat}/K_m$ value.
¶This peptide was assayed as a competitive substrate with VSQLY ↓ PIVQ to determine the specificity constant.
¶Residues that were added to the cleavage-site sequences to enhance the solubility of the peptides are underlined.
#The HIV-1 PR cleaves this peptide at the original MMTV cleavage site (L ↓ L), whereas the BLV PR cleaves one residue upstream, at the V ↓ L bond.
**This peptide is also cleaved by the BLV PR at the L ↓ L site, with a $k_{cat}/K_m$ of 9.0 mM$^{-1}$ s$^{-1}$.
††This peptide was cleaved at the C ↓ L site by the HIV-1 PR.
‡‡These data were taken from Zahuczky et al. (2000).

2004). In most cases, the lack of hydrolysis could not be attributed to the lack of binding of the peptides, as they were able to inhibit the enzyme activity when tested with the HTLV-1 P1 Phe-substituted CA/NC peptide (data not shown). The specificity results on these peptides indicated a substantially broader specificity of the BLV and HIV-1 PRs compared with the HTLV-1 PR. Interestingly, one residue shift from the authentic site of cleavage was observed in three cases (Table 2). Peptide 31 was cleaved at the authentic site (DLVI ↓ LSAE) by HIV-1 PR, but at a shifted site (SDLV ↓ LLLSA) by the BLV PR. Peptide 42 was also cleaved at a shifted site (TSTL ↓ LIEN) besides the concomitant cleavage at the authentic site (STLL ↓ IENS) by the BLV PR, whilst this peptide was not a substrate for the HIV-1 PR. On the other hand, peptide 49 was cleaved at a shifted site by the HIV-1 PR (ELEC ↓ LLSI) compared with the authentic one (LECL ↓ LSIP). Although these shifts are in agreement with the substantially more hydrophilic nature of the S4, S2 and S2′ subsites of HIV-1 (as discussed below), they cannot be explained readily by the specificity of the PRs, due to its strong sequence-context dependence. Only a few peptides were efficiently cleaved substrates with significant specificity constants ($k_{cat}/K_m > 1$ mM$^{-1}$ s$^{-1}$) for all three PRs: these include the peptides representing the HIV-1 reverse transcriptase/integrase (RT/ IN), HIV-2 RT/IN, RSV p3/NC, MMTV p3/p8 and HTLV-1 matrix/capsid (MA/CA), CA/NC and PR/pX cleavage sites. Common characteristics of these generally efficient substrates are that all of them contain β-branched residues (Val or Ile) at P2 and also at P2′, with the exception of the two HIV sites, and, with one exception, they contain Leu at P1 (Table 2).

Characterization of the specificity of the BLV PR with a series of peptides representing the CA/NC cleavage site of HTLV-1

A peptide series representing the CA/NC cleavage site of HTLV-1 was previously utilized successfully to compare the specificity of the HIV-1 and HTLV-1 PRs (Tőzsér et al., 2000), as well as to determine the specificity changes exerted by HIV-1 PR-like mutations in the HTLV-1 PR (Kádas et al., 2004). This set contains peptides with N-terminally shortened sequences and peptides with single amino acid substitutions in the P4–P1′ positions, and proved useful in characterizing the specificity of the BLV PR in this study. The kinetic constants are given in Table 3, together with the specificity constants ($k_{cat}/K_m$) determined previously for the HTLV-1 and HIV-1 PRs (Tőzsér et al., 2000). The specificity constants obtained for the BLV PR were in the same range as those determined for the other two PRs; however, hydrolysis with the BLV PR generally resulted in relatively low $K_m$ and $k_{cat}$ values (Table 3), as was also observed for the natural cleavage-site peptides (data not shown).

Size of the substrate-binding site. The original unmodified peptide was a good substrate for all three PRs. It is important to note that this peptide was also a good substrate for several other retroviral PRs (unpublished data), therefore it can be considered as a general PR substrate. Interestingly, the shortened peptides were even better substrates than the full-length peptide for the BLV PR. In this aspect, the BLV PR appears to be more similar to the HIV-1 PR than to the HTLV-1 PR, for which the best substrate was the full-length decapeptide. This result suggested that the BLV PR and HIV-1 PR have less extended substrate-binding sites than does the HTLV-1 PR.

S4 binding site. All of the P4-substituted peptides were good substrates for the BLV PR. The best substrates were obtained when the original Thr was exchanged for hydrophobic amino acid residues, such as Val or Leu, similar to the results with the HTLV-1 PR. However, unlike these two enzymes, the HIV-1 PR preferred the more hydrophilic Ser (and Thr) in this position.

The S4 subsite of retroviral PRs is close to the surface and more or less open to the solvent. A side chain at P4 may either interact with the solvent molecules or form interactions with residues of the binding pockets. A unique feature of the HIV-1 PR and other primate lentiviral PRs is that they contain Asp30, whereas most of the retroviral PRs, including the BLV and HTLV-1 PRs, contain
Table 3. Comparison of the specificity of the BLV, HTLV-1 and HIV-1 PRs with shortened as well as substituted analogues of the HTLV-1 CA/NC cleavage-site peptide

ND, Not determined; a small degree of hydrolysis was observed when incubated with concentrated PR. NC, Not cleaved; no hydrolysis was observed when incubated with concentrated PR for 20 h at 37°C.

<table>
<thead>
<tr>
<th>Substrate sequence*</th>
<th>BLV PR</th>
<th>HTLV-1 PR†</th>
<th>HIV-1 PR†</th>
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<tr>
<td></td>
<td>(K_m) (mM)</td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>(k_{cat}/K_m) (mM(^{-1}) s(^{-1}))</td>
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<td>VVQPK</td>
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<td>0.27</td>
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<tr>
<td>KTKSL</td>
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<td>0.35</td>
</tr>
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<td></td>
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</tr>
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</tr>
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<td>KTKVL</td>
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</tr>
<tr>
<td>KTKVL</td>
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<tr>
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<td>PVQPK</td>
<td>0.063</td>
<td>0.85</td>
</tr>
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</table>

*Substituted residues are in bold.
†The corresponding specificity constants were published previously (Tószér et al., 2000).
‡This peptide was assayed as competitive substrate with VSQLY ↓ PIVQ to determine the \(k_{cat}/K_m\) value.
§In the studied concentration range, the \([S]\) versus \(v\) curve was linear and was used to determine the \(k_{cat}/K_m\) value.
||This peptide was assayed as competitive substrate with KTKVL ↓ KVQPK to determine the \(k_{cat}/K_m\) value.
uncharged residues at the equivalent position (Table 1; Fig. 1). The BLV PR has Asn at this position, which still may form hydrogen bonds with the Thr residue of the original HTLV-1 substrate, whereas the corresponding HTLV-1 PR residue is Met. Besides the critical Asn residue, the other residues of S4 form a more hydrophobic pocket in the BLV PR compared with the HIV-1 PR (Table 1). Therefore, BLV PR S4 resembles the S4 subsite of other (non-primate) retroviral PRs more closely (Tozsér, 1997; Bagossi et al., 2005). Interestingly, the Asp 30 to Ser mutation occurs frequently in the HIV-1 PR with drug resistance to PR inhibitors (Fig. 1).

**S3 binding site.** All of the studied P3-substituted peptides were hydrolysed well by the BLV PR (Table 3). The range of specificity constants was smaller for the BLV PR than for the other two enzymes: the only large deviation was with the Ala substitution. Based on these results, various amino acid residues can be accommodated at this subsite by the enzymes. The S3 subsites of all retroviral PRs characterized so far consist of a relatively open and deep pocket that is near the surface. The side chains of amino acids in the P3 position have freedom of movement to interact with either hydrophobic residues near the S1 subsite or polar residues at the surface of the enzyme (Cameron et al., 1993). Furthermore, in the BLV PR, Leu 58 and Trp 99′ can interact with hydrophobic P3 residues (as predicted for the identical residues in the HTLV-1 PR). Val 82′ of HIV-1 PR, which is structurally equivalent to the Trp residues of the deltaretroviral PRs, provides a deeper pocket, which explains why substrates containing P3 Phe or Leu were much better for the HIV-1 PR than substrates with small- or medium-sized side chains, such as Ala or Val.

**S2 binding site.** By using the HTLV-1 cleavage-site peptide series, substitution of Val by other hydrophobic residues provided similar or even better substrates for the BLV PR (Table 3). The best substrates were obtained with Phe and Ile substitutions. The same substitutions exerted various effects on the other two PRs: they were typically very unfavourable for the HIV-1 PR, but some of them were tolerated well by the HTLV-1 PR (Table 3). The preference for larger hydrophobic P2 residues is consistent with the presence of smaller S2 residues Ala and Val in both the BLV and HTLV-1 PRs, instead of Ile 47 and Ile 50 in HIV-1 PR. Unlike the HTLV-1 and HIV-1 PRs, the BLV PR tolerated the hydrophilic Asn residue at this position well, even though Asn is the typical P2 residue in type 1, but not in type 2, cleavage sites. In a recent comparative study of 11 retroviral PRs using a type 1 substrate series based on the MA/CA cleavage site of HIV-1, the S2 binding site of the BLV PR appeared to be a relatively large pocket, and a preference for Leu was observed (Bagossi et al., 2005). However, it is important to note that the specificity of retroviral PRs appears to be strongly context-dependent, as reviewed for HIV-1 PR (Tózsér & Oroszlan, 2003).

The S2 subsites of all PRs are more restricted sterically than the S4 and S3 subsites and are predicted to accommodate mainly hydrophobic residues. Based on the side chains forming S2 (Table 1), the BLV PR is expected to have a somewhat larger pocket than the HIV-1 PR. The ability of the HIV-1 PR to accommodate more polar residues may be related to the presence of Asp 30 (Cameron et al., 1993). Except for Asn 38 and Tyr 68, which are Met and Phe, respectively, in the HTLV-1 PR, all other residues forming the S2 subsite in the BLV PR S2 are identical to those in the HTLV-1 PR, but substantially different from those forming the S2 site in the HIV-1 PR. However, due to the presence of Asn 38 and Tyr 68, the S2 subsite of BLV PR is more hydrophilic than the corresponding S2 subsite of the HTLV-1 PR, in good agreement with the relatively good kinetic efficiencies obtained for peptides with Asn, Asp or Ser at the P2 position.

**S1 binding site.** Interestingly, various peptides with P1 substitutions, including charged or hydrophilic residues, in the HTLV-1 cleavage site peptide set provided fairly good substrates for the BLV PR, whereas the HTLV-1 and HIV-1 PRs tolerated only hydrophobic residues and Gly at this position. P1 Tyr or Phe substitutions were the best for the PRs, followed by other hydrophobic residues in varied order (Table 3). Based on structural analysis, the S1 subsite is mainly hydrophobic and buried inside the protein. Many of the residues forming the S1 site of the retroviral PRs are conserved. However, there are some non-conserved changes (Table 1). The Thr 80′–Pro 81′–Val 82′ part of the HIV-1 PR and the corresponding regions of other retroviral PRs provide a ‘roof’ over the S1 binding site and these residues are involved in determining the size of the pocket. In the HTLV-1 and BLV PRs, these three residues are replaced by a Trp residue, as the other two side chains cannot interact directly with the substrate. Trp 99′ of the BLV PR renders the S1 pocket smaller than that of the HIV-1 PR, which has Val 82 in the equivalent position. It is an interesting feature of the retroviral PRs that a bulky P1 side chain is predicted to occupy a region that is also a part of the S3 pocket. The S1 and S3 subsites seem to overlap much more than the S4–S2, S2–S1′, S1–S2′ and S1′–S3′ pockets (Tózsér et al., 1996). This could be a major factor in the sequence-context dependence of the results of specificity studies based on different peptide sequences (Tózsér & Oroszlan, 2003).

**S1′ binding site.** Hydrophilic amino acid residues at the P1′ position were tolerated relatively well by the BLV PR compared with the other two PRs. The Pro substitution provided a large increase in $K_m$ for the HTLV and HIV-1 PRs (Tózsér et al., 2000), whereas the relatively large $k_{cat}$ in the case of BLV PR yielded a relatively high specificity constant (Table 3). Due to the symmetry of the PR, the S1′ subsite is structurally very similar to the S1 subsite, which is formed by the corresponding residues of the other subunit of the dimer. Therefore, the better tolerance of the BLV PR for various P1′ residues is in good agreement with...
the predicted participation of Trp 99 in this binding site, rather than the loop of Thr 80, Pro 81 and Val 82 as in the HIV-1 PR.

**Sensitivity of the BLV PR towards substrate-binding-site mutations**

To further understand the specificity of the BLV PR and its tolerance to mutations, several residues in the substrate-binding site were mutated to the corresponding HIV-1 PR residue, individually or in combination (shown underlined in Fig. 2). The self-processing (cis) activity and activity on an oligopeptide substrate (trans-activity) of these mutants are summarized in Table 4. Many of the mutants appeared to be defective in self-processing ability, indicating the lack of proper folding or activity of the folded mutants (Fig. 3). The mutant containing Asp in place of Asn 38 behaved aberrantly, as a partial self-processing resulted in a truncated PR; this is predicted to be due to the creation of a new cleavage site by the mutation (Fig. 3). The sensitivity towards mutation may extend to residues outside the substrate-binding site. Based on the molecular models, Asn 97 and Lys 98 of BLV PR cannot participate directly in ligand binding, unlike the corresponding Thr 80 and Pro 81 of HIV-1 PR (Fig. 1; Table 1); nevertheless, mutation of Lys 98 to Pro also blocked the activity of the enzyme completely (Fig. 3). The enzyme with all six mutations (Table 4).

**Table 4.** Description of the mutant BLV PRs, altered substrate-binding sites, their self-processing ability, folding efficiency and kinetic parameters for hydrolysis of the HTLV-1 CA/NC cleavage-site peptide (KTKVL ↓ VVQPK)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Affected site or region*</th>
<th>Self-processing/folding efficiency (%)†</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLV PR (wt)‡</td>
<td>–</td>
<td>100/100</td>
<td>0.0110</td>
<td>0.270</td>
<td>24.50</td>
</tr>
<tr>
<td>BLV PR (N38D fusion form)</td>
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<td>0.023</td>
<td>0.13</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
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<tr>
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<td>All</td>
<td>0/ND</td>
<td>NC</td>
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</tr>
</tbody>
</table>

*It should be noted that, due to the symmetrical, homodimeric nature of the PR, the respective primed binding sites (S4’, S2’ etc.) are also affected by the mutation(s), although these sites were not probed in this study.
†Self-processing efficiency was calculated from densitometric analysis of immunobLOTS of small culture expressions, whilst folding efficiency was calculated from the ratio of active enzyme determined by using active-site titration and total protein amount.
‡These data are taken from Table 3.
§The enzyme was assayed by using a competitive substrate (KTKVA ↓ VVQPK) to determine the $k_{cat}/K_m$ value.

**Fig. 3.** Expression of the BLV PR mutants as MBP-fusion proteins. (a) Schematic representation of the fusion proteins used in this study. The 8 aa N-terminal flanking sequence allowing the PR to process itself out of the fusion protein is indicated above the boxes representing the Gag–Pro polyprotein. (b) Self-processing capability of mutant BLV PRs. Fusion proteins were expressed in E. coli cells and the self-processing capability of the PRs was analysed by immunoblotting using anti-BLV PR antisera. Mutations in the BLV PR coding regions are indicated above the lanes. ‘Flap’ refers to the triple flap mutant V57I/L58G/A60I, ‘Roof’ refers to the triple mutant D97T/K98P/W99V and ‘Flap+Roof’ refers to the enzyme with all six mutations (Table 4).
highly purified, concentrated MBP–PR fusion proteins with an oligopeptide substrate (data not shown). Only one mutant, W99V, was able to self-process itself completely from the fusion protein. Previous studies with the HTLV-1 PR also indicated a high sensitivity towards mutations (Kádas et al., 2004), whereas HIV-1 PR activity showed much better tolerance of mutations of the substrate-binding residues.

**Inhibition profile of the BLV PR in comparison with that of the HTLV-1 and HIV-1 PRs**

Several potent inhibitors that target the HIV-1 PR are currently used in clinical therapy. We have tested the clinical inhibitors saquinavir, ritonavir, nelfinavir, amprenavir and indinavir, as well as other retroviral PR inhibitors, on the BLV PR (Table 5). Most of these inhibitors were tested previously on HIV-1 and HTLV-1 PRs by using the same fluorescent assay (Bagossi et al., 2004) or by the more conventional HPLC-based assay (Louis et al., 1999); for comparison, the data obtained for the HTLV-1 and HIV-1 PRs with the fluorescent measurements are also shown. Whilst all of the HIV-1 PR inhibitors (compounds 1–11) inhibited the HIV-1 PR with a $K_i$ value of $<30$ nM, most of them were weak inhibitors or practically inactive on the BLV PR: only amprenavir and compound 3 inhibited this enzyme to an appreciable extent. Interestingly, all four HTLV-1 PR sequence-based inhibitors inhibited the BLV PR more strongly than they did the HTLV-1 PR (Table 5), whilst they were inactive against the HIV-1 PR. This effect appears to correlate with the lower $K_m$ values of the corresponding substrates for the BLV PR (data not shown). One of these inhibitors, IB-268, was very potent against the BLV PR, having an unusually low $K_i$ value considering the moderate ionic strength used in the assay, and is predicted to be useful for *in vivo* inhibition studies on the BLV PR. This compound was even more potent at the high-ionic-strength conditions used in the HPLC-based assay and was utilized to perform the active-site titration of the enzyme (see Methods). This strong dependence of $K_i$ values on ionic strength has been observed previously (Bagossi et al., 2004). With the exception of amprenavir, which discriminated substantially between the BLV and HTLV-1 PRs, there is a correlation ($R=0.91$) between the inhibition values obtained for the two deltaretrovirus PRs (data not shown).

**DISCUSSION**

Broad-spectrum PR inhibitors, developed based on the understanding of the specificity features of retroviral PRs, may reduce the possibility of selection for viable HIV-1 mutants when utilized in AIDS therapy. Studies on the

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>BLV PR</th>
<th>HTLV-1 PR</th>
<th>HIV-1 PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Saquinavir*</td>
<td>&gt;20 000</td>
<td>&gt;20 000†</td>
<td>0.001†</td>
</tr>
<tr>
<td>2 Ritonavir</td>
<td>11 472</td>
<td>&gt;20 000†</td>
<td>0.366†</td>
</tr>
<tr>
<td>3 Nelfinavir</td>
<td>&gt;20 000</td>
<td>&gt;20 000†</td>
<td>0.088†</td>
</tr>
<tr>
<td>4 Amprenavir</td>
<td>114</td>
<td>&gt;20 000†</td>
<td>0.001†</td>
</tr>
<tr>
<td>5 Indinavir</td>
<td>4 475</td>
<td>3 475†</td>
<td>0.440†</td>
</tr>
<tr>
<td>6 DMP-323</td>
<td>&gt;20 000</td>
<td>&gt;20 000†</td>
<td>0.914†</td>
</tr>
<tr>
<td>7 Compound 3‡</td>
<td>63</td>
<td>244</td>
<td>5.5</td>
</tr>
<tr>
<td>8 HBY-793§</td>
<td>1 005</td>
<td>439</td>
<td>26</td>
</tr>
<tr>
<td>9 UK 88,947‖</td>
<td>&gt;20 000</td>
<td>&gt;20000</td>
<td>1.5</td>
</tr>
<tr>
<td>10 KH 164¶</td>
<td>1 715</td>
<td>5 605</td>
<td>28</td>
</tr>
<tr>
<td>11 LP-149#</td>
<td>12 959</td>
<td>12 582</td>
<td>0.54</td>
</tr>
<tr>
<td>12 Ala-Pro-Gln-Val-Val-Met-His-Pro*</td>
<td>181</td>
<td>2 300†</td>
<td>530†</td>
</tr>
<tr>
<td>13 Lys-Thr-Lys-Val-Sta-Gln-Pro-Ly*</td>
<td>10 857</td>
<td>&gt;20 000†</td>
<td>&gt;20 000†</td>
</tr>
<tr>
<td>14 IB-269: Ala-Pro-Gln-Val-Leu-r-Pro-Val-Met-His-Pro</td>
<td>88</td>
<td>&gt;465†</td>
<td>20 000†</td>
</tr>
<tr>
<td>15 IB-268: Lys-Thr-Lys-Val-Leu-r-Pro-Val-Gln-Pro-Lys</td>
<td>13</td>
<td>298†</td>
<td>11 215†</td>
</tr>
</tbody>
</table>

*These inhibitors were tested previously in the HPLC-based assay system (Zahuczky et al., 2000).
†These data were taken from Bagossi et al. (2004).
§Compound 3 (Grobelyn et al., 1990) is a potent inhibitor of the HIV-1, HIV-2 and EIAV PRs.
¶This compound inhibits the EIAV PR with a $K_i$ of 0.1 nM (Powell et al., 1996).
‖This compound (Baboonian et al., 1991) has been designed against the HIV-1 PR.
¶¶A modest inhibitor of the MMLV PR with a $K_i$ of 62 nM (Menendez-Arias et al., 1993).
#This statine-based HIV-1 PR inhibitor has been shown to inhibit the FIV PR with a $K_i$ of 260 nM (Wlodawer et al., 1995).
BLV PR may aid the development of such advanced inhibitors.

The specificity of the BLV PR was characterized by using a large set of oligopeptides representing naturally occurring cleavage sites in various retroviruses. Based on the results, the BLV PR appears to have a broad substrate specificity, similar to that of HIV-1 PR, but unlike that of the related HTLV-1 PR (Kádas et al., 2004). Interestingly, this broad specificity is not entirely apparent from the analysis of maturation cleavage sites of BLV, although the sites are more varied within the P2–P2′ positions compared with the HTLV-1 cleavage sites (see Table 2). The sequences processed by all three PRs appear to resemble those observed in distantly related retro-elements, including Ty3/Gypsy and Ty1. One of these substrates, based on the CA/NC site of HTLV-1, was used previously to develop a fluorescent assay for the HTLV-1 PR (Bagossi et al., 2004) and was applied successfully to the BLV PR (Table 5).

The broader specificity of the BLV PR than the HTLV-1 PR was also verified by mapping the individual substrate-binding sites using a set of oligopeptides with single amino acid substitutions. Furthermore, the substrate-binding site of the BLV PR appeared to be less extended than that of the HTLV-1 PR. Whilst both the BLV and HTLV PRs showed a preference for larger hydrophobic P2 and P1 residues, the BLV PR tolerated hydrophilic or even charged residues at these positions much better. Nevertheless, in most aspects, the specificity of individual subsites of the BLV PR resembled that of the HTLV-1 PR more closely, in good agreement with these positions much better. Nevertheless, in most aspects, the specificity of individual subsites of the BLV PR resembled that of the HTLV-1 PR more closely, in good agreement with the more similar sets of residues predicted to be involved in substrate binding compared with those of the HIV-1 PR.

The inhibition profile of the BLV PR resembles that of the HTLV-1 PR more than that of the HIV-1 PR. However, the BLV PR was inhibited substantially better than the HTLV-1 PR. This effect appears to correlate with the generally lower $K_m$ values observed for the BLV PR compared with the HTLV-1 PR.

It appears to be a common characteristic of the BLV and HTLV-1 PRs that their folding capability and/or catalytic efficiency are much more sensitive to mutations than those of many other retroviral PRs, especially HIV PRs. It is important to note that, whilst HIV has a high mutation rate, HTLV-1 (and predictably BLV) do not utilize the error-prone reverse transcriptase to generate substantial sequence diversity, as the virus maintains its high proviral load chiefly through clonal expansion of HTLV-infected cells (Lemey et al., 2005). As a consequence, BLV, like HTLV-1, has not undergone the extensive mutational changes that might have optimized the HIV-1 PR for high catalytic efficiency, as well as flexibility in tolerating mutations under selective pressure. In conclusion, based on our studies, despite the specificity differences, in terms of mutation intolerance and inhibitor susceptibility of the PR, BLV and the corresponding animal-model systems may provide good models for testing PR inhibitors that would be developed by in vitro studies against the PR of HTLV-1.

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REFERENCES


Substrate specificity of BLV protease


