Inhibition of Human Endogenous Retrovirus-K10 Protease in Cell-free and Cell-based Assays*

A full-length and C-terminally truncated version of human endogenous retrovirus (HERV)-K10 protease were expressed in Escherichia coli and purified to homogeneity. Both versions of the protease efficiently processed HERV-K10 Gag polyprotein substrate. HERV-K10 Gag was also cleaved by human immunodeficiency virus, type 1 (HIV-1) protease, although at different sites. To identify compounds that could inhibit protein processing dependent on the HERV-K10 protease, a series of cyclic ureas that had previously been shown to inhibit HIV-1 protease was tested. Several symmetric bisamides acted as very potent inhibitors of both the truncated and full-length form of HERV-K10 protease, in subnanomolar or nanomolar range, respectively. One of the cyclic ureas, SD146, can inhibit the processing of in vitro translated HERV-K10 Gag polyprotein substrate by HERV-K10 protease. In addition, in virus-like particles isolated from the teratocarcinoma cell line NCCIT, there is significant accumulation of Gag and Gag-Pol precursors upon treatment with SD146, suggesting the compound efficiently blocks HERV-K Gag processing in cells. This is the first report of an inhibitor able to block cell-associated processing of Gag polyptides of an endogenous retrovirus.

The human genome contains a large number of endogenous retroviral sequences that are virtually all highly defective because of multiple termination codons, deletions or the lack of a 5’ long terminal repeat (1, 2). It is assumed that at some time during the course of human evolution, exogenous progenitors of human endogenous retroviruses (HERVs) integrated into the cells of the germ line and thereby obtained the ability to be inherited by offspring of the host as a mendelian trait (3).

HERVs are grouped into at least a dozen single and multiple copy number families and are classified according to the tRNA that they use as primer for reverse transcription (1, 4). The retroviral element that carries a primer binding site complementary to the 3’ end of a lysine tRNA is called HERV-K. HERV type K represents the biologically most active form of a variety of retroviral elements present in the human genome (1, 5). Although the HERV-K group comes closest of all known HERVs to containing infectious virus, no corresponding replication-competent virus has so far been described (1, 3). Although humans harbor several dozen proviral copies of HERV type K per haploid genome (4, 6, 7), some of which code for the characteristic retroviral proteins Gag, Pol, and Env (8, 9), recent studies raised a suggestion that no complete proviral copy of HERV-K exists (10, 11); the issue remains to be clarified. In terms of infectious virion production, HERV-K could be defective at multiple levels, including the observed arrest during budding, inefficient RT enzyme activity, and incomplete Env expression and processing (1).

HERV-K elements exhibit restricted cell type expression, observed mainly in germ cell tumors (including testicular teratocarcinoma cell lines) and their testicular precursor lesions (8, 12, 13). Typically the coding regions of HERV-K elements are far less disrupted by mutations than other HERV families, and protein synthesis has been observed for all the main retroviral genes. The HERV-K Gag precursors are cleaved into major core, matrix, and nucleocapsid components (14–16), presumably by HERV-K protease, because functional activity has been demonstrated for this enzyme (15, 17).

Detailed electron microscopic surveys have revealed the existence of retrovirus-like particles in breast carcinoma and teratocarcinoma cell lines (18–20). The phenotype of human teratocarcinoma-derived retrovirus particles has been correlated with complex mRNA expression of HERV-K sequences in those cells, reminiscent of the mRNA expression pattern observed after exogenous retrovirus infection with, for example, lentiv- or spumavirus strains (8, 9).

Several hypotheses have so far been proposed about possible implication of HERV expression in certain pathogeneses, including autoimmune diseases such as insulin-dependent diabetes mellitus (21), tumor development, and even cardiovascular disease (22). In addition, numerous possible roles have been proposed for HERVs in reproductive physiopathology (reviewed in Ref. 23). In the study published by Sauter et al. (16), authors reported that HIV-1-infected patients and especially patients with seminomas exhibit elevated titers of anti-HERV-K10 Gag antibodies. Towler et al. (25) reported that HERV-K10 protease is highly resistant to a number of clinically used HIV-1 protease inhibitors, including ritonavir, indinavir, and saquinavir.
They reported the protease to be a homodimer with a pH optimum at 4.5 and with a higher enzymic activity and stability at elevated ionic strengths. The authors raised an interesting speculation that HERV-K protease might somehow complement HIV-1 protease under conditions where the latter activity is impaired because of either the presence of drug resistance mutations or the presence of potent HIV-1 protease inhibitors.

The aim of this study was to identify potent inhibitors of HERV-K10 protease and to demonstrate their action in virus-producing cells. The results shown in this report indicate that some members of the cyclic urea class can act as very potent inhibitors of this protease in a nanomolar range and are capable of blocking processing of HERV Gag in vitro as well as in the teratocarcinoma cell line NCCIT.

**EXPERIMENTAL PROCEDURES**

**Cloning of Truncated Version of HERV-K10 Protease**—Genomic DNA was extracted from the buffalo cow fraction of fresh human blood (24). DNA coding for core region of HERV-K10 protease (25) was then amplified by polymerase chain reaction with Taq DNA polymerase (PerkinElmer Life Sciences). Oligonucleotides 5′-CTAGGAGACCTCA-TATGACTATAAAGGGCAATACTCA-3′ (PR-A) and 5′-GCTGTTGGATCCTCTACATGGATTTGCCACC-3′ (PR-B) were used as sense and antisense primer, respectively. PCR product was cloned into mammalian expression vector pcDNA3.1(+) (Invitrogen) via HindIII and BamHI restriction sites. DNA sequencing of several clones revealed presence of substantial polyporphism. The clone with the DNA sequence identical to that published by Ono et al. (6) was chosen for further experiments. This clone was subjected to another round of PCR amplification, this time with oligonucleotides 5′-AGACTGAGTCCGACTTAAAGGGCAATACTCA-3′ and 5′-AGACATCTGGAGCTTATGGCACC-3′. The amplification product was cloned into Escherichia coli expression plasmid pET21a(+) (Novagen) via BamHI and XhoI restriction sites.

**Cloning of the Full-length Version of HERV-K10 Protease**—The cloning of the full-length version of HERV-K10 protease into pET21a(+) and its site-directed mutagenesis was described previously (25).

**Cloning of HERV-K10 gag—Plasmid pcG3 gag** (a gift from Dr. Ralf R. Tonjes, Paul-Ehrlich-Institut, Langen, Germany) (26) was used as a template for PCR amplification of HERV-K10 gag region. Oligonucleotides 5′-TACCGGAGTGGGAGAATTCA-3′ and 5′-AGATGAATTCCTACTGCTGCACTGCCGCTTG-3′ were used as sense and antisense primer, respectively. PCR product was cloned into pcDNA3.1(+) (Invitrogen) via BamHI and EcoRI restriction sites.

**Expression and Purification of 13-kDa Form of HERV-K10 Protease**—Luria-Bertani broth (1 L) supplemented with ampicillin (200 μg/ml) was inoculated with 5 ml of overnight culture of E. coli BL21(DE3) expression strain (Novagen) harboring pET21a(-) HERV-K10 protease construct. When an A_{600} value of 0.6 was reached, the expression of HERV-K10 protease was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (Sigma) to a final concentration of 0.4 mM. After 3 h at 37 °C the bacterial cells were pelleted by centrifugation at 6000 × g for 10 min. The cells were resuspended in 50 ml of 5× TE buffer (0.1 M Tris/Cl, 5 mM EDTA, pH 7.5) and subjected to sonication (6 × 30 s, 40 W, microtip). The soluble fraction was discarded. Inclusion bodies were washed twice with 20 ml of 5× TE buffer and then dissolved in 100 ml of 8 mM urea, 0.1 mM Tris/Cl, pH 7.5, 1 mM DTT. Refolding of HERV-K10 protease was achieved by dialyzing the solution against 4 liters of 20 mM Pipes, pH 6.5, 1 mM NaCl, 1 mM DTT, at 4 °C for 3 h and then against 4 liters of fresh buffer overnight. During the renaturation procedure the precursor form of HERV-K10 protease (20 kDa) completely autoprocessed to give rise to the mature, catalytically active 13-kDa form. The solution was centrifuged for 10 min to eliminate the precipitated proteins and then further clarified by filtration through a 0.45-μm membrane. The solution was then mixed 1:1 with buffer A (50 mM Pipes, pH 6.5, 1 mM NaCl, 0.5 mM EDTA, 1 mM NaK tartrate, 10% glycerol). Pepstatin A-agarse suspension (Sigma) was then added, and the lysate was incubated for 60 min at 30 °C and 100,000 g, and then applied to an Amersham Pharmacia Biotech MonoS HR 5/5 column and allowed to enter by gravity flow. 20 ml of lysis/wash buffer were used to wash the protease. The protease was eluted with 6 ml of elution buffer (40 mM phosphate buffer, pH 7.0, 0.3 mM NaCl, 300 mM imidazole) and then further purified by ion exchange chromatography. Protein concentration was determined via UV spectrophotometry. Molar absorption coefficients of 33,690 and 34,970 m^{-1} cm^{-1} were used for wild type and active site mutant forms, respectively.

**Expression and Purification of HIV-1 Protease**—HIV-1 protease was expressed in E. coli and then renatured from inclusion bodies as described previously (28).

**N-terminal Amino Acid Sequence Analysis**—The N-terminal sequence was determined using the Hewlett Packard G1005A protein sequencing system with on-line PTH analysis. All methods, reagents, and consumables used were those recommended by the manufacturer.

**Mass Spectrometry**—Matrix-assisted laser desorption ionization mass spectrometry data were obtained on a PerSeptive Biosystems Voyager DE-Pro mass spectrometer. The spectra were acquired in the linear mode with delayed extraction. External calibration was performed using calibrant 3 supplied by the manufacturer. The sample was diluted 1:10 in sinapinic acid matrix solution. The matrix was prepared by dissolving 10 mg/ml sinapinic acid in aqueous 30% acetonitrile containing 0.3% trifluoroacetic acid.

**Generation of Anti-HERV-K10 Protease Antiserum**—1 mg of truncated version of HERV-K10 protease was loaded on SDS-PAGE, and the band was excised from the gel. The gel slice was covered with phospholipid and saline and emulsified with a syringe through a 25-gauge needle. The emulsion was then used directly to immunize rabbits with 100 μg/dose.

**Enzyme Assay**—To measure the inhibitory potency of compounds, the discontinuous HPLC method described in Erickson-Vitaniæt al. (34) was used. The synthetic fluorescent cationic peptide substrate 2-amino-benzoyl-Ala-Thr-His-Gln-Val-Tyr-Phe(NO2)-Val-Arg-Lys-Ala (34) was used. The synthetic fluorescent cationic peptide substrate was added to the reaction solution containing substrate from the fluorescent substrate. The mobile phase buffer A was incubated with truncated or full-length HERV-K10 at 25 °C in an assay buffer containing 50 mM MES, pH 5.0, 1 mM NaCl, 20% glycerol, 1 mM EDTA. The synthesis of the substrate has been described elsewhere (28). The enzymatic reaction was terminated with 0.2 mM ammonium hydroxide. Enzymatic hydrolysis of the substrate yielded the fluorescent anionic product, (2-amino-benzoyl)-ATHQVY. The extent of hydrolysis was determined using anion-exchange HPLC. An Amersham Pharmacia Biotech HR5/5 MonoQ column eluted at 1.0 ml/min with 0%–70% buffer B for 10 min was used to separate the fluorescent cleavage product from the fluorescent substrate. The mobile phase buffer A contained 20 mM Tris/Cl, 0.02% sodium azide, and 10% acetonitrile at pH 9.0, whereas buffer B consisted of buffer A plus 0.5 mM ammonium formate at pH 9.0. The column was washed with 100% buffer B for 5 min, then buffer A was added for the next injection. The cleavage product was measured at an emission wavelength of 430 nm and excitation wavelength of 330 nm. Linearity of enzymatic activity with time was first established, and based on the results, reactions involving the truncated or full-length HERV-K10 protease were quenched after 20 in or 40 min, respectively (Fig. 1).
Subnanomolar Inhibitors of HERV-K10 Protease

Effect of SD146 on the Cleavage of in Vitro Translated HERV Gag Polyprotein by HERV-K10 Protease—Plasmid pCDNA3.1(+)HERV-K10 gag was used as template in TnT® Quick Coupled Transcription/Translation (Promega) reactions to produce [35S]methionine-labeled HERV Gag polyprotein that then served as a substrate for HERV-K10 protease. The in vitro translation product was incubated together with 0.54 mM HERV-K10 protease (truncated form) and various concentrations of SD146 (0–1 μM) in 20 mM PIPES, pH 6.5, 0.1 mM NaCl, 1 mM DTT, 10% glycerol, for 1 h at 37 °C. The substrate and cleavage products were separated on NuPage SDS-polyacrylamide gel (Novex) and autoradiographed. Subsequently, the dried gel was scanned for radioactivity with a Bio-Rad Molecular Imager FX, and the HERV Gag polyprotein bands were quantitated using QuantityOne software (Bio-Rad).

Expression and Purification of HERV-K10 Proteases.—Two versions of HERV-K10 protease were expressed in E. coli. The amino acid sequences of polypeptide chains that were expressed are shown in Fig. 3. The C-terminal boundary of the truncated version was chosen on the basis of sequence homology with the mature HIV-1 protease (25). An additional 58 amino acid residues included at the N-terminal end of the protein were expected to be cleaved off in an autocatalytic manner. This N-terminal flanking portion was expressed to allow us to readily monitor the processing activity (28). The nucleotide sequence of the clone that was chosen for E. coli expression was in complete agreement with the cDNA sequence of HERV-K10 protease ORF published in Ono et al. (6). The truncated, “core” protease was expressed as a 185-amino acid precursor at a high level in form of insoluble cytoplasmic inclusion bodies (29). The inclusion bodies were recovered by ultracentrifugation, washed, and resuspended in buffer containing 50 mM MES, pH 5.0, 1 mM EDTA, and 20% glycerol, for 1 h at 37 °C. The substrate and cleavage products were separated on NuPage SDS-polyacrylamide gel (Novex) and autoradiographed. Subsequently, the dried gel was scanned for radioactivity with a Bio-Rad Molecular Imager FX, and the HERV Gag polyprotein bands were quantitated using QuantityOne software (Bio-Rad).

Mammalian Cell Cultures and Collection of Particulate Material—Human teratocarcinoma cell lines NCCIT, PA-1, and NTERA-2, as well as the embryonic kidney line 293 (all purchased from American Type Culture Collection) were cultivated in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). Cell cultures were subcultured routinely twice per week. NCCIT cell line was treated with several concentrations of SD146 (up to 2 μM) or left untreated, and aliquots of culture supernatants were taken at time 0 and after 1 day. HERV-K particles were recovered by ultracentrifugation. After a 100,000 rpm centrifugation in a Sorvall RT6000B table top centrifuge at 1500 rpm to remove unbroken cells and large cell debris, the samples were centrifuged for 3 h at a Sorvall RC60 ultracentrifuge at 78,000 × g at 8 °C. Medium was discarded. The virus pellets were resuspended in a minimal volume of 1% SDS, 1% mecaptothanol, and 7% glycerol, heated at 56 °C for 1 min, and loaded onto 10% polyacrylamide gels.

Immunoblotting—Protein samples were separated with SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) using semidyed method. The membranes were probed with either anti-HERV-K10 protease antiserum at a dilution of 1:250 or polyclonal anti-HERV-K Gag antiserum (15) at a dilution of 1:10,000. Blots were stained indirectly by using horseradish peroxidase-conjugated donkey anti-rabbit antibodies and subsequent chemiluminescence detection (PerkinElmer Life Sciences).

Viral RNA Isolation and RT-PCR—RNA was isolated from infected virus particles with QIAamp Viral RNA Mini Kit (Qiagen). Eluted RNA was treated with RNase-free DNase I to digest any contaminating cell genomic DNA and repurified with the same kit. RNA was eluted in 60 μl of diethylpyrocarbonate-treated water. Reverse transcription was performed in a volume of 20 μl containing 5 μl of viral RNA, 0.5 mM dNTP mix, 10 units of RNasin, 100 ng of primer PRT-B, and 4 units of Omniscript reverse transcriptase (Qiagen). The reaction was carried out for 1 h at 37 °C. 5 μl of RT reaction was used in PCR amplification, together with 0.1 μM primers PRT-A and PRT-B, 1.5 mM MgCl2, 0.2 mM dNTP mix, and 1 unit of Taq DNA polymerase (PerkinElmer Life Sciences). The reaction mix was initially denatured at 94 °C for 5 min and then subjected to 30 cycles of denaturation at 94 °C, annealing at 50 °C, and extension at 72 °C. An aliquot of PCR reaction was used directly in DNA sequencing, with either PRT-A or PRT-B as a primer.

Molecular Modeling of HERV-K10 Protease—The three-dimensional homology model of the truncated version of HERV-K10 protease was constructed using coordinates of HIV-1 protease complexed with SD146 as a template (Protein Data bank file 1QBT.pdb; Ref. 36) with the program Molecular Operating Environment (Chemical Computing Group Inc.). The sequence was aligned initially maximizing the homology but later adjusted to accommodate the insertion at position 39 (HIV numbering) at the elbow of the flap and the insertion at position 80 (HIV numbering) at the active site, mimicking the three-dimensional structure of feline immunodeficiency virus protease. The homology algorithm of the Molecular Operating Environment software created 10 models, each of which was generated by making a series of Boltzmann-weighted choices of side chain rotamers and loop conformations from a set of protein fragments of high resolution protein structures. An average model was potential energy-minimized using AMBER forcefield.

RESULTS

Inhibition Kinetics—Samples of the HIV-1 protease inhibitors indinavir (MK-639), saquinavir (Ro 31–8895), and ritonavir (ABT-538) were synthesized at DuPont Pharmaceuticals. Peptatin A was purchased from Sigma-Aldrich. The cyclic ureas were prepared as described elsewhere (29–32). All inhibitors were dissolved in dimethyl sulfoxide and stored at −20 °C. Their chemical structures are shown in Fig. 2. The activity of the proteases was measured in the absence and presence of seven different concentrations of inhibitor at a fixed concentration of both enzyme and substrate. The proteases were preincubated 5 min at 25 °C with inhibitors. Substrate was then added to the final concentration of 2 μM, and the assay was carried out as described above. Fractional activities ranging from 0.2 to 0.8 relative to uninhibited control were fitted directly to the following Morrison equation (33).

\[
\frac{v_i}{v_0} = 1 - \frac{([E_i] + [I] + K_{i\text{app}}) - \sqrt{([E_i] + [I] + K_{i\text{app}})^2 - 4[E_i][I]}}{2[E_i][I]} \tag{Eq. 1}
\]

In this equation, [I] is inhibitor concentration, [Ei] is the concentration of active enzyme, vi is the activity at a particular inhibitor concentration, v0 is activity of uninhibited enzyme, v/Ei is fractional activity, and Ki(app) is the estimated apparent inhibition constant.

On the basis of previous studies with HIV-1 protease (34), the mode of inhibition was assumed to be competitive. To verify this assumption, the dose response data were obtained for SD146 as a representative compound at four substrate concentrations; IC50 values increased linearly with increasing substrate concentration, indicating the competitive nature of inhibition (35).

In Fig. 3, the enzyme kinetics of the in vitro expressed HERV-K10 protease was studied at different concentrations of SD146, and their time course was determined. The overall Michaelis-Menten constants, Km and Vmax, were calculated as described in Experimental Procedures. The apparent Michaelis-Menten constants for the protease inhibition by SD146 were calculated at different concentrations of substrate and protease. In Fig. 3c, the Michaelis-Menten constant (Km) for protease inhibition by SD146 was 7.8 μM, whereas the Vmax value was 21.1 nM/min. The enzyme kinetics for HERV-K10 protease inhibition by SD146 is represented by an initial rapid reaction followed by a slower reaction, as depicted in Fig. 3c. The apparent Michaelis-Menten constants (Km) for the protease inhibition by SD146 were calculated at different concentrations of substrate and protease. In Fig. 3c, the Michaelis-Menten constant (Km) for protease inhibition by SD146 was 7.8 μM, whereas the Vmax value was 21.1 nM/min.
The expression plasmid for full-length HERV-K10 protease was constructed so that only five additional amino acids (in addition to T7 tag) were present at N terminus because the presence of longer flanking region was observed not to be necessary for proper autoprocessing. At the C terminus the protease extends all the way to the termination codon that is present in full-length HERV-K10 provirus (6), which accounts for additional 50 amino acid residues not present in the truncated, core protease version. Nucleotide and deduced amino acid sequence of the clone that coded for full-length protease differed from that published by Ono et al. (6) as described previously (25). The full-length version differs from the truncated form also in the residue at position 65 (mature HERV protease numbering; Fig. 3); this residue is not positioned close to the active site or in the flaps and is believed not to be important for substrate or inhibitor binding. Metal chelation chromatography and subsequent ion exchange chromatography were used to purify both mature wild type full-length HERV-K10 protease and its active site mutant (D26N). Soluble fraction of E. coli cells was applied to nickel resin, and His tag-containing protease was bound. After elution with high imidazole buffer, the protease was further purified to homogeneity with cationic ion exchange chromatography on MonoS column. The mature wild...
type enzyme had a molecular mass of 18.2 kDa (including His tag), whereas active site mutant showed a molecular mass of 20 kDa because of the presence of T7 tag and remaining N-terminal pentapeptide that was not cleaved off because of lack of enzymatic activity of the protein.

**Enzymatic Activity of HERV-K10 Proteases**—Enzymatic activity of the enzymes was qualitatively assessed by determining kinetic constants for the hydrolysis of 2-aminobenzoyl-Ala-Thr-His-Gln-Val-Tyr-Phe(NO2)-Val-Arg-Lys-Ala. First, the \( K_m \) values were determined. After identifying compounds with potent inhibitory activity, the active sites of the protein preparations were titrated, and the \( k_{cat} \) values were then calculated from \( v_{max} \). As can be seen in Table I, the \( K_m \) value for the truncated version of HERV-K10 protease was about 20 times lower than that of the full-length counterpart. Similar ratio was observed previously for the hydrolysis of a different peptide substrate and under slightly different reaction conditions; in that report the \( K_m \) for the truncated version was about 10 times lower than that of the full-length enzyme (25). The turnover capacity (\( k_{cat} \)) of the full-length protease was about 10 times higher than that of the truncated form, resulting in a catalytic efficiency that was twice higher for the 13-kDa protein than what one could observe with the 18-kDa form. The ratio of \( k_{cat} \) values of both protease forms differs from that in a previous report (25); the difference is probably to be attributed to different substrates and to slightly different reaction conditions that were used in the assays.

**Identification of Potent HERV-K10 Inhibitors**—To evaluate the capacity of potent HIV-1 protease inhibitors to inhibit HERV-K10 protease, \( K_{(app)} \) values for a series of P2,P2'-substituted cyclic ureas were determined. In addition, pepstatin and three Food and Drug Administration-approved HIV-1 protease inhibitors, ritonavir, saquinavir, and indinavir, were tested. The apparent inhibition constants for both versions of HERV-K10 protease are shown in Table II, together with previously reported values for inhibition of wild type HIV-1 protease (39). Although potent inhibitors of wild type HIV-1 protease activity, the three Food and Drug Administration-approved compounds turned out to be weak inhibitors of both versions of HERV-K10 protease. The linear peptide mimetic inhibitors had \( K_{(app)} \) values ranging from 0.6 to 5.7 \( \mu M \).

A series of 13 compounds of the cyclic urea class was tested, all of them being P2,P2'-substituted. The symmetric substituted cyclic ureas in general fared better in inhibition assay than the five asymmetric compounds tested. From the latter, compound Q8467 exhibited the weakest activity, with the apparent inhibition constants being 16 and 61 nM for truncated and full-length HERV-K10 protease, respectively. The remaining asymmetric ureas (SD152, SD145, XW805, and XV651) did not differ significantly from each other, their \( K_{(app)} \) values being in the range of about 3–8 nM for 13-kDa protein and about 30–40 nM for 18-kDa form. Among cyclic \( C_6 \) symmetric ureas the compound with the smallest, cyclopropyl side groups, XK234, fared the worst, the \( K_{(app)} \) values being about 0.7 and 1.9 \( \mu M \). This compound had also turned out to be less efficient in inhibiting HIV-1 protease than the bulkier members of this group. XM412, also known as DMP450, containing \( m \)-amino-methylbenzyl groups, exhibited more inhibitory potency toward HERV-K10 proteases, although with apparent inhibition constants of about 90 and 400 nM, it was still much less potent than the remaining six cyclic ureas. XV643, XV644, SD146, XV648, and XV652 were capable of inhibiting 13-kDa protease in subnanomolar range, with the \( K_{(app)} \) values ranging from 0.10 nM for XV648 to 0.52 nM for XV652. The group of these five compounds inhibited the 18-kDa enzyme in nanomolar range; apparent inhibition constants were 2.3–4.3 nM. In general, \( K_{(app)} \) values for the full-length form of HERV-K10 protease were about 3–20 times higher than those for the truncated counterpart; however, the compounds that acted as weak inhibitors with one version of the protease were also weak with the other and vice versa. The differences in \( K_{(app)} \) values between both versions of the protease were consistent with the lower \( K_m \) value obtained for the 13-kDa form and were ob-

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**TABLE I**

**Kinetic parameters for the cleavage of substrate (2-aminobenzoyl)-\( \text{ATHQYYPNO2VRKAA} \)**

| Protease          | \( k_m \) (\( \mu M \)) | \( k_{cat} \) (s\(^{-1} \)) | \( k_{cat}/k_m \) (M\(^{-1} \)s\(^{-1} \)) |
|-------------------|--------------------------|----------------------------|----------------------------------|
| 13-kDa HERV-K10   | 2.3 ± 0.3                | 0.130 ± 0.007 (5.7 ± 0.8) × 10\(^4 \) |
| 18-kDa HERV-K10   | 41 ± 6                   | 1.37 ± 0.13 (3.3 ± 0.6) × 10\(^4 \) |

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**FIG. 3.** Amino acid sequence alignment of mature HIV-1 protease and all three versions of HERV-K10 protease expressed. The HERV proteins shown here represent the E. coli expression products. N-terminal T7 tag and C-terminal His tag are underlined. The DTG signature motif of aspartic proteases is shown in gray. Sites of N-terminal autoprocessing of HERV-K10 protease are denoted with an arrow. ASM, active site mutant; WT, wild type. Because of two insertions, the numberings of amino acid residues of mature HIV-1 and HERV-K10 proteases differ.
erved also in a previous report where compounds KNI-227 and KNI-272 were measured (25). The differences are very likely to be attributed to 50-amino acid C-terminal extension present in the full-length enzyme; however, in the absence of x-ray data it is not possible to provide a more detailed explanation.

Inhibition of HERV-K10 Gag Processing—SD146 was previously reported (40) to have potent activity in cells to block HIV-1 Gag processing by a variety of HIV-1 protease mutants. Because of this and its excellent potency against HERV-K10 protease (Table II), it was chosen for detailed studies of HERV-K10 Gag processing. To estimate the range of concentrations of SD146 as described under “Experimental Procedures.”

TABLE II

| Inhibition of HERV-K10 protease and HIV-1 protease activity |
|------------------------------------------------------------|
| $K_{app}$       | 13-kDa HERV | 18-kDa HERV | HIV-1 wild type$^a$ |
|-----------------|-------------|-------------|---------------------|
| Linear peptidomimetics |             |             |                     |
| Pepstatin       | 2570 ± 790  | 4900 ± 370  | NA$^b$              |
| Saquinavir      | 3580 ± 750  | 5740 ± 800  | 0.15 ± 0.03         |
| Ritonavir       | 1130 ± 4300 | 1210 ± 80   | 0.17 ± 0.07         |
| Indinavir       | 610 ± 340   | 940 ± 50    | 0.14 ± 0.01         |
| Asymmetric cyclic ureas |         |             |                     |
| Q648            | 16.2 ± 3.7  | 61 ± 5      | 0.14 ± 0.02         |
| SD152           | 7.9 ± 1.3   | 27 ± 1      | 0.04 ± 0.03         |
| SD145           | 5.9 ± 1.3   | 30 ± 9      | 0.08 ± 0.04         |
| XM412           | 3.6 ± 0.8   | 31 ± 4      | NA                  |
| XM420           | 3.2 ± 0.6   | 43 ± 10     | 0.036 ± 0.01        |
| Symmetric cyclic ureas |      |             |                     |
| XK334           | 670 ± 160   | 1870 ± 720  | 5.8 ± 0.4           |
| XM412           | 91 ± 48     | 390 ± 70    | 0.41 ± 0.04         |
| XV638           | 2.2 ± 0.4   | 20 ± 4      | 0.11 ± 0.02         |
| XV652           | 0.52 ± 0.08 | 3.3 ± 0.3   | 0.05 ± 0.02         |
| XV644           | 0.22 ± 0.08 | 2.9 ± 0.4   | 0.10 ± 0.04         |
| XV643           | 0.17 ± 0.06 | 2.9 ± 0.5   | 0.14 ± 0.04         |
| SD145           | 0.15 ± 0.02 | 4.3 ± 1.4   | 0.10 ± 0.02         |
| XV648           | 0.10 ± 0.01 | 2.3 ± 0.3   | 0.18 ± 0.01         |

$^a$ Values for HIV-1 protease as previously reported by Klabe et al. (39).

$^b$ NA, not available.

Asymmetric cyclic ureas

| Linear peptidomimetics | Pepstatin       | Saquinavir      | Ritonavir       | Indinavir       |
|-------------------------|-----------------|-----------------|-----------------|-----------------|
|                         | 2570 ± 790      | 3580 ± 750      | 1130 ± 4300     | 610 ± 340       |
| Asymmetric cyclic ureas | Q648            | SD152           | SD145           | XM412           |
|                         | 16.2 ± 3.7      | 7.9 ± 1.3       | 5.9 ± 1.3       | 3.6 ± 0.8       |
| Symmetric cyclic ureas  | XK334           | XM412           | XV638           | XV652           |
|                         | 670 ± 160       | 91 ± 48         | 2.2 ± 0.4       | 0.52 ± 0.08     |

**Note:** The table provides the $K_{app}$ values for the inhibition of HERV-K10 protease and HIV-1 protease activity for various compounds. The data suggests that SD146 has a subnanomolar inhibitory effect on both HERV-K10 and HIV-1 protease activity.

**Fig. 4.** HERV-K10 Gag polyprotein cleavage. Radioactively labeled HERV-K10 Gag was prepared by in vitro transcription/translation reaction as described under “Experimental Procedures.” A, HERV-K10 Gag was incubated in 20 mM PIPES, pH 6.5, 0.1 mM NaCl, 1 mM DTT, 10% glycerol for 1 h with 0.4 μg of either HIV-1 protease (lane 2), 13- or 18-kDa version of HERV-K10 protease (lanes 3 and 4, respectively), or active site mutant of full-length HERV-K10 protease (lane 5). Lane 1 represents noncleaved HERV-K10 Gag. B, effect of SD146 on the cleavage of HERV-K10 Gag polyprotein by HERV-K10 protease. The in vitro translated HERV Gag was incubated with 13-kDa form of HERV-K10 protease in presence of various concentrations of SD146 as described under “Experimental Procedures.” Lanes 1–7, HERV Gag incubated with the protease and different concentrations of SD146 (from left to right: 0, 0.1, 0.2, 0.3, 0.5, 0.7, and 1.0 μM); lane 8, HERV Gag incubated without the protease.
Gag processing and 0.42 μm in the case of protease maturation. Taken together, the results in Fig. 5 show that the HIV-1 protease inhibitor SD146 is able to effectively block HERV-K10 Gag processing, both in a teratocarcinoma cell line and in the released particles, as predicted from our enzyme inhibition cell-free results (Table II and Fig. 4B).

**RT-PCR and DNA Sequencing of NCCIT-derived Virions**—To verify that the particles derived from NCCIT cell line are indeed HERV-K encoded, viral RNA was isolated from the cell culture medium and its protease region RT-PCR amplified. A single product of expected size (~500 base pairs) was obtained. Direct DNA sequencing of the PCR product resulted in a single sequence and revealed that this region differs from the HERV-K10 clone published by Ono et al. (6) in 2 nucleotides. Neither of the substitutions (T3545C and C3572T; numbering as in Ref. 6) lead to an amino acid change. When BLAST search was performed against all nucleotide sequences deposited in GenBank™ to that date, the amplified region of RNA of NCCIT-derived HERV particles completely matched only the HERV protease region of a recently deposited *Homo sapiens* chromosome 5 clone CTB-69E10 (GenBank™ accession number AC016577).

**DISCUSSION**

Retroviral proteins are synthesized in the form of Gag or Gag/Pol precursors that are then processed by the action of a virus-encoded aspartic protease. The existence of a functional HERV-K protease was inferred from the presence of processed Gag proteins in teratocarcinoma cells (5). Direct evidence for a functional protease activity came from expression of different clones in *E. coli* (15, 17, 25, 38).

Recently, a hypothesis has been proposed that HERV-K10 encoded aspartic protease might complement HIV-1 protease during infection and thereby interfere with clinical antiviral therapy because it is highly resistant to currently approved HIV-1 protease inhibitors (25). To identify low molecular weight compounds that could inhibit proteolytic activity of this enzyme, we first expressed two versions of this enzyme in an *E. coli* expression system. The N termini of both enzymes were the result of autocatalytic processing by the protease. The C terminus of smaller, core form was chosen on the basis of sequence homology with mature HIV-1 protease. The C-terminal boundary of full-length version corresponds to that found in *prt*-ORF of proviral DNA (6); this version has 50 additional amino acids on its C terminus. Whether it is the full-length enzyme that is biologically relevant or additional C-terminal processing occurs to give rise to smaller molecular species remains to be seen. Initial studies suggest that some limited cleavage of 13 amino acid residues at the C terminus occurs after prolonged incubation (25, 38).

The DNA sequence of HERV-K10 protease ORF strongly suggests that this protease belongs to the group of aspartic proteases, because the ORF contains sequence motif LVDT-GAXX(T/S)(V/I). Furthermore, a sequence GLVGIG, a so-called “flap,” is found downstream of the active center. In addition, the sequence GRDDL conserved in aspartic proteases, is found at nucleotide position 3723–3737 (Ref. 15; numbering as in Ref. 6). Schommer et al. (17) showed that presence of high concentration of HIV-1 protease inhibitor Ro 31-8959 (saquinavir) can inhibit autoprocessing of HERV-K10 protease in *E. coli* expression broth, suggesting a similarity between active sites of the two viral proteases. We therefore decided to test a series of our cyclic ureas, second generation HIV protease inhibitors (reviewed in Ref. 41), for their ability to inhibit HERV-K10 protease. Although as a whole the cyclic urea class has relatively poor pharmacokinetic properties, mostly because of low water and oil solubility (41), these compounds are extremely potent against HIV-1 protease *in vitro*, and some of them have very good resistance profiles. At least one of the cyclic ureas,
DMP450 (42), is presently in human clinical trials versus HIV. Several symmetric bisamides exhibited high potency against both versions of HERV-K10 protease. In the absence of any available structural data, we built a homology three-dimensional model of the 13-kDa form of this enzyme to be able to understand the mode of action of the compounds.

The cyclic urea substituents at P1, P1', P2, and P2' are optimized for good potency against HIV-1 protease. In this enzyme, P1 and P1' residues form van der Waals' contacts with Pro30', Val53, and Ile54, whereas P2 and P2' groups form contacts with Ile50', Ile50, and Ile44. Cyclic urea inhibitors with smaller P2 and P2' were shown to be less potent against HERV-K10 protease than HIV-1 protease (XK234, XM412). However, cyclic urea amides containing P3 and P3' groups are as potent against HERV-K10 protease as HIV-1 protease (e.g., XV652, XV643, XV644, SD146, and XV648). Most of the hydrophobic bond contacts between the cyclic urea amide and HERV-1 protease complexes are predicted to be maintained in the cyclic urea amide and HERV-K10 protease complexes (Fig. 6).

The potency of cyclic ureas increase with the increasing potential of forming hydrogen bonds. For example, SD146 (HERV-K10 $K_{\text{IC}}$ (app) = 0.15 nM), which is capable of forming 12 hydrogen bonds, is ~4500 times more potent than XK234 (HERV-K10 $K_{\text{IC}}$ (app) = 670 nM). Besides the interaction with hydrogen bonds, the hydrophobic interaction is predicted to be important for the good potency of the cyclic urea amides. For instance, the substitution of Ile50' in HIV-1 protease for Leu52' in HERV-K10 protease is predicted to result in loss of van der Waals' interactions between Leu52'HERV and P3, P3' groups of cyclic urea amides. A similar effect caused by the hydrophobic interactions was observed previously in case of double mutant V82F/I84V of HIV-1 protease (40, 42).

The question of activity of the cyclic ureas in cells was addressed. In this study we demonstrated that HERV-K Gag processing in a cell environment can be blocked by synthetic protease inhibitors, as could be seen by substantially reduced proportion of HERV Gag precursor being cleaved to smaller polypeptides in NCCIT cell line treated with SD146 (Fig. 5A). To our knowledge, this is the first report of inhibition of HERV-K Gag maturation in cell milieu. Given the inability of cyclic ureas to inhibit cellular proteases (34), our results strongly support a model in which the aspartic protease of HERV-K10 processes homologous Gag polypeptides in human teratocarcinoma cells. Much of the HERV-K10 Gag within NCCIT cells is unprocessed. This is different from the case with HIV-1-infected cells, where a significant percent of HIV-1 Gag is cleaved. In contrast, processing of extracellular HERV Gag appears to be efficient, implying the HERV-K10 protease is inactive or unavailable except in maturing virions. HIV-1 protease is toxic to a variety of mammalian cells (43), but clearly human cells, including some teratocarcinoma cell lines, are not damaged by endogenous retroviral proteases. The question of whether the cells have evolved to resist the action of the endogenous viral proteases or the enzymes are sequestered/inactive until packaging/exit should be addressed.

The extracellular particle yield, as estimated by Western blotting of total viral proteins, was roughly the same in presence of the protease inhibitor, indicating that HERV-K Gag polypeptide processing is not a limiting step for particle release. Similar results obtained with viral RNA isolation from the particulate material of NCCIT cell culture medium and its subsequent quantification with RT-PCR amplification support this observation (data not shown). These data are consistent with the observation that HIV-1 protease inhibitors block the processing of Gag and Gag-Pol precursor polypeptides in HIV-1-infected cells but do not markedly alter either the number of particles released from the infected cells (44, 45) or the amount of packaged viral RNA (46, 47). In addition to using antigen-specific immunoblotting, we verified the identity of NCCIT released virions by checking the nucleotide sequence of packaged RNA. The sequence of the 500-nucleotide protease region that was RT-PCR amplified unequivocally shows that the virions belong to HERV-K family. However, additional regions would have to be sequenced for an exact clone number to be assigned, especially with regard to the fact that the recent estimates based on BLAST searches and phylogenetic analyses show that there could be as many as 170 HERV-K elements present in human genome (4). The protease amino acid sequence deduced from the obtained nucleotide sequence was identical to that of HERV-K10 clone (6).

Although cyclic ureas act as potent inhibitors of HIV-1 and HERV-K protease, they do not inhibit mammalian, nonretroviral cellular aspartic proteases (34). However, a question arises whether cell processes could be affected because of HERV-K protease inhibition. The fact that HERVs remain a constitutive part of the genome and the notion that ORFs for all major viral proteins exist and have retained coding capacity despite extensive deleterious effects normally associated with endogenization of retroviruses suggest that they may confer certain positive traits to the host (48). HERV encoded proteins, including HERV-K protease, might well be involved in normal cell physiology and pathophysiology. Our results in which the activity of HERV protease and inhibition of viral protein processing could be efficiently accomplished in teratocarcinoma cells may help to clarify the role of HERVs in cell physiology.

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Subnanomolar Inhibitors of HERV-K10 Protease

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