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Mutations in the Nonstructural Protein 3A Confer Resistance to the Novel Enterovirus Replication Inhibitor TTP-8307

Armando M. De Palma,¹ Hendrik Jan Thibaut,† Lonneke van der Linden,²† Kjerstin Lanke,² Ward Hleggermont,¹ Stephen Ireland,³ Robert Andrews,³ Murty Arimilli,³ Taleb H. Al-Tel,³ Erik De Clercq,¹ Frank van Kuppeveld,² and Johan Neyts¹*  
Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium;¹ Department of Medical Microbiology, Radboud University Nijmegen, Nijmegen Centre for Molecular Life Sciences, 6500 HB Nijmegen, The Netherlands;² and Transtech Pharma, Inc., 4170 Mendenhall Oaks Parkway, High Point, North Carolina 27265³  

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A novel compound, TTP-8307, was identified as a potent inhibitor of the replication of several rhino- and enteroviruses. TTP-8307 inhibits viral RNA synthesis in a dose-dependent manner, without affecting polypeptide synthesis and/or processing. Drug-resistant variants of coxsackievirus B3 were all shown to carry at least one amino acid mutation in the nonstructural protein 3A. In particular, three mutations located in a nonstructured region preceding the hydrophobic domain (V45A, I54F, and H57Y) appeared to contribute to the drug-resistant phenotype. This region has previously been identified as a hot spot for mutations that resulted in resistance to enviroxime, the sole 3A-targeting enterovirus inhibitor reported thus far. This was corroborated by the fact that TTP-8307 and enviroxime proved cross-resistant. It is hypothesized that TTP-8307 and enviroxime disrupt proper interactions of 3A(B) with other viral or cellular proteins that are required for efficient replication.

Enteroviruses comprise several pathogens that are implicated in an large variety of clinical manifestations that range from mild illnesses to more serious or even life-threatening diseases, such as meningitis, encephalitis, myocarditis, pancreatitis, acute paralysis, or neonatal sepsis (30, 40). Enteroviruses are small, nonenveloped, and spherical in shape, with a diameter of about 30 nm. The icosahedrally shaped capsids are assembled from 60 protomers, each composed of four structural proteins, designated VP1 (for viral protein 1), VP2, VP3, and VP4 (38, 39). The enteroviral genome consists of a single-stranded, positive-sense RNA of approximately 7,500 bases in length. The coding region of the viral genome is divided into the coding ends of plus- and minus-stranded RNA when it is complexed with the 3Dpol complex (26, 28, 35–37). 3AB but not 3A shows nonspecific RNA-binding activity but binds specifically to the 5’ RNA cloverleaf of the viral RNA genome when it is complexed with 3Dpol (20, 28, 48). The protein also binds to the 3’ untranslated region but, in contrast to binding at the 5’ cloverleaf, binding at the 3’ untranslated region can also occur in the absence of other proteins (20). Moreover, 3AB has been shown to induce membrane permeability in bacterial but not mammalian cells (1, 24, 25, 29), to induce membrane alterations in the endoplasmic reticulum (ER) (15), to form homodimers (26, 42, 47), and to be involved in host range pathology (2, 27, 33, 34).

Apart from these features that are directly or indirectly associated with viral replication, 3A and 3AB are also involved in processes that specifically affect the host cell. It is well documented that protein 3A is able to interfere with cellular protein secretion via inhibition of ER-to-Golgi transport, causing accumulation of proteins otherwise destined for export (4, 13, 14). Determinants for this feature are located at the N terminus (4, 13, 44–46). This inhibition of ER-to-Golgi transport has been shown to reduce or inhibit the secretion of antiviral cytokines such as interleukin-6, interleukin-8, and beta interferon (12), the concentration of tumor necrosis factor receptor on the surfaces of infected cells (32), and the presen-

* Corresponding author. Mailing address: Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Phone: 32(0)16-33-73-53. Fax: 32(0)16-33-73-40. E-mail: johan.neyts@rega.kuleuven.be.
† These two authors made equal contributions.
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tation of antigen in the context of major histocompatibility complex class I molecules (7). In doing so, ER-to-Golgi transport inhibition in infected cells might help in evading the host cell’s immune response and, hence, promote viral replication in an indirect way, although this inhibition is not required for efficient viral replication per se (12, 13).

The indispensable presence of 3A(B) during viral replication makes this protein an attractive candidate as a target for inhibition of viral replication. Thus far, only one compound (environixone) has been reported to target protein 3A (8, 22, 23). Despite its potent antiviral activity, however, the development of this compound was halted, mainly because of toxicity and an unfavorable pharmacokinetic profile (6). We here report on a novel compound (TTP-8307) that was identified in a screening campaign for inhibitors of the replication of enteroviruses. The compound appeared to be a potent inhibitor of the replication of several rhino- and enteroviruses.

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL-81), Buffalo green monkey (BGM) cells (ECACC 90092601), and HeLa cells (ATCC CCL-2) were grown in minimal essential medium (Gibco, Mérieux, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Integro, Leuvenheim, The Netherlands), 5% bicarbonate (Gibco), and 5% l-glutamine (Gibco). Cells were grown at 37°C in a 5% CO2 incubator. Coxackievirus B3 (CVBS) was derived from plasmid pCB53/T7, which contains a full-length cDNA of CVB3 strain Nancy behind a T7 RNA promoter (45). For assays involving virus growth, 2% fetal bovine serum was used instead of 10% fetal bovine serum. Rhinoviruses were provided by K. Andries, and the poliovirus Sabin 1, 2, and 3 strains were from B. Rombaut (Vrije Universiteit Brussels, Brussels, Belgium). Enterovirus 71 (BrCr) and coxsackievirus A16 (Coe) and A21 (Coe) were obtained from the Rijksinstituut voor Volksgezondheid en Milieu (The Netherlands).

Compounds. The synthesis of TTP-8307 (436 g/mol) will be reported elsewhere. The purity of the compound used in the present study was determined to be >96% by spectroscopic (nuclear magnetic resonance) and chromatographic (liquid chromatography-mass spectrometry) techniques. TBZ-209 and environixone were synthesized as reported elsewhere (10). Guanidine hydrochloride was from Sigma (Bornem, Belgium). All compounds were solubilized in dimethyl sulfoxide at 20 mM and stored at 4°C. For working solutions, the dimethyl sulfoxide stock solutions were diluted in minimal essential medium to the desired concentration.

In vitro RNA transcription and transfection. Prior to in vitro RNA transcription, plasmid pCB53/T7 was linearized with Sall (Promega, Leiden, The Netherlands). The linearized plasmid was modified (gel and phenol extraction; Qiagen), and 2.5 μg of DNA was used for in vitro RNA transcription (Ribomax large-scale RNA production system; Promega). The transcription reaction was carried out at 37°C for 4 h, after which the reaction mixture was DNase treated, and RNA was purified (RNA Cleanup System; Qiagen, Venlo, The Netherlands). Transfection reaction was carried out at 37°C for 4 h, after which the reaction mixture was DNase treated, and RNA was purified (RNA Cleanup System; Qiagen, Leiden, The Netherlands). Transfection reactions were carried out in 25-cm² culture flasks in Vero cells, grown to ~75% confluence. Reaction mixtures, containing 2 ml of OptiMEM (Gibco), 2.5 μg of purified RNA, and 10 μl of DMRIE-C transfection reagent (Invitrogen) were incubated for 4 h at 37°C. Subsequently, the medium was replaced with fresh growth medium and incubated until the cultures exhibited an extensive cytopathic effect (CPE). At this point, the flasks were subjected to three rounds of freezing-thawing, and the collected supernatant was titrated for infectious virus content by endpoint titration.

Multicycle CPE reduction assays. The antiviral activity of the selected compound was initially determined by using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]-based CPE reduction assay. Briefly, cells grown to confluence in 96-well plates were infected with 100 50% cell culture infective doses (CCID50) of virus. After an adsorption period of 2 h at 37°C (35°C for rhinovirus), the virus was removed, and serial dilutions of the compounds were added. The cultures were further incubated at 37°C for 3 days, until complete CPE was observed in the infected and untreated virus control (VC). After removal of the medium, 90 μl of culture medium and 10 μl of MTS/PMS (Promega) were added to each well. After an incubation period of 2 h at 37°C, the optical density at 498 nm (OD498) of each well was read in a microplate reader. CPE values were calculated as follows: % CPE = 100 × (ODvirus – ODuntreated-compound) / (ODvirus – ODuntreated). In these formulas, ODvirus corresponds to the optical density of the uninfected and untreated cell cultures, ODuntreated represents the infected and untreated cell cultures, and ODvirus-compound are virus-infected cell cultures treated with a given concentration of compound. The 50% effective concentration (EC50) was defined as the concentration of compound that resulted in 50% protection against virus-induced CPE and was calculated by using logistic interpolation.

Viral plaque assays. For determination of viral plaques, Vero cells, grown to confluence in six-well plates, were infected with CVB3 at 37°C with slight shaking at 55 rpm. After 2 h, the virus was removed, the cells were washed twice with phosphate-buffered saline (PBS), and the growth medium was replaced with agar (final concentration, 0.4%) in the presence or absence of compound. After 3 to 4 days, plaques were visualized. Briefly, cells were fixed with 2 ml of a solution containing 4% formaldehyde, after which the agar was removed. A 2% Giemsa solution was used to stain the cells.

Analysis of viral RNA accumulation with subgenomic replicon pCB53/T7-Luc. Accumulation of viral (+)RNA was monitored by transfecting cells (in the presence or absence of 25 μM TTP-8307) with RNA derived from the Sall-linearized plasmid pCB53/T7-Luc, which contains a subgenomic CVB3 replicon, carrying a luciferase gene in place of the capsid coding P1 region. At the indicated times posttransfection, the cells were washed three times with PBS and lysed with 75 μl of lysis buffer. The luciferase activity was measured in a liquid scintillation counter with the luciferase assay system according to the recommendations of the manufacturer (Promega). The luciferase activity was expressed in (relative) light units.

Quantitative analysis of CVB3 RNA by real-time qRT-PCR assays. Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed with an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Primers and probes were developed using Primer Express software (Applied Biosystems) as described elsewhere (9). The following primers and probe were used: a forward primer specific for nucleotides 2937 to 2957 (5'-AGC AAT CCC AGT GTG TTT TGG-3'), a reverse primer specific for nucleotides 3030 to 2982 (5'-TGC TCA AAA ACG GTA TGG ACA T-3'), and a TaqMan probe specific for nucleotides 2960 to 2977 (5'-FAM-CAG GGA AAG CCG CCC GCC- MRRCA AGA-3'). Each reaction was carried out in 25 μl of a One-step qRT-PCR mix (Eurogentec, Seraing, Belgium) containing 900 nM concentrations of each primer and 200 nM concentrations of the specific Taqman probe. The PCR consisted of an RT step (30 min at 48°C), a Taq activation step (10 min at 95°C), and 50 cycles of denaturation (15 s at 94°C) and annealing/extension (1 min at 60°C). The RNA copy number in each sample was determined by a standard curve generated from increasing copy numbers of a synthetic transcript corresponding to 67 nucleotides of the CVB3 genome.

Time of drug addition studies. Vero cells, grown to confluence in 24-well culture plates, were infected with 104 CCID50 of coxsackievirus B3. After an adsorption period of 1 h at 37°C, virus was removed and replaced with 500 μl of growth medium. At 1-h intervals, 500 μl of medium containing a 2× compound solution was added (final concentration, 25 μM). At 8 h postinfection, the supernatant, as well as infected RNA, of A549 cells treated with the compound, and viral RNA was quantified by means of real-time qRT-PCR. The levels of viral RNA were compared to their untreated controls.

Analysis of viral polyprotein processing in vivo. BGM cells, grown to confluence in 24-well plates, were infected with coxsackievirus B3 at a multiplicity of infection of 50. At 5 h postinfection, the medium was replaced with 300 μl of methionine-free medium. Thirty minutes later, the cultures were pulse-labeled in methionine-free medium containing 1 μl of [35S]methionine (final concentration, 25 μM). After 2 to 4 h of labeling, the cells were washed once with PBS and lysed in 75 μl of cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.05% sodium dodecyl sulfate (SDS). Translation products were analyzed on a 12.5% polyacrylamide gel containing SDS. The gels were fixed in 30% methanol-10% acetic acid, rinsed in dimethyl sulfoxide, fluorographed with 20% 2,5-diphenyloxazole in dimethyl sulfoxide, dried, and exposed to Kodak XAR film.

Generation of TTP-8307-resistant coxsackievirus. Drug-resistant virus was generated by growing virus in the presence of gradually increasing concentrations of TTP-8307 on confluent Vero cultures in 48-well culture plates. After 4 to 5 days of culture, culture supernatant was collected from cultures that exhibited full CPE in the presence of the highest concentration of compound used. This virus was used for a successive round of infection, a procedure that was repeated until full CPE was noticed at concentrations of TTP-8307 (20 μM) that did not allow replication of wild-type virus. Subsequently, the resistant virus pool was subjected to plaque purification (in the presence of 20 μM compound), and individual clones were sequenced for.
Site-directed mutagenesis. Four mutant CVB3 clones were constructed, containing single amino acid replacements at positions 8, 45, 54, and 57 in protein 3A. The four clones were designated mutant I (3A [I8T]), mutant II (3A [V45A]), mutant III (3A [I54F]), and mutant IV (3A [H57Y]). The corresponding synthetic oligonucleotides (and their complementary reverse oligonucleotides) were used for site-directed mutagenesis: (i) 5′-GGA AAA GGA TGG TTG GCT CCT GAG ATC AAC TCC ACC CAA TTG GAG AAA CAT GTC AGT CGG GCT TTC-3′, and (ii) 5′-CC CTC CAA ATT GAG AAA TAT GTC AGT CGG GCT TTC-3′. The mutated sequences are underlined. Site-directed mutagenesis was performed with plasmid pCB53/T7 using the XL-Blue large site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The four clones were designated mutant 1 (3A [I8T]), mutant 2 (3A [V45A]), mutant 3 (3A [I54F]), and mutant 4 (3A [H57Y]). The corresponding synthetic oligonucleotides (and their complementary reverse oligonucleotides) were used for site-directed mutagenesis: (i) 5′-GGA AAA GGA TGG TTG GCT CCT GAG ATC AAC TCC ACC CAA TTG GAG AAA CAT GTC AGT CGG GCT TTC-3′, and (ii) 5′-CC CTC CAA ATT GAG AAA TAT GTC AGT CGG GCT TTC-3′. The mutated sequences are underlined. Site-directed mutagenesis was performed with plasmid pCB53/T7 using the XL-Blue large site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands).

Sequencing. PCR fragments that cover the entire CVB3 genome were generated as described earlier. Sequencing, PCR fragments that cover the entire CVB3 genome were generated and analyzed by using the cycle sequencing method (ABI Prism BigDye terminator cycle sequencing ready reaction kit). Both DNA strands were sequenced. Sequencing data were obtained with an ABI 373 automated sequence analyzer (Applied Biosystems), and sequences were analyzed with the Vector NTI software (Invitrogen).

RESULTS

TTP-8307 inhibits replication of several enteroviruses. TTP-8307 was identified in a screening campaign as a selective inhibitor of CVB3 replication in Vero cells. The effect of TTP-8307 (Fig. 1A) was next evaluated against a selection of enteroviruses and rhinoviruses in an MTS-based CPE reduction assay. TTP-8307 inhibits the replication of cosackievirus B3 and the three poliovirus Sabin strains, as well as cosackieviruses A16 and A21 (Table 1). TTP-8307 inhibits human rhinoviruses (HRVs) 2, 29, 39, 45, 63, and 85, but not other rhinovirus serotypes (HRV serotypes 9, 14, 15, 41, 42, 70, 72, 86, and 89) or enterovirus 71. Cosackievirus B3 (CVB3), the prototype of the non-polio enteroviruses, was used to further study the particular characteristics of the antiviral activity of TTP-8307. TTP-8307 inhibited CVB3 replication in a dose-dependent manner (Fig. 2) when monitored either by (i) CVB3-induced CPE formation (Fig. 2A) or (ii) CVB3-induced plaque formation (Fig. 2B).

TTP-8307 acts at a stage that coincides with CVB3 viral RNA replication and polyprotein synthesis/processing. Time of drug addition studies were carried out to obtain a first indication about the stage in the viral replication cycle where TTP-8307 exerts its antiviral activity. Maximal inhibition of viral replication was maintained when the drug was added within the first 3 h postinfection (Fig. 3A). The addition of the drug at a time point later than 3 h postinfection, which coincides with the onset of viral RNA synthesis, resulted in a gradual decrease in antiviral activity. It can thus be concluded that TTP-8307 does not hamper early (attachment, entry, and uncoating) or late (assembly and release) events but rather interferes with intermediate processes, such as viral RNA replication, viral polyprotein synthesis, and/or processing.

TTP-8307 inhibits accumulation of CVB3 viral RNA. The accumulation of viral RNA in the absence or presence of TTP-8307 was monitored upon transfection of BGM cells with an infectious subgenomic replicon of CVB3 (in which the PI region was replaced with a luciferase marker). Transfection in the presence of TTP-8307 (25 μM) led to a complete inhibition of viral RNA accumulation, whereas an increase in luciferase activity was measured in the absence of compound (Fig. 3B). The replication inhibitor guanidine hydrochloride (2 mM) was included as a reference compound and resulted in the inhibition of viral RNA accumulation as well.

TTP-8307 does not affect CVB3 polyprotein synthesis or processing. Viral protein synthesis and polyprotein processing were monitored in a pulse-labeling experiment in the presence (25 μM) or absence of TTP-8307. From Fig. 3C it is evident that TTP-8307 does not hamper early (attachment, entry, and uncoating) or late (assembly and release) events but rather interferes with intermediate processes, such as viral RNA replication, viral polyprotein synthesis, and/or processing.

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FIG. 1. Structural formulae of TTP-8307 (A) and enviroxime (B).

![Image](https://example.com/image1)

![Image](https://example.com/image2)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean EC&lt;sub&gt;50&lt;/sub&gt; (μM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTP-8307</td>
<td>0.85 ± 0.08 (0.12 ± 0.01)</td>
</tr>
<tr>
<td>Enviroxime</td>
<td>0.05 0.06 (0.005 0.02)</td>
</tr>
</tbody>
</table>

*Data are mean values for at least three independent experiments. The cytotoxicity of TTP-8307 was determined on confluent Vero and HeLa cells, and the TC<sub>50</sub> values (i.e., the 50% cytotoxic concentration) were >100 μM. The compound solubility limit in assay medium is 100 μM.*
Inhibition of enterovirus protein 3A

We identified a novel compound, TTP-8307, that potently inhibits the replication of several enteroviruses, including coxsackievirus B3 and poliovirus by interfering with the synthesis of viral RNA. Contemplating the need for antivirals in the end stages of the worldwide polio eradication (5, 11), TTP-8307 might be considered as an interesting compound for lead

**DISCUSSION**

We identified a novel compound, TTP-8307, that potently inhibits the replication of several enteroviruses, including coxsackievirus B3 and poliovirus by interfering with the synthesis of viral RNA. Contemplating the need for antivirals in the end stages of the worldwide polio eradication (5, 11), TTP-8307 might be considered as an interesting compound for lead op-
timization, given its potent inhibitory effect on the replication of the three PV Sabin strains.

To determine the viral target of TTP-8307, resistant CVB3 variants were selected. Each clone carried at least one of the following mutations in protein 3A: I8T, V45A, I54F, or H57Y.

<table>
<thead>
<tr>
<th>Passage line (pool)</th>
<th>Clone</th>
<th>Amino acid mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2A</td>
<td>2B</td>
</tr>
<tr>
<td>1</td>
<td>A I8T, V45A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B N26D H57Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C N26D H57Y</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>A E59Q I112V</td>
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</tr>
<tr>
<td></td>
<td>B E64A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C E120Q S109G I54F D397E</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A R41K</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B R41K</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C R41K</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>A L47Q, K51N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B L47Q, K51N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C L47Q, K51N</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>A I92V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B I92V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C I92V</td>
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</table>

These mutations were reintroduced in an infectious CVB3 full-length clone and the antiviral sensitivity of the resulting viruses was studied. Mutants 2, 3, and 4 (carrying 3A mutations V45A, I54F, or H57Y) were shown to form plaques in the presence of concentrations of TTP-8307 that did not allow replication of wild-type virus, confirming that these mutations contribute to the observed resistance phenotype. The calculated EC90 values of TTP-8307 for inhibition of these mutant viruses were ca. 8- to 10-fold higher than for wild-type CVB3. Moreover, cross-resistance was observed with enviroxime. The specificity of this resistance profile was corroborated by the fact that a 2C-targeting compound (9, 10) inhibited the replication of the mutants as efficiently as that of the wild-type virus. Mutant I8T allowed for some very low replication in the presence of TTP-8307 and enviroxime. The viruses carrying the engineered mutations proved phenotypically (formation of plaques) comparable to the wild-type virus, suggesting no deleterious effects of the 3A mutations on viral replication. It should be noted, however, that the reconstructed mutants carrying single amino acid mutations in 3A did not exhibit the same high degree of resistance that we observed with the naturally selected clones. In fact, all naturally selected clones
were selected and plaque purified in the presence of 20 μM TTP-8307 and, hence, had EC<sub>90</sub> values of >20 μM. None of the reconstructed viruses carrying single amino acid mutations, however, reached EC<sub>90</sub> values of ≈20 μM. It may therefore be assumed that other sequence variations (in proteins different than 3A) may contribute to a further increase in the level of resistance to TTP-8307.

An amino acid sequence alignment of the 3A proteins of CVB3, PV1, HRV2, and HRV14 is depicted in Fig. 6. Globally, two major regions were reported to be important for resistance to enviroxime (3, 22, 23). The first region involves residues in the hydrophobic domain of 3A, located near its C terminus, whereas the second region is located between amino acids 40 and 60. This latter region, preceding the hydrophobic domain (underlined in green in Fig. 6), has been predicted to be unstructured, based on the nuclear magnetic resonance structure of the soluble domain of PV protein 3A (42). Remarkably, the 3A mutations that we identified in the present study as major determinants for resistance to TTP-8307 (V45A, I54F, and H57Y) were also located in this region (arrows in Fig. 6A; residues highlighted in Fig. 6C). Moreover, the very same H57Y mutation that was identified in TTP-8307-resistant CVB3 was also detected in enviroxime-resistant CVB3. In contrast to enviroxime, no mutations were detected in the hydrophobic domain of 3A in TTP-8307-resistant CVB3. This may be explained by the fact that TTP-8307 and enviroxime interact with the same region of 3A but that, given their different chemical structure, the precise molecular interactions with the various amino acids in this region may be (partially) different. Single amino acid mutations in the nonstructured region (amino acids 40 to 60) were shown to be sufficient to confer a certain degree of resistance to enviroxime (22, 23), corroborating our present observations. Florez de Sessions et al. observed that a chimeric CVB3 carrying an HRV2 internal ribosome entry site and that was adapted for growth in a neuroblastoma cell line carried the mutation V45A in 3A (16). Similar observations were described by Harris and Racaniello (19), who identified mutations in 3A at amino acid positions that correspond to CVB3 residues 42 and 44 in HRV39 that was adapted for growth in mouse cells. None of these mutations substantially affected viral plaque formation (16, 19), which is in line with our observations. The unstructured region preceding the hydrophobic domain may thus allow for adaptation of the virus to selective pressure of different kinds (e.g., antivirals or a changing host) without compromising viral growth.

A question that remains to be answered is what determines the spectrum of activity of TTP-8307. An alignment of the 3A amino acid sequence of TTP-8307-sensitive and -resistant viruses did not reveal any particular amino acids or regions in 3A that determined whether or not a virus would be inhibited by TTP-8307 (data not shown). For example, CVA16 and enterov-
virus 71 (TTP-8307 sensitive and resistant, respectively) share the N-terminal 3A amino acid sequence entirely, despite their difference in sensitivity. A possible explanation might be that 3A is a key protein in the replication inhibition, but that proteins other than 3A are involved in this inhibition as well.

Therefore, despite the fact that 3A was identified as a prime target for TTP-8307 as well as enviroxime, the precise mechanism of action for inhibition of viral inhibition by these compounds remains to be elucidated. In contrast to 2CATPase, 3C(D)pro, and 3Dpol, protein 3A is not thought to be associated with any enzymatic activity and, hence, the inhibition of this protein by a compound cannot be assessed in a simple in vitro enzymatic assay. Rather than hampering a catalytic reaction, 3A-targeting drugs are likely to mediate hindrance of certain interactions of 3A with other (viral or cellular) proteins in the viral replication complex, an idea that can be supported given the multitude of interactions that have been ascribed to this protein (43, 50). For enviroxime, it was recognized that replication inhibition is probably not only occurring through targeting of 3A but may also depend on interactions with other proteins in the replication complex (3). More precisely, a HRV14 mutant was identified that carried, in addition to 3A mutations, mutations in 3Dpol. These additional mutations resulted in a higher degree of resistance. These findings are in line with our observations that several of the identified mutants carry amino acid changes in proteins other than 3A (Table 2). However, these mutations were identified in various proteins (2A, 2B, 2C, and 3D) and at different residue positions. Based on the hypothesis that TTP-8307 and enviroxime interfere with 3A through inhibition of certain interactions with other proteins, one could postulate that mutations in 3A are necessary for resistance, but that the level of resistance is increased by additional mutations in other proteins (that interact with 3A). Interestingly, recently CVB3 was selected that was resistant to amiloride; resistance was shown to map to the 3Dpol, but the resistant variant carried also a mutation at 3A residue I54 (similar to clones 4 and 6 identified in the present study) (21). The contribution of this 3A mutation to the resistant phenotype was however, not further determined.

In conclusion, we identified a novel enterovirus replication inhibitor that targets the nonstructural protein 3A. Mutations conferring the highest levels of resistance mapped to the non-structured region preceding the hydrophobic domain, a region that was also reported to contain mutations in enviroxime-resistant viruses. The precise mechanism of viral inhibition by targeting 3A remains to be addressed in future studies.

ACKNOWLEDGMENTS

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REFERENCES


Dodd, D. A., T. H. Giddings, Jr., and K. Kirkegaard. 2001. Poliovirus 3A protein limits interleukin-6 (IL-6), IL-8, and beta interferon secretion during viral infection. J. Virol. 75:1585–1596.


