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A pyrazolotriazolopyrimidinamine inhibitor of bovine viral diarrhea virus replication that targets the viral RNA-dependent RNA polymerase

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ABSTRACT

[7-[3-(1,3-Benzodioxol-5-yl)propyl]-2-(2-furyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine] (LZ37) was identified as a selective inhibitor of *in vitro* bovine viral diarrhea virus (BVDV) replication. The EC_{50} values for inhibition of BVDV-induced cytopathic effect (CPE) formation, viral RNA synthesis and production of infectious virus were $4.3 \pm 0.7\,\mu M$, $12.9 \pm 1\,\mu M$ and $5.8 \pm 0.6\,\mu M$, respectively. LZ37 proved inactive against the hepatitis C virus and the flavivirus yellow fever. LZ37 inhibits BVDV replication at a time point that coincides with the onset of intracellular viral RNA synthesis. Drug-resistant mutants carried the F224Y mutation in the viral RNA-dependent RNA polymerase (RdRp). LZ37 showed cross-resistance with the imidazopyrrolopyridine AG110 [which selects for the E291G drug resistance mutation] as well as with the imidazopyridine BPIP [which selects for the F224S drug-resistant mutation]. LZ37 did not inhibit the *in vitro* activity of purified recombinant BVDV RdRp. Molecular modelling revealed that F224 is located near the tip of the finger domain of the RdRp. Docking of LZ37 in the crystal structure of the BVDV RdRp revealed several potential contacts including: (i) hydrophobic contacts of LZ37 with A221, A222, G223, F224 and A392; (ii) a stacking interaction between F224 side chain and the ring system of LZ37 and (iii) a hydrogen bond between the amino function of LZ37 and the O backbone atom of A392. It is concluded that LZ37 interacts with the same binding site as BPIP or VP32947 at the top of the finger domain of the polymerase that is a "hot spot" for inhibition of pestivirus replication.

1. Introduction

The bovine viral diarrhea virus (BVDV) is the prototype of the pestivirus genus of the *Flaviviridae* family. Apart from BVDV, the genus contains other important animal pathogens such as the classical swine fever virus (CSFV) that causes disease in pigs and the border disease virus (BDV) that causes disease in sheep. Two biotypes of BVDV exist: (i) the cytopathogenic (cp) biotype that result in lysis of *in vitro* infected cells and (ii) the non-cytopathogenic (ncp) biotype that does not lyse the infected cell (Lindenbach and Rice, 2001).

BVDV is a major pathogen of cattle and is able to cross-species barriers (Avalos-Ramirez et al., 2001; Becher et al., 1997, 1999, 2003; Grondahl et al., 2003; Kim et al., 2006; Mattson et al., 2006; Stewart et al., 1980; Uttenthal et al., 2005). In cattle BVDV can cause a range of clinical manifestations varying from subclinical to death (Lindberg, 2003). Infection of pregnant heifers can lead to the birth of persistently infected calves. High risk of unnoticed BVDV contamination of biological products arise from the use of contaminated bovine serum in cell culture (Erickson et al., 1991). Hence, contamination of interferon preparations (Harasawa and Sasaki, 1995) and vaccines (Harasawa and Tomiyama, 1994; Levings and Wessman, 1991) for medical use have been reported. It could thus be important to have selective anti-BVDV compounds at hand in particular circumstances (i) to treat valuable animals in zoologic collections, (ii) to treat expensive animals in breeding programs and *in vitro* embryo production (Stringfellow et al., 2005), (iii) to prevent virus

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spread during local outbreaks of the infection, or (iv) to cure established cell lines from contaminating pestiviruses (Durantel et al., 2004; Givens et al., 2004).

The pyrazolotriazolopyrimidinamine derivative LZ37 was initially discovered as a selective antagonist of the adenosine A_{2A} receptors that belong to the family of G protein-coupled receptors (Baraldi et al., 1996). A_{2A} receptors are predominantly located in the brain and appear to play a major role in the control of motor behaviour and in the modulation of dopamine-mediated responses. Hence, this class of compounds may hold potential for the treatment of Parkinson's disease. Unexpectedly, we identified LZ37 as an inhibitor of pestivirus replication. Here we describe the mechanism by which LZ37 exerts its anti-BVDV activity and demonstrate that this is unrelated to the antagonistic activity of the compound on adenosine A_{2A} receptors.

2. Materials and methods

2.1. Compounds

The synthesis of LZ37 [7-[3-(1,3-benzodioxol-5-yl)propyl]-2-(2-furyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine] (Baraldi et al., 1996), BPIP (5-[(4-bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-C]pyridine (Puerstinger et al., 2006) and ethyl 2-methylimidazo[1,2-a]pyrrolo[2,3-c]pyridin-8-carboxylate (AG110) (Paeshuyse et al., 2007) were reported earlier. 3′-Deoxyguanosine-5′-triphosphate (3′-dGTP) and 2′-C-methylguanosine-5′-triphosphate (2′-C-me-GTP) were purchased from Trilink (San Diego, CA).

2.2. Cells and viruses

Madin–Darby bovine kidney (MDBK) cells were grown in MEM supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Integro, Zaandam, The Netherlands). FBS was shown to be free of BVDV-1 and BVDV-2 by RT-PCR (Letellier et al., 1999). Porcine kidney cells (PK15) were grown in MEM supplemented with 10% heat-inactivated FBS. First-passage BVDV NADL stock was generated from pNADLp15a as previously described (Vassilev and Donis, 2000). BPIP-resistant BVDV ($BPIP^r$) derived from a pNADLp15a plasmid containing the F224S mutation in the NS5B gene was generated as described previously (Paeshuyse et al., 2006). The CSFV strain Alfort was obtained from the Institut für Virologie, Hannover, Germany. BVDV-2 strains 890 and Q4812 are American field isolates kindly provided by Dr S. Bolin and E. Dubovi to the Veterinary and Agrochemical Research Center (Ukkel, Belgium). Human hepatoma cells (Huh 7) containing subgenomic HCV replicons I_{389}luc-ubi-neo/NS3-3′/5.1 (Huh 5-2) were kindly provided by Prof. R. Bartenschlager (University of Heidelberg, Germany) and were used to assess activity against HCV (Lohmann et al., 1999). Huh 5-2 cells were grown in Dulbecco's modified Eagle's Medium (DMEM; Gibco) supplemented with 10% FBS, 1× non-essential amino acids (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 250 μg/ml Geneticin (Gibco). Yellow fever virus (YFV) 17D was the vaccine strain Stamaril® from Aventis Pasteur S.A.

2.3. Antiviral assays

Antiviral assays were performed as described previously (Paeshuyse et al., 2006). In brief, the appropriate cells were seeded at a density of 5×10^3 per well in 96-well cell culture plates. Following 24 h incubation, at 37 °C and 5% CO_2, medium was removed and 3-fold serial dilutions of the test compounds were added in a total volume of 100 μL, after which the cells were infected with the appropriate virus (except for Huh 5-2 replicon containing cells). After 3 days, medium was removed and (i) the cytopathic effect (CPE) induced by BVDV cp strains was quantified using the MTS/PMS method (Promega, Leiden, The Netherlands), (ii) the number of foci were assessed by means of an immunohistochemical method for BVDV ncp strains and CSFV and (iii) the Steady-Glo Luciferase assay system (Promega) was used to assess the effect on HCV replicon replication. The 50% effective concentration (EC_{50}) was defined as the concentration of compound that offered 50% protection of the cells against virus-induced CPE or a 50% reduction in foci or luciferase signal and was calculated using linear interpolation or for the immunohistochemical assays using the method of Reed–Muench (Reed and Muench, 1938).

Antiviral assays against a selection of DNA and RNA viruses were based on inhibition of virus-induced cytopathicity in either E6SM cells (HSV-1, HSV-2, VV, VSV), human embryonic lung (HEL) cells [Varicella-zoster virus VZV), HCMV], HeLa cells (respiratory syncytial virus) or Vero cells (yellow fever virus, Coxsackie B4 virus, parainfluenza-3 virus, sindbis virus, Punta Toro virus, reovirus-1), according to previously established procedures (De Clercq et al., 1980, 1986; De Clercq, 1985; Neyts et al., 1996).

2.4. Cytostatic assays

MDBK or Huh 5-2 cells were seeded at a density of 5×10^3 cells per well of a 96-well plate in MEM containing 5% FBS; 24 h later, serial dilutions of the test compounds were added. Cells were allowed to proliferate for 3 days at 37 °C, after which the cell number was determined by means of the MTS/PMS method (Promega). The % cell growth was calculated as follows: ($OD_{treated}/OD_{control}$); in which ($OD_{treated}$) = the $OD_{490\,nm}$ of cells treated with a certain dilution of compound ($OD_{control}$) = the $OD_{490\,nm}$ of cells left untreated. The 50% cytostatic concentration (CC_{50}) was defined as the concentration that inhibited the proliferation of exponentially growing cells by 50% and was calculated using logarithmic interpolation.

2.5. Time-of-(drug)-addition studies

MDBK cells (3.5×10^4 cells per well) were seeded in 24-well culture plate. Cultures were inoculated with BVDV (strain NADL, MOI = 2). The inoculum was removed following a 1 h incubation period and cells were washed three times with prewarmed PBS. The test compound (at 15 μM) was added at different time-points after infection. Cultures were further incubated until 24 h postinfection, at which time cell culture supernatant was collected and stored at −80 °C until further analysis by means of RT-qPCR.

2.6. Virus yield assay

MDBK cells were seeded at a density of 5×10^3 cells per well of a 96-well plate in MEM-FBS and were, 24 h later, infected with 10-fold serial dilutions of culture supernatant of which the infectious virus titer needed to be determined. After 4 days, medium was removed and cultures were fixed with 70% ethanol, stained with Giemsa solution, washed and air-dried. Virus-induced CPE was recorded microscopically and the viral titer was quantified according to the method of Reed and Muench (1938). Viral titers were expressed as the 50% cell culture infectious dose ($CCID_{50}$/ml).

2.7. Isolation of LZ37-resistant BVDV

LZ37-resistant virus was generated by culturing wild-type BVDV in MDBK cells in the presence of increasing concentrations of the compound in a 48-well plate. After 3 days of cultivation, cultures were freeze-thawed. Lysates of infected and treated cultures that exhibited cytopathic effect under drug pressure were used to infect new cell monolayers. These were further incubated in the presence of increasing concentrations of the compound. The procedure was

repeated for 25 consecutive passages until drug-resistant virus was selected.

2.8. RNA isolation

Viral RNA was isolated from cell culture supernatant using the QIAamp viral RNA minikit (Qiagen, Venlo, The Netherlands). Total cellular RNA was isolated from cells using the RNeasy minikit (Qiagen).

2.9. RT-qPCR

A 25 μL RT-qPCR reaction contained 12.5 μL 2× reaction buffer (Eurogentec, Seraing, Belgium), 6.3 μL H_2O, 300 nmol/L forward primer [5′-TGA GCT GTC TGA AAT GGT CGA TT], 300 nmol/L reverse primer [AGA AAT ACT GGG TCA TCT GAT GCA A], 300 nmol/L TaqMan probe [6-FAM-CGA AGC AGG TTA CCA AGG AGG CTG TTA GGA-TAMRA] and 5 μL total cellular or viral RNA extract. The RT step was performed at 48 °C for 30 min, 15 min at 95 °C and subsequent PCR amplification of 40 cycles of denaturation at 94 °C for 20 s and annealing and extension at 60 °C for 1 min in an ABI 7000 sequence detector. The 50% EC_{50} was defined as the concentration of compound that reduced the amount of viral RNA by 50% as compared to an infected untreated control and was calculated using linear interpolation.

2.10. Sequencing

PCR fragments that cover the entire non-structural protein coding region of the BVDV genome were generated and analyzed using the cycle sequencing method (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit). Both DNA strands were sequenced. Sequence data were obtained using an ABI 373 Automated Sequence Analyser (Applied Biosystems) and sequences were analyzed using the Vector NTI software package (Invitrogen, Merelbeke, Belgium).

The presence of a tyrosine residue at position 224 in the NS5B protein of BVDV type-2 strains was confirmed by sequencing the corresponding genome fragment using the primers described by Gilbert et al. (1999) in the sequencing protocol mentioned above.

2.11. RNA-dependent RNA polymerase reaction

BVDV (NADL) RNA-dependent RNA polymerase (RdRp) was expressed and purified as described before (Zhong et al., 1998). The purified BVDV polymerase (100 nM) was mixed with 100 μM GTP (containing 8.3 μM of [^{3}H]GTP, Amersham) and an increasing concentrations of the inhibitor (0.1 μM, 10 μM, 100 μM or 500 μM) in 50 mM Hepes pH 8.0, 10 mM KCl, 10 mM DTT, 1 mM $MgCl_2$, 2 mM $MnCl_2$ and 0.5% igepal (Sigma). Enzyme mix and inhibitors were pre-incubated in order to favor enzyme–inhibitor interactions before RNA binding, in case of competition for RNA binding site. Reactions were started by the addition of 100 nM of poly(C) (about 500 nt in size) template. Reactions were incubated at 30 °C and stopped by addition of 50 mM EDTA after 1 min, 5 min or 15 min. Samples were transferred onto DE-81 filters, washed with 0.3 M ammonium formate solution and dried. Radioactivity bound to the filter was determined by liquid scintillation counting.

2.12. Molecular modelling

The published X-ray structure of the BVDV RdRp (PDB entry 1S48 (Choi et al., 2004)) was used in all docking experiments. Selenium atoms in the selenomethionine residues were modified back to sulphur atoms to get methionine residues. The inhibitor LZ37 was drawn using jchempaint (Krause et al., 2000) and BUILD3D (Smith et al., 1981). The molecular geometry was fed into gamess for geometry optimization using the AM1 force field (Schmidt et al., 1993). Polar hydrogen atoms were added to the enzyme and inhibitor structures using autodocktools (Rogers et al., 2006). LZ37 was docked in the region of the finger domain in which F224 is located by means of the Autodock 3.05 software (Morris et al., 1998). The top 10 of docked ligand conformations was examined and finally the conformation with the highest autodock score was selected being a good representative of these 10 docked conformations. Interactions (H-bonds and hydrophobic) were calculated using Ligplot and HBPlus (McDonald and Thornton, 1994; Wallace et al., 1995).

3. Results

3.1. Antiviral activity

LZ37 (Table 2) was identified, in a multiple infection cycle assay in MDBK cells, as an inhibitor of BVDV (NADL) replication. The EC_{50}, as assessed by monitoring CPE reduction by the MTS method, was 4.3 ± 0.7 μM (Table 1). The compound inhibited virus-induced CPE formation in a dose-dependent manner (Fig. 1A). The anti-BVDV activity of LZ37 was further confirmed by means of RT-qPCR for viral RNA synthesis (Fig. 1B) and determination of infectious virus yield (Fig. 1C). Comparable inhibition patterns for CPE reduction, viral RNA synthesis and infectious virus yield were observed (Fig. 1A–C). The EC_{50} for inhibition of viral RNA production in culture supernatant was 12.9 ± 1 μM and for inhibition of infectious virus yield was 5.8 ± 0.6 μM (Table 1). In contrast, the BVDV-2 strains (890 and Q4812) were about 10-fold less susceptible to inhibition by LZ37 (EC_{50} ~58 μM). LZ37 did not inhibit the *in vitro* replication of other pestiviruses (classical swine fever virus), the hepatitis C virus or the yellow fever virus. Likewise, LZ37 did not inhibit the replication of a panel of unrelated viruses, i.e. DNA viruses (herpes simplex virus 1 and 2, vaccinia virus and cytomegalovirus) and RNA viruses (respiratory syncytial virus, vesicular stomatitis virus, Coxsackie virus B4, sindbis virus, reovirus-1 and parainfluenza-3 virus) (data not shown).

The concentration of LZ37 that reduced the proliferation of exponentially growing MDBK cells by 50% (50% cytostatic concentration or CC_{50}) was >100 μM (Fig. 1A). Hence, a selectivity index (the ratio CC_{50}/EC_{50}) of about 25 was calculated.

3.2. Time-of-(drug)-addition studies

To determine at what time during the viral replication cycle LZ37 interfered with viral replication, detailed time-of-(drug)-addition experiments were carried out. We previously determined that one

Table 1
Effect of LZ37 on the *in vitro* replication of BVDV type-1 strain NADL.

Virus	Strain or genotype	Biotype	EC_{50} (μM)			
			CPE	IMC	RNA yield	Virus Yield
BVDV type-1	NADL	cp	4.3 ± 0.7	6*	12.9 ± 1	5.8 ± 0.6

The 50% effective concentration (EC_{50}) for inhibition of viral replication as assessed by either a CPE reduction assay, an immunohistochemical assay (IMC), an RNA or virus reduction assay and a luciferase assay. Data are mean values ± S.D. for three or more independent experiments [values marked with *are from a single experiment]. cp = cytopathic.

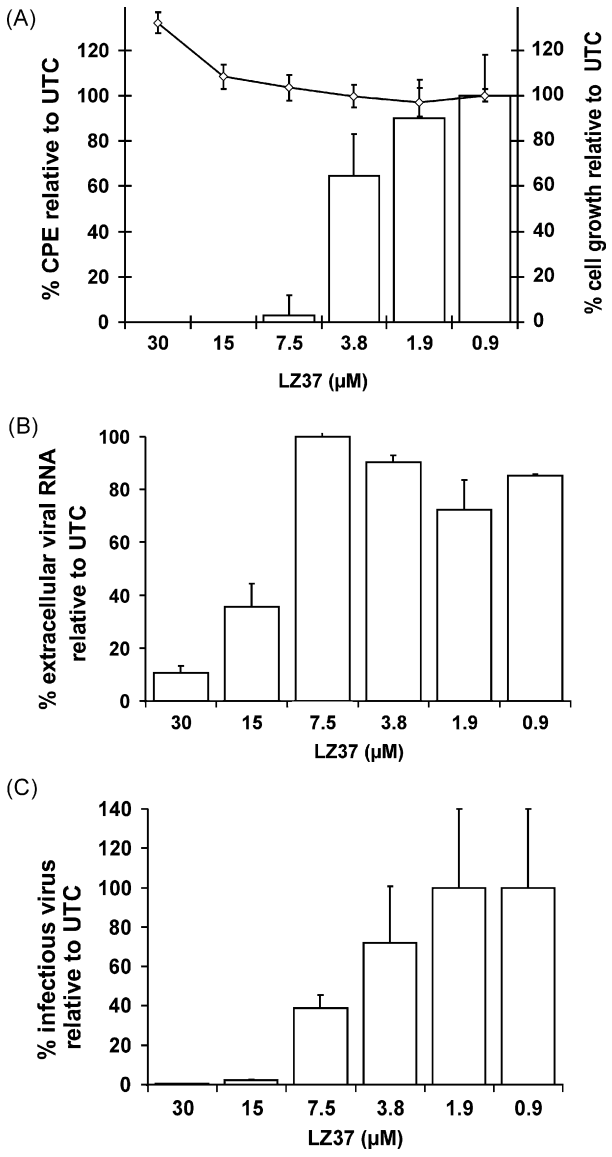


Fig. 1. Panel A: effect of LZ37 on BVDV-(NADL)-induced CPE formation in MDBK cells (open bars) and on the proliferation of exponentially growing MDBK cells (open diamonds). Panel B: inhibitory effect of LZ37 on release of extracellular viral RNA. Panel C: inhibitory effect of LZ37 on infectious virus yield. Data are mean values ± S.D. for three independent experiments. UTC = untreated infected control.

replication cycle of BVDV (by means of RT-qPCR and viral yield assay) takes on average 13 h, and from 6 to 8 h postinfection, a gradual increase of intracellular viral RNA is noted (Paeshuyse et al., 2006). This increase must coincide with the formation of functional replication complexes (RC). When LZ37 was added during the first 10 h postinfection, it resulted in marked reduction of intra- and extracellular (Fig. 2) viral RNA yield as detected at 24 h postinfection and when compared with the untreated infected control. A gradual increase of intracellular viral RNA is noted at time-points later than 6 h postinfection and of extracellular viral RNA at time-points later than 8 h postinfection. The addition of LZ37, however, prevents the virus from replicating to the same extend as the untreated control does.

3.3. Isolation and characterization of drug-resistant viruses

LZ37-resistant virus (LZ37^r) was obtained by propagating BVDV (NADL) for 25 passages at increasing drug concentrations [0.1–33 μM]. The LZ37^r virus proved at least 13-fold less susceptible to the inhibitory effect of LZ37 than the parent wild-type strain (Table 2). The resistant virus replicated about as efficiently as the parent wild-type virus (data not shown). BPIP (Paeshuyse et al., 2006) and AG110 (Paeshuyse et al., 2007), that were included as reference drugs, did not inhibit the replication of LZ37^r virus. *Vice versa*, LZ37 had no inhibitory effect on the replication of BPIP resistant virus (BPIP^r) and was about 2-fold less active against AG110 resistant virus (AG110^r). 2′-*C*-me-cytosine, a nucleoside inhibitor of HCV (Carroll et al., 2003; Eldrup et al., 2004) that is also active against BVDV, proved equally active against wild-type and LZ37^r and BPIP^r (Table 2).

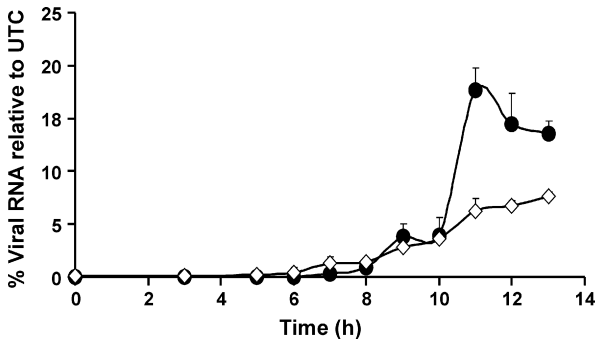


Fig. 2. Effect of time-of-(drug)-addition on the antiviral activity of LZ37. Intracellular (diamonds) or extracellular (circles) viral RNA was monitored by RT-qPCR at 24 h postinfection [in cells treated with LZ37 (at 15 μM) starting at different times postinfection] as compared to untreated infected cells (UTC). Values are expressed as percentage viral RNA of untreated infected cells. Data are mean values ± S.D. for three independent experiments.

3.4. Molecular characterization of LZ37-resistant virus

A single mutation [A to T substitution at position 10863 [F224Y] in the RdRp] was detected in the genome of the LZ37^r virus by comparing the LZ37^r genomic sequences with the corresponding sequence of the parent wild-type strain. Interestingly, both BVDV-2 viruses, which were less susceptible to the LZ37 product, also carried the F224Y substitution.

3.5. In vitro RdRp assay

LZ37 and the nucleotide analogues 3′-dGTP or 2′-*C*-me-GTP (that were included as positive controls) were studied for their effects on the polymerase activity of highly purified BVDV RdRp, using poly(C) as a template. LZ37 had no effect on the activity of the viral polymerase (Fig. 3). IC_{50} values for inhibition of BVDV polymerase

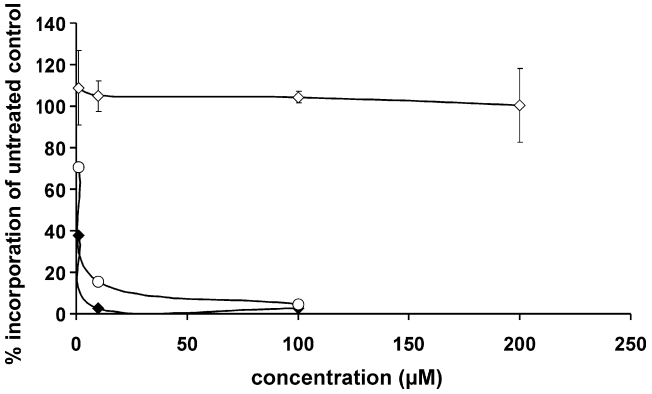
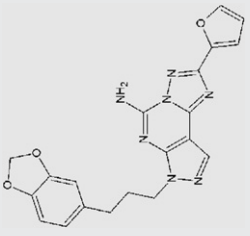
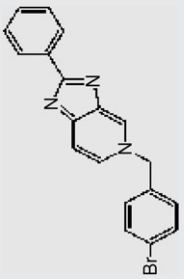
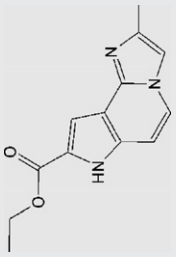
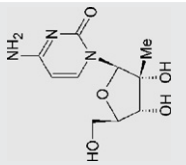


Fig. 3. Effect of LZ37 (open diamonds), 3′-dGTP (filled diamonds) and 2′-*C*-me-GTP (open circles) on the activity of purified BVDV RdRp using poly(C) as a template. Data are from a typical experiment and are expressed as % incorporation relative to untreated control.

Table 2
Susceptibility of wild type and LZ37^r BVDV to LZ37, BPIP, AG110 and 2′-C-methylcytosine.

Virus	EC_{50} (μM)				
					
	LZ-37	BPIP	AG110	2′-C-methylcytosine	
BVDV (NADL)	8 ± 1	0.2 ± 0.1	5 ± 3		1.2 ± 0.2
BVDV (selected LZ37^r)	>100 (>13)	34 ± 14 (170)	43 ± 18 (9)		1.2 ± 0.2 (1)
BVDV (recombinant BPIP^r)	75 ± 30 (9)	53 ± 17 (265)	94 ± 17 (19)		1.2 ± 0.4 (1)
BVDV (selected AG110^r)	16 ± 3 (2)	4 ± 2 (20)	97 ± 4 (12)		2.1 ± 0.6 (2)

Effective concentration 50% values (EC_{50}) are mean values ± S.D. for 4 independent experiments. Values between brackets represent fold-resistance. Values are obtained using the MTS/PMS method.

activity were: >200 μM for LZ37, <1 μM for 3′-dGTP, and ~1 μM for 2′-C-me-GTP.

3.6. Docking of LZ37 in the BVDV RdRp crystal structure

Docking of LZ37 close to F224 revealed possible interactions between the polymerase and LZ37 (Fig. 4). Ligplot analysis revealed the following potential interactions: (i) hydrophobic contacts of LZ37 with A221, A222, G223, F224 and A392; (ii) a stacking interaction between F224 side chain and the ring system of LZ37 (with distances of 3.0–4.2 Å between the 2 ring systems and a plane angle of 45° (Hunter, 1994)) and (iii) a hydrogen bond between the amino function of LZ37 and the O backbone atom of A392.

4. Discussion

LZ37, a known A_{2A} adenosine receptor antagonist (Baraldi et al., 1996), was identified in a large screening effort as an inhibitor of BVDV-1 induced CPE formation. LZ37 proved to be inactive against other *Flaviviridae* (i.e. the pestiviruses CSFV and BDV, the flavivirus YFV and the hepacivirus HCV) and against a panel of DNA and RNA viruses. The anti-BVDV activity was further corroborated by means of RT-qPCR and viral yield assays.

Detailed time-of-(drug)-addition studies revealed that the time-point at which LZ37 exerts its activity coincides with the onset of viral RNA synthesis, which is at 6–8 h postinfection. Addition of compound at a time-point before onset of intracellular viral RNA synthesis resulted in a complete inhibition of viral RNA production whereas addition at later time-points resulted in a gradual loss of antiviral activity. These data suggest that LZ37 interferes with the formation or functioning of the RC of the virus.

In vitro generated LZ37^r carried one mutation in the viral RdRp, i.e. F224Y. As is evident from Fig. 4, binding of LZ37 is mainly stabilized by stacking with F224. The stacking interaction is in agreement with our hypothesis that this inhibitor should bind close to the F224 residue, near the tip of the finger domain, with diminishing activity when replacing the F224 by Y. The hydrophobic and aromatic side chain of F224 is at the surface of the enzyme and thus has some flexibility. Introduction of a polar hydroxyl group by replacing phenylalanine with tyrosine may create a new local energy minimum in the conformational energy surface of the rotamers of residue 224. For instance, the introduction of the hydroxyl group may allow for additional hydrogen bonding possibilities (Fig. 4). This could lock Y224 temporarily in a more stable conformation, however with unfavorable stacking possibilities for the inhibitor LZ37 (Fig. 4). The same unfavorable stacking possibilities might also explain the loss of activity observed for the BPIP against LZ37^r. Previously we hypothesized that stacking between the aromatic rings of F224 and imidazopyridine BPIP were essential in the interaction between BPIP and the viral RdRp (Paeshuyse et al., 2006). A possible explanation why AG110 is also less active against LZ37^r may be that the F224Y mutation (selected by antiviral pressure with LZ37) perturbs the structure of the RdRp in the vicinity of residue 224 in such a way that it afflicts the conformation of the E291 residue which is only 7 Å away. This structural perturbation may than affect the interaction between AG110 and the amino acid residue E291.

Both BVDV type-2 strains used in this study were far less susceptible to LZ37 than the BVDV-1 strain used. Both BVDV type-2 strains were sequenced and were found to carry a tyrosine at position 224 of the viral RdRp. Thus the lack of activity of LZ37 against BVDV-2 strains 890 and Q4812 replication can be explained by the natural presence of Y224 in the RdRp. However, Y224 is not conserved throughout all known strains of BVDV-2. In contrast to some BVDV type-2 strains, the inability of LZ37 to inhibit CSFV replication cannot be explained as intrinsic resistance due to the presence of a Y residue at position 224 of the viral RdRp. A plausible explana-

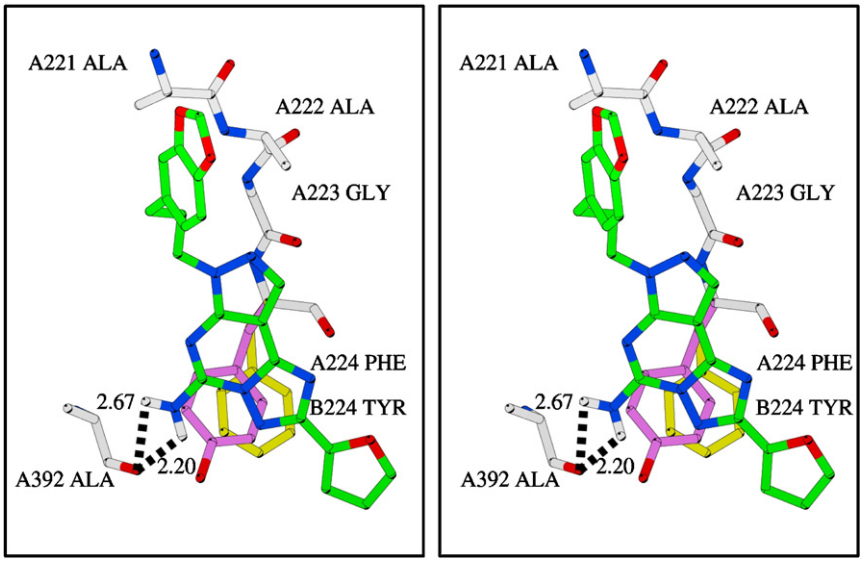


Fig. 4. Stereo picture of the docking of LZ37 near F224 (yellow). All surrounding amino acids having hydrophobic contact and stacking interaction with the inhibitor are shown, as well as the H-bonds to the main chain C=O of A392. In pink is shown the mutated F224Y residue. One rotamer of this residue places the hydroxyl group within hydrogen bonding distance to the carbonyl group of A392 illustrating the conformational locking possibility, which is detrimental for the stacking interaction with the inhibitor.

tion might be that other AA residues in the vicinity of F224 residue are of more importance in the interaction between polymerase and inhibitor(s). Previously it was shown that the T259S mutation in the RdRp of CSFV resulted in BPIP resistance (Vrancken et al., 2008) in contrast to F224S mutations that result in BPIP resistance in BVDV (Paeshuyse et al., 2006).

Although the genotyping and resistance profile of LZ37^r strongly suggests interference with the viral polymerase activity, LZ37 had very limited effect on the activity of the highly purified RdRp. Similarly, (i) the cyclic urea derivative (Sun et al., 2003), (ii) VP32947 (Baginski et al., 2000) and (iii) BPIP (Paeshuyse et al., 2006) had limited, if any, inhibitory effect on the highly purified RdRp. A possible explanation for the lack of activity of effect inhibitors on the purified polymerase, may therefore, be that the compound, following its interaction with NS5B, disturbs the formation/stability/function of the replication complex.

In conclusion, we here report on the inhibitory activity of LZ37 on the replication of BVDV-1. Previously we and others reported compounds with very different chemical structures that all appear to interact with a single drug-binding pocket within the finger domain region of the BVDV RdRp with two separate but potentially overlapping binding sites rather than two distinct drug-binding pockets (Paeshuyse et al., 2007). Here we show that LZ37 interacts with the same binding site as BPIP (Paeshuyse et al., 2006) or VP32947 (Baginski et al., 2000). LZ37^r carries, in contrast to BPIP^r or VP32947-resistant virus, a F224Y mutation instead of the F224S mutation (Baginski et al., 2000; Paeshuyse et al., 2006). This also explains the reduced activity of LZ37 against BVDV-2 strains that possess a tyrosine at position 224 of the RdRp. The fingertip of the BVDV-RdRp thus appears to be a "hot spot" for inhibitors of BVDV replication.

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