Antiviral Research 106 (2014) 71–79

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Substituted 2,6-bis(benzimidazol-2-yl)pyridines: A novel chemical class of pestivirus inhibitors that targets a hot spot for inhibition of pestivirus replication in the RNA-dependent RNA polymerase

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ARTICLE INFO

Article history:
Received 28 February 2013
Revised 17 March 2014
Accepted 19 March 2014
Available online 27 March 2014

Keywords:
Bovine viral diarrhea virus
RNA-dependent RNA polymerase
Substituted 2,6-bis(benzimidazol-2-yl)pyridine inhibitors

ABSTRACT

2,6-Bis(benzimidazol-2-yl)pyridine (BBP/CSFA-0) was identified in a CPE-based screening as a selective inhibitor of the *in vitro* bovine viral diarrhea virus (BVDV) replication. The EC_{50}-values for the inhibition of BVDV-induced cytopathic (CPE) effect, viral RNA synthesis and the production of infectious virus were 0.3 ± 0.1 μM, 0.05 ± 0.01 μM and 0.3 ± 0.04 μM, respectively. Furthermore, BBP/CSFA-0 inhibits the *in vitro* replication of the classical swine fever virus (CSFV) with an EC_{50} of 0.33 ± 0.25 μM. BBP/CSFA-0 proved *in vitro* inactive against the hepatitis C virus, that belongs like BVDV and CSFV to the family of *Flaviviridae*. Modification of the substituents on the two 1*H*-benzimidazole groups of BBP resulted in analogues equipotent in anti-BVDV activity (EC_{50} = 0.7 ± 0.1 μM), devoid of cytotoxicity (S.I. = 142). BBP resistant BVDV was selected for and was found to carry the I261M mutation in the viral RNA-dependent RNA polymerase (RdRp). Likewise, BBP-resistant CSFV was selected for; this variant carries either an I261N or a P262A mutation in NS5B. Molecular modeling revealed that I261 and P262 are located in a small cavity near the fingertip domain of the pestivirus polymerase. BBP-resistant BVDV and CSFV proved to be cross-resistant to earlier reported pestivirus inhibitors (BPIP, AG110 and LZ37) that are known to target the same region of the RdRp. BBP did not inhibit the *in vitro* activity of recombinant BVDV RdRp but inhibited the activity of BVDV replication complexes (RCs). BBP interacts likely with the fingertip of the pestivirus RdRp at the same position as BPIP, AG110 and LZ37. This indicates that this region is a "hot spot" for inhibition of pestivirus replication.

1. Introduction

The *Flaviviridae* family consists of three genera: the genus flavivirus (including human pathogens such as dengue virus, West Nile virus and yellow fever virus), the genus hepacivirus (hepatitis C virus, HCV), and the genus pestivirus (including veterinary pathogens such as the bovine viral diarrhea virus, BVDV, and the classical swine fever virus, CSFV).

Pestivirus infections of domesticated livestock (cattle, pigs, and sheep) cause significant economic losses worldwide, (Houe, 2003; Hasler et al., 2012; Stegeman et al., 2000).

Classical swine fever is a highly infectious viral disease that affects domestic and wild pigs. CSFV is included in the list of diseases notifiable to the OIE (www.oie.int). CSFV is considered to cause one of the most devastating diseases for the pig industry throughout the world both from an economical and sanitary point of view (Le Potier, 2012).

The disease is endemic in Asia and is prevalent in many countries of central and South America. In contrast to North America, where CSFV was eradicated several decades ago, the European Union (EU) still has an ongoing progressive eradication program that started in the early 1990s (Dong and Chen, 2007; European Union, 1980; Pereda et al., 2005). The most efficient vaccines currently available against CSFV are live attenuated vaccines (Le Potier, 2012). However, recently many efforts went into the development of new and safer marker vaccines against CSFV, along with improved diagnostic tools (Vannie et al., 2007; Beer et al., 2007).

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http://dx.doi.org/10.1016/j.antiviral.2014.03.010
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BVDV is ubiquitous and causes a range of clinical manifestations (including abortion, teratogenesis, respiratory problems, chronic wasting disease, immune system dysfunction, and predisposition to secondary viral and bacterial infections). Certain BVDV strains can cause acute fatal disease with mortality rates of 17–32% (Colloff et al., 2012; Hessman et al., 2012; Friedgut et al., 2011). BVDV is also able to establish a persistent infection in fetuses (Peterhans et al., 2010). When born, these persistently infected animals remain viremic throughout their lifespan and serve as continuous virus reservoirs. Persistently infected animals may also succumb to fatal mucosal disease if they are superinfected with a closely related BVDV strain. Vaccines are used in some countries in an attempt to control pestivirus disease with varying degrees of success (Kalaycioglu, 2007). Other containment strategies comprise quarantine and animal culling (Stahl and Alenius, 2012). Currently, there are no antiviral drugs to control pestivirus infections.

Pestiviruses show greater similarity in genome structure and translation strategy to hepaciviruses than to flaviviruses (Lindenbach and Rice, 2001). Despite of the relatively low levels of sequence identity between BVDV and HCV, the BVDV model was widely used in the early days of HCV drug discovery as a surrogate for HCV (Beaulieu and Tsantrizos, 2004; Buckwold et al., 2003). Several classes of inhibitors (Finkielsztein et al., 2010) with anti-BVDV activity are known; targeting either a cellular target, i.e. α-glycosidase [which is involved during the maturation of virions (Branza-Nichita et al., 2001)] as well as viral encoded enzymes such as the NS3 protease and helicase/NTPase (Bukhtiyarova et al., 2001) or the NS5B RNA-dependent RNA polymerase (RdRp). Polymerase inhibitors include nucleoside (Finkielsztein et al., 2010) and non-nucleoside inhibitors such as *N*-propyl-*N*-[2-(2*H*-1,2,4-triazino[5,6-*b*]indol-3-ylthio)ethyl]-1-propanamine (VP32947) (Baginski et al., 2000), a thiazole urea derivative (King et al., 2002), a cyclic urea derivative (Sun et al., 2003), imidazopyridines (BPIP) (Paeshuyse et al., 2006), ethyl 2-methylimidazo[1,2-*a*]pyrrolo[2,3-*c*]pyridin-8-carboxylate (AG110) (Paeshuyse et al., 2007) and pyrazolotriazolopyrimidinamine (LZ37) (Paeshuyse et al., 2009). BVDV strains that are resistant to most of these non-nucleosidic RdRp inhibitors all carry mutations in the fingertip domain of the viral RdRp. However, most of these inhibitors do not inhibit the *in vitro* activity of the recombinant viral polymerase but are able, in a dose dependent manner, to inhibit the activity of the BVDV replicase complex (RC) (Paeshuyse et al., 2006, 2007). The fingertip domain of the polymerase is crucial for the function of the polymerase and the viral RC. This domain is thus apparently a "hot spot" binding site for selective inhibitors of pestivirus replication (Paeshuyse et al., 2009).

Here we report on a series of benzimidazolylpyridine analogues as a *novel* chemical class of inhibitors of the replication of pestiviruses. In particular, the antiviral characteristics and mode of action of 2,6-bis(benzimidazol-2-yl)pyridine (BBP/CSFA-0) and analogues will be discussed in detail.

2. Materials and methods

2.1. Compounds

Most of the analogues were prepared by reaction of (substituted) pyridine-2,6-dicarboxylic acid with 2 equivalents of (a substituted) 1,2-phenylenediamine in PPA (polyphosphoric acid) at 160 °C for 6 h as depicted in Fig. 1. The synthesis of LZ37 [7-[3-(1,3-benzodioxol-5-yl)propyl]-2-(2-furyl)-7*H*-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-5*H*-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 minimal essential medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Integro, Zaandam, The Netherlands). BVDV NADL strain was obtained from the Veterinary and Agrochemical Research Center (CODA-CERVA, Ukkel, Belgium). BPIP-resistant BVDV ($BPIP^{res}$) was produced from a pNADLp15a plasmid containing the F224S mutation in the NS5B gene as described previously (Paeshuyse et al., 2006). Porcine kidney cell line (PK15), in conjunction with the CSF reference-strain $Alfort_{187}$ (subgroup 1.1) were cultured as described before (Vrancken et al., 2008).

2.3. Antiviral assay

The experiments were performed as previously described (Paeshuyse et al., 2006, 2007, 2009). In brief, MDBK 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 BVDV NADL virus (100 $CCID_{50}$). After 3 days, medium was removed and the cytopathic effect (CPE) was quantified using the MTS/PMS method (Promega, Leiden, The Netherlands). The 50% effective concentration (EC_{50}) was defined as the concentration of compound that offered 50% protection of the cells against virus-induced CPE and was calculated using linear interpolation. To assess the antiviral activity against CSFV, candidate inhibitors were evaluated using a virus-cell-based assay as described before (Vrancken et al., 2008). In brief, the cell culture medium, obtained after incubation with BBP, was used for the quantification of excreted viral RNA and infectious progeny virus. Both the EC_{50} and CC_{50} values were calculated using linear interpolation and viral titers of the medium were expressed as the 50% tissue culture infectious dose ($TCID_{50}$/ml) using the method of Reed and Muench (Reed and Muench, 1938).

2.4. Cytotoxicity assay

Assays were carried out as described previously (Paeshuyse et al., 2006, 2007, 2009). In brief, MDBK cells were seeded at a density of 5×10^3 per well in 96-well cell culture plates in MEM containing 5% of FBS; 24 h later, serial dilution of the test compounds were added. Cells were allowed to proliferate for 5 days at 37 °C, after which the overall metabolic activity of the cells was quantified by means of the MTS/PMS method (Promega, Leiden, The Netherlands). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of compound that inhibited the proliferation of exponentially growing cells by 50% and was calculated using linear interpolation.

2.5. Isolation of BBP^{res} BVDV

BBP-resistant (BBP^{res}) virus was selected as previously described (Paeshuyse et al., 2006, 2007, 2009). In brief, BBP^{res} BVDV 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

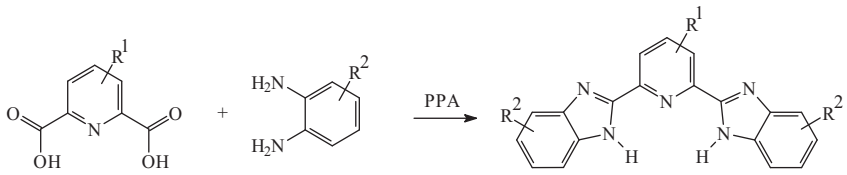


Fig. 1. Schematic representation of the synthesis of analogues 0–25.

increasing concentrations of the compound. The procedure was repeated for 28 consecutive passages until drug-resistant virus was obtained. Selection of BBP^{res} CSFV was done as described before (Vrancken et al., 2008). To select for BBP^{res} CSFV semi-confluent PK15 cells infected with $Alfort_{187}$ were passaged at increasing concentration of BBP. The selected CSFV $Alfort_{187}$ was regarded as resistant when a 15-fold decrease in sensitivity (increase in EC_{50}) to the antiviral effect of BBP was observed.

2.6. RNA isolation

Viral RNA was isolated from cell culture supernatant using a QIAamp viral RNA minikit (Qiagen, Venlo, The Netherlands). Total cellular RNA was isolated from cells using an RNeasy minikit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. To extract CSFV RNA, the total cellular RNA content was isolated from infected cell cultures using the RNeasy Mini kit (QIAgen, Venlo, Netherlands) according to the manufacturer's instructions.

2.7. RT-qPCR

RT-quantitative PCR (RT-qPCR) was performed as previously described (Paeshuyse et al., 2006, 2007, 2009). In brief, a 25 μL RT-qPCR reaction was composed of 12.5 μL 2× reaction buffer (Eurogentec, Seraing, Belgium), 6.3 μL H_2O, 300 nmol/L forward and reverse primer, 300 nmol/L TaqMan probe and 5 μL total cellular or viral RNA extract. The RT step was performed at 48 °C for 30 min, denatured for 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 7500 FAST Real-Time PCR System. The 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. CSFV RNA levels were quantified by a CSFV-specific, probe-based real-time RT-PCR assay using the TaqVet Kit (LSI, Lissieu, France) as described before (Vrancken et al., 2009a,b). The assay detects a 90 bp sequence located in the 5′-UTR, with a limit of detection of 2.2 ± 1.2 equivalent genome copies (EGC) and uses β-actin as an internal control. In each assay, positive and negative controls were included.

2.8. Sequencing

PCR fragments that cover the entire nonstructural 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). To sequence the genomic region of the CSFV polymerase, a fragment of 2419 b, comprising the NS5B gene was amplified by combining the forward primer [5′-GGA GGT TGG TGC AAA AGT GT-3′] (designed using Primer 3; (Rozen and Skaletsky, 2000)), in combination with the reversed primer HB32 (Bjorklund et al., 1998) using the SuperScript™ One-Step RT-PCR System for Long Templates (Invitrogen, Merelbeke, Belgium). The sequences of the PCR fragments were determined by an ABIPRISM Sequence Analyser 310 (Applied Biosystems, Foster City, CA, USA) using the Big Dye Termination v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed using the Chromas 2.3 application (http://www.technelysium.com.au/chromas.html).

2.9. RNA-dependent RNA polymerase assay

BVDV (NADL) RNA-dependent RNA polymerase (RdRp) was expressed and purified as described before (Zhong et al., 1998). These experiments were performed as previously described (Paeshuyse et al., 2006, 2007, 2009). In brief, the purified BVDV polymerase (100 nM) was mixed with 100 μM GTP (containing 8.3 μM of [^{3}H]GTP, Perkin Elmer) and increasing concentration of the inhibitor (0.2 μM, 0.7 μM, 2 μM, 6 μM, 19 μM, 56 μM, 165 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, was allowed to proceed, 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. 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.

2.10. Replication complex assay

The replication complex (RC) assay is similar to the one described before (Paeshuyse et al., 2007, 2009). In brief, BVDV-infected MDBK cells were suspended in ice-cold hypotonic buffer A (10 mM Tris, 10 mM NaCl pH 7.8) and were further disrupted by 50 strokes with a Dounce homogenizer. The disrupted cells were pelleted by centrifugation at 1000×*g* for 5 min at 4 °C. The supernatant fraction, containing cytoplasmic material and plasma membranes, was concentrated by high-speed centrifugation at 200,000×*g* for 20 min at 4 °C. The pellet was resuspended in 120 μL of hypotonic buffer B (10 mM Tris, 10 mM NaCl pH 7.8 and 15% glycerol) and used for an RNA polymerase assay. Replicase reactions were carried out as described before with some modification (Paeshuyse et al., 2007, 2009). The [α-^{33}P]UTP (3000 mCi/mmol) was replaced by [α-^{33}P]CTP (3000 mCi/mmol). The following incubation, RNA extraction, RNA analysis and quantification were performed as previously described (Paeshuyse et al., 2007, 2009).

2.11. Molecular modeling

The published X-ray structure of the BVDV RdRp [PDB entry 1S48 (Choi et al., 2004)] was used for the docking experiments. Selenium atoms in the selenomethionine residues were modified back to sulfur atoms to get methionine residues. Target structure and ligand BBP was prepared for docking by Autodocktools (Gasteiger charges were added, atom types assigned for use with

autodock 4.2). The docking site for BBP was defined as a cube centered at Ile261A.CD1, dimensions 60 × 60 × 60, spacing 0.375 Å (Morris et al., 1998). Flexible dockings of BBP with 2 active torsion angles were performed using Autodock 4.2. Several runs (100) using a Lamarckian genetic algorithm were performed and the best docking (lowest energy score) was selected.

3. Results

3.1. Antiviral activity of BBP (CSFA-0)

BBP (CSFA-0) was identified as an inhibitor of BVDV (NADL) replication in a multiple infection cycle assay in MDBK cells. BBP (CSFA-0) was found to inhibit virus-induced CPE formation in a dose-dependent manner (Fig. 2A) with an EC_{50} of 0.3 ± 0.1 μM. To further characterize the anti-BVDV activity of BBP, the effect of the compound on viral RNA synthesis (Fig. 2B and C) and infectious virus yield (Fig. 2D) was determined. Overall, comparable inhibitory effects on viral RNA synthesis and infectious viral yield production were obtained (Fig. 2A–D). The EC_{50} for inhibition of intracellular viral RNA replication and release of viral RNA in the culture supernatant was 0.05 ± 0.01 μM and 0.05 ± 0.02 μM respectively. The inhibitory effect of BBP (CSFA-0) produced an EC_{50} of 0.3 ± 0.04 μM for the inhibition of infectious virus yield in culture supernatant. BBP (CSFA-0) did also inhibit the *in vitro* replication of the CSFV strain $Alfort_{187}$ in a dose-dependent manner. An EC_{50} of respectively 0.33 ± 0.25; 0.37 ± 0.19 and 0.38 ± 0.12 μM was calculated for a standard anti-CSFV assay or when viral RNA or infectious virus yield was quantified, respectively. A CC_{50} of >100 μM (maximum tested concentration) was determined. BBP (CSFA-0) did not inhibit the hepatitis C virus, the yellow fever virus (YFV), the dengue virus (DENV) nor the replication of a panel of unrelated DNA viruses (herpes simplex virus 1 & 2, vaccinia virus and cytomegalovirus) or RNA viruses (respiratory syncytial virus, vesicular stomatitis virus, Coxsackie virus B4, Sindbis virus, reovirus-1 and parainfluenza-3 virus) (data not shown).

3.2. Structure–activity relationship

In an attempt to optimize the antiviral activity of BBP (CSFA-0), additional analogues were synthesized. First, the importance of the benzimidazolyl substituents was assessed by either omitting one of the benzimidazolyl side chains (1) or by modification of the benzimidazolyl ring system (2–7). From Table 1, it is apparent that both the nature of the benzimidazolyl side chain and the number, i.e. two, of side chains on position 2 and 6 of the pyridine ring are essential for the anti-BVDV activity of this class of compounds. Next, the importance of the central pyridine ring for the anti-BVDV activity was assessed. As shown in Table 2, the substitution of the pyridine by benzene (8) results in reduced anti-BVDV activity. The introduction of a hydroxyl group on position 4 of the pyridine (9) or the substitution of the pyridine ring by a thiophene (10) also yields inactive derivatives. For the pyridine N-oxide (11) a reduced cytotoxicity as compared to 8 and 9 and a 5-fold increase in antiviral activity were observed.

To further understand the structural requirements for the antiviral activity within this class of compounds, a third set of analogues was synthesized. Here, different substituents (R_1 and R_2) were introduced onto the two 1*H*-benzimidazole groups (compounds 12–25, Table 3). Some substituents that were introduced on position 4 and 5 (compound 12) and in position 4(7) (compounds 13 and 14, Table 3) resulted in a decrease in anti-BVDV activity as compared to the parent compound [BBP (CSFA-0)], with compound 12 being cytotoxic. Further modifications of the two 1*H*-benzimidazole groups were introduced at position 5(6) of the benzimidazole group (compounds 15–24, Table 3); in particular a fluorine in position 5(6) of the benzimidazole groups (16) resulted in a 10-fold increase in anti-BVDV activity as compared to BBP (CSFA-0) but is still associated with

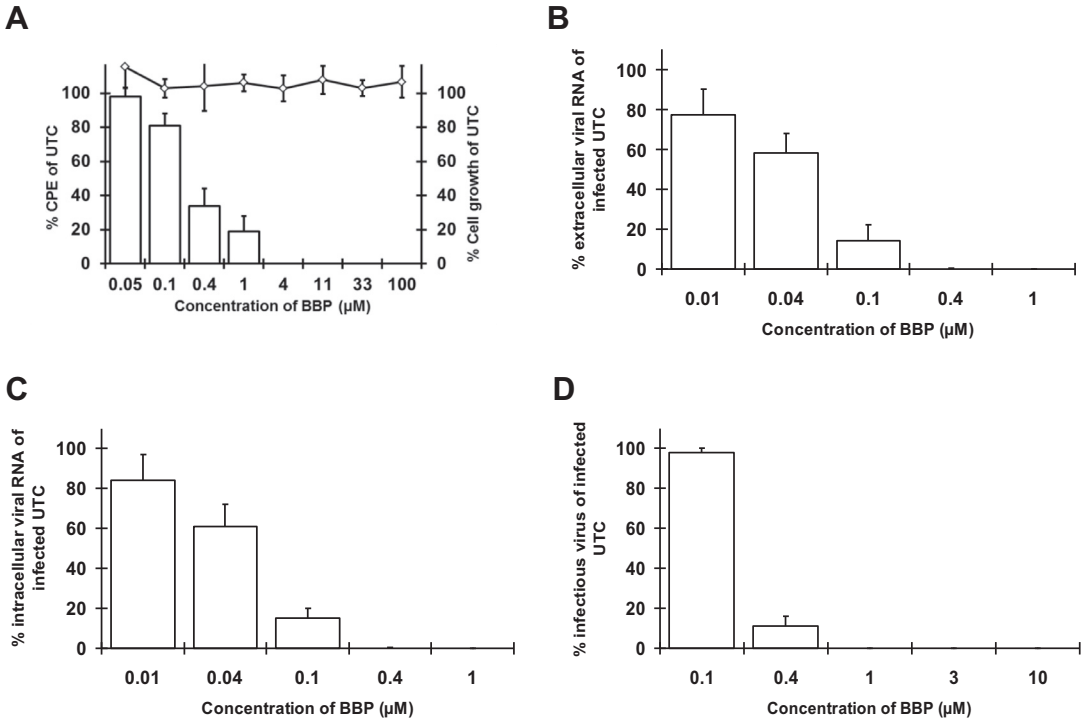
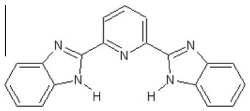
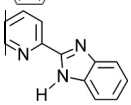
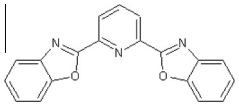
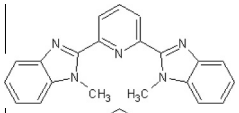
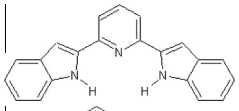
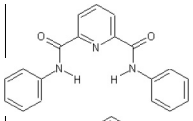
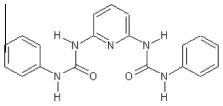
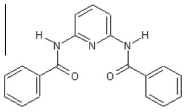


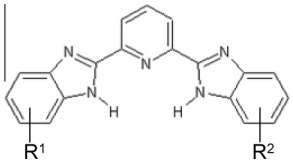
Fig. 2. (A) Effect of BBP on BVDV (NADL)-induced CPE formation in MDBK cells (bars) and on the proliferation of exponentially growing MDBK cells (diamonds). (B) Inhibitory effect of BBP on release of intracellular viral RNA. (C) Inhibitory effect of BBP on release of extracellular viral RNA. (D) Inhibitory effect of BBP on infectious virus yield. Data are mean values ± standard deviations from three independent experiments. UTC, untreated control. Antiviral activity and cytotoxicity were obtained in separate experiments.

Table 1
Structure, *in vitro* anti-BVDV activity and cytotoxicity of compounds 0–7.

Compound	Structure	EC_{50}[a] (μM)	CC_{50}[a] (μM)	S.I.[b]
0		0.5 ± 0.2	>100	200
1		>33	>100	n.a.
2		>33	>100	n.a.
3		>33	>100	n.a.
4		>33	73 ± 5.6	n.a.
5		>33	>100	n.a.
6		>33	>100	n.a.
7		>33	>100	n.a.

The EC_{50} (50% effective concentration) is the concentration of compound that inhibits virus-induced cytopathic effect by 50%. The CC_{50} (50% cytostatic concentration) is the concentration that inhibits the proliferation of exponentially growing cells by 50%.
[a] Data are mean values ± SD for at least 4 independent experiments.
[b] *In vitro* selectivity index (CC_{50}/EC_{50}); n.a., not applicable.

Table 3
Structure, *in vitro* anti-BVDV activity and cytotoxicity of compounds 12–25.



Compound	R_1 and R_2	EC_{50}[a] (μM)	CC_{50}[a] (μM)	S.I.[b]
12	4,5-$(CH_3)_2$	7.3 ± 1.1	7.3 ± 1.1	1
13	4(7)-CH_3	1.1 ± 0.1	15 ± 2.9	13.3
14	4(7)-Cl	6.7 ± 2.7	21 ± 4.3	3.1
15	5(6)-CH_3	1.0 ± 0.5	20 ± 3.2	21
16	5(6)-F	0.05 ± 0.02	5.5 ± 0.8	110
17	5(6)-Cl	0.05 ± 0.02	2.5 ± 0.4	50
18	5(6)-Br	0.8 ± 0.2	3.0 ± 0.5	3.8
19	5,6-$(CH_3)_2$	2.5 ± 0.1	18 ± 2.4	7.3
20	5,6-Cl_2	3.4 ± 2.3	20 ± 3.8	5.8
21	5(6)-CF_3	0.7 ± 0.5	0.7 ± 0.5	1
22	5(6)-OCH_3	0.7 ± 0.1	>100	142
23	5(6)-I	14 ± 6.2	21 ± 4.1	1.5
24	5-Cl, 6-F	0.5 ± 0.1	2.6 ± 0.5	5.3
25	5,6-phenyl	5.9 ± 3.9	60 ± 7.2	10

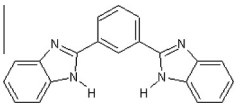
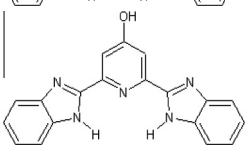
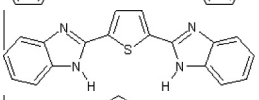
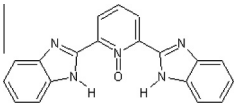
The EC_{50} (50% effective concentration) is the concentration of compound that inhibits virus-induced cytopathic effect by 50%. The CC_{50} (50% cytostatic concentration) is the concentration that inhibits the proliferation of exponentially growing cells by 50%.
[a] Data are mean values ± SD for at least 4 independent experiments.
[b] *In vitro* selectivity index (CC_{50}/EC_{50}); n.a., not applicable.

cytotoxic effects. Analogue 22 had an equipotent anti-BVDV as BBP (CSFA-0) and was not cytotoxic to the cells. The addition of 5(6)-methoxy substituents on the two 1*H*-benzimidazole groups (22) was well tolerated by the cells, much better than other substituents (–CH_3, –Cl, –CF_3, –I, 5,6-benzo).

3.3. Isolation and characterization of drug-resistant viruses

To identify the target of BBP, we isolated and characterized BBP-resistant virus (BBP^{res}). BBP^{res} virus was selected by propagating BVDV (strain NADL) for 28 passages in increasing concentration

Table 2
Structure, *in vitro* anti-BVDV activity and cytotoxicity of compounds 8–11.

Compound	Structure	EC_{50}[a] (μM)	CC_{50}[a] (μM)	S.I.[b]
8		4.0 ± 1.4	4.6 ± 0.9	1.1
9		2.6 ± 0.5	2.6 ± 0.5	1
10		>33	>100	3.0
11		0.1 ± 0.6	15 ± 3.2	150

The EC_{50} (50% effective concentration) is the concentration of compound that inhibits virus-induced cytopathic effect by 50%. The CC_{50} (50% cytostatic concentration) is the concentration that inhibits the proliferation of exponentially growing cells by 50%.
[a] Data are mean values ± SD for at least 4 independent experiments.
[b] *In vitro* selectivity index (CC_{50}/EC_{50}); n.a., not applicable.

of the drug (from 1.8 to 30 μM). Drug resistant BVDV variants selected have comparable replication kinetics and no gross differences in replication fitness (Supplemental file). The resulting drug-resistant virus proved 55-fold less susceptible to the inhibitory effect of BBP as compared to the parent wild-type strain and about 6.7-fold, >11.3-fold and 186-fold less susceptible to the known BVDV inhibitors AG110, LZ37 or BPIP, respectively (Table 4). Conversely, BBP proved to be about 6-fold less active on the replication of recombinant BVDV carrying only the single mutation F224S in the polymerase. By comparing the BBP^{res} virus genome sequence to the parental wild-type strain (GenBank Accession No. AJ781045), we identified a transition of A to G at position 10,975 in the viral RNA. This point mutation results in an amino acid change of isoleucine (I) to methionine (M) at amino acid residue 261 in the RdRp. For CSFV, three independently selected cultures of BBP^{res} CSFV were obtained following 11 consecutive passages of the wild type virus (strain Alfort$_{187}$) in the presence of gradually increasing concentrations of BBP. All three drug-resistant virus cultures proved almost completely refractory to inhibition of viral replication by BBP (EC_{50} > 100 μM) and were thus regarded as BBP-resistant (Table 4). Susceptibility of BBP-resistant viruses and other resistant variants studied to 2′-*C*-methylcytidine was comparable to or more than WT (Table 4).

Moreover, BBP proved to be cross-resistant with the earlier selected BPIP^{res}-CSFV (Vrancken et al., 2008) (EC_{50} > 100 μM, Table 4). Since the molecular target of BPIP in CSFV replication has been shown to be the RdRp, the sequence of the CSFV NS5B-gene of the selected BBP^{res} CSFV was determined and aligned to the sequence of the viral population before the start of the resistance selection and with wild-type CSFV strain Alfort$_{187}$ (NCBI Accession No. X87939). Two of the three independently selected, drug-resistant viruses carried a T to A transversion at position 10,695 resulting in the substitution of an isoleucine (I) to asparagine (N) at position 261 of the NS5B protein. The other drug-resistant virus carried a C to G transversion at position 10,697 resulting in the substitution of a proline (P) to an alanine (A) at position 262 of the NS5B protein.

3.4. Effect of BBP (CSFA-0) on the BVDV RdRp and replication complexes

Since the BBP-resistant virus carries mutations in RdRp, the inhibitory effect of BBP on the BVDV polymerase activity was evaluated using a recombinant BVDV RdRp making use of a poly(C) template. For the reference inhibitor 3′-dGTP, the 50% inhibitory concentration for the BVDV polymerase activity was <1 μM. BBP however had no effect on the activity of the viral polymerase (Fig. 3). Because BBP did not inhibit the activity of the purified BVDV RdRp, the effect of the compound on viral replication complexes (RCs), isolated from MDBK cells that had been infected with the wild-type virus or with the selected BBP^{res} BVDV strain, was assessed. BBP inhibited the activity of the BVDV WT replication complexes in a dose-dependent manner with a maximum inhibition of ~80% at 5 μM (Fig. 4A and B). By contrast, the activity of BBP against RCs isolated from MDBK cells that had been infected with the BBP^{res} BVDV, was 4-fold less potent compared to the inhibitory effect of BBP on the RCs derived from cells infected with BVDV WT (Fig. 4A and B).

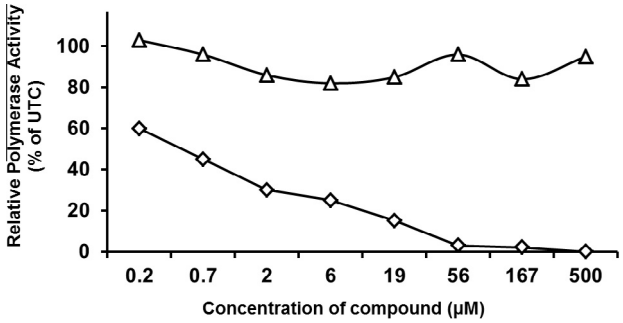


Fig. 3. Effect of BBP (open triangles) and 3′-dGTP (open diamonds) on the activity of the purified BVDV RdRp. Data are from a typical experiment and are expressed as the percentage of untreated control.

3.5. Computational docking of BBP in the BVDV RdRp crystal structure

A closer examination of the BVDV RdRp crystal structure revealed that the mutation I261M is located in a small cavity near the fingertip domain of the BVDV polymerase at a distance of only 8 Å from F224 and 12 Å from E291, (Fig. 5). The larger side chain of methionine M261 may be involved in resistance against VP32947, BPIP, LZ37 and AG110, respectively (Baginski et al., 2000; Paeshuyse et al., 2006, 2007, 2009). Docking studies in this cavity

Table 4
Susceptibility of wild type and BBP^{res} BVDV and CSFV to BBP, BPIP, AG110 and LZ37.

Virus	EC_{50} (μM)				
	CSFA-0/BBP	BPIP	AG110	LZ-37	2′-CMC
BVDV (NADL)	0.3 ± 0.1	0.05 ± 0.006	1.6 ± 0.4	8.8 ± 1.7	1.9 ± 0.5
BVDV (selected BBP^{res})	>100 (55)	9.3 ± 1.3 (186)	11 ± 6.0 (6.7)	>100 (>11.3)	0.9 ± 0.3 (0.5)
BVDV (recombinant BPIP^{res})	1.8 ± 0.4 (6)	23 ± 5.3 (456)	>100 (>62)	32 ± 8.5 (3.7)	0.24 ± 0.08 (0.1)
CSFV (Alfort$_{187}$)	0.33 ± 0.25	1.6 ± 0.4	10 ± 0.6	n.d.	n.d.
CSFV (selected BBP^{res})	>100 (>300)	n.d.	n.d.	n.d.	n.d.

Effective concentration 50% values (EC_{50}) are mean values ± SD for 4 independent experiments. Values between brackets represent fold-resistance.
n.d.: not determined.

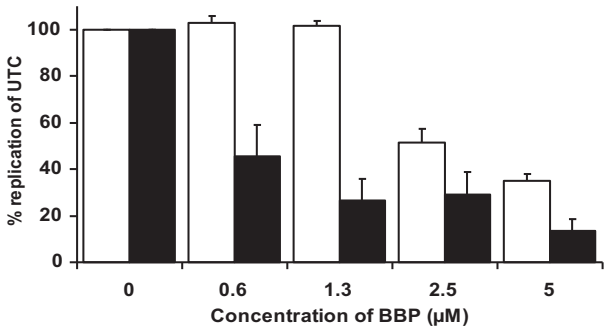


Fig. 4. Effect of BBP on the activity of RCs isolated at 14 h post-infection from MDBK cells that had been infected with wild-type BVDV (NADL) or with the selected BBP^{res} BVDV strain. Densitometric analysis of the autoradiographs; black bars represent activity of wild-type BVDV (NADL) RCs, and open bars represent activity of RCs from cell infected with BBP^{res} virus. Data are mean values ± SD of 2 independent experiments. UTC, untreated control; WT, wild-type.

revealed the following possible interactions between the polymerase and BBP: (*i*) hydrophobic contacts of BBP with T160, F224, P262, K263, N264, I287 and A392; and (*ii*) a hydrogen bond from a N in the 5-membered ring to the main chain O atom in P262 (Fig. 5). The larger side chain of methionine may clash with the BBP, thereby interfering with its binding.

4. Discussion

In a large scale screening effort aimed to identify *novel* pestivirus inhibitors, we identified 2,6-bis(benzimidazol-2-yl)pyridine [BBP (CSFA-0)], as a selective *in vitro* inhibitor of the replication of pestiviruses. BBP proved active against BVDV-1 as well as against CSFV, but proved inactive against the related HCV and flaviviruses (YFV and DENV) or a panel of unrelated RNA and DNA viruses.

In an attempt to improve the antiviral activity/selectivity, 25 analogues were synthesized. A structure–activity relationship was derived from the data obtained with these compounds in virus-cell-based assays. We did not succeed in optimizing the potency of this class of molecules. In particular analogue 22 was shown to have similar activity/selectivity as the lead compound.

To unravel the mechanism of antiviral activity, the genotype of *in vitro* selected BBP^{res} BVDV was determined. An isoleucine (I) to methionine (M) mutation was detected at amino acid residue 261 (I261M) of the NS5B, the gene that encodes the viral RNA-dependent RNA polymerase. Similar observations were made for CSFV for which two individual point mutations, i.e. I261N and P262A, were identified in the viral polymerase. The crystal structure of the RdRp reveals a right-handed structure made up of fingers, a palm and a thumb domain. A special feature of the pestivirus RdRp is that the fingers domain contains an extension called fingertips domain, which interacts with the thumb domain, with which it forms an entrance to the template-binding channel. The fingertip domain is believed to be involved in the template/product translocation, dimerization of RdRp within the RC, or protein–protein interactions, enabling the assembly of an active RC (Choi et al., 2004, 2006; Uttenthal et al., 2005; Morris et al., 1998). Moreover, the fingertip region contains the polymerase motifs I and II, which are involved in RNA template and nucleoside triphosphate (NTP) binding. Motif I has been reported to be located near the initiation NTP binding site and to bind the incoming NTP (Choi et al., 2004). Interestingly, the I261M mutation is located in the fingertip region within this motif. This mutation, selected under antiviral pressure with BBP, might thus cause conformational changes in the fingertip region of RdRp, disturbing one of the aforementioned functions.

Despite the fact that only (a) mutation(s) in the RdRp was (were) found in both BBP^{res} BVDV and CSFV, provides strong evidence that the viral polymerase is the target, BBP had no effect on the activity of the purified recombinant BVDV RdRp. The viral polymerase assay was validated by demonstrating that 3′-dGTP

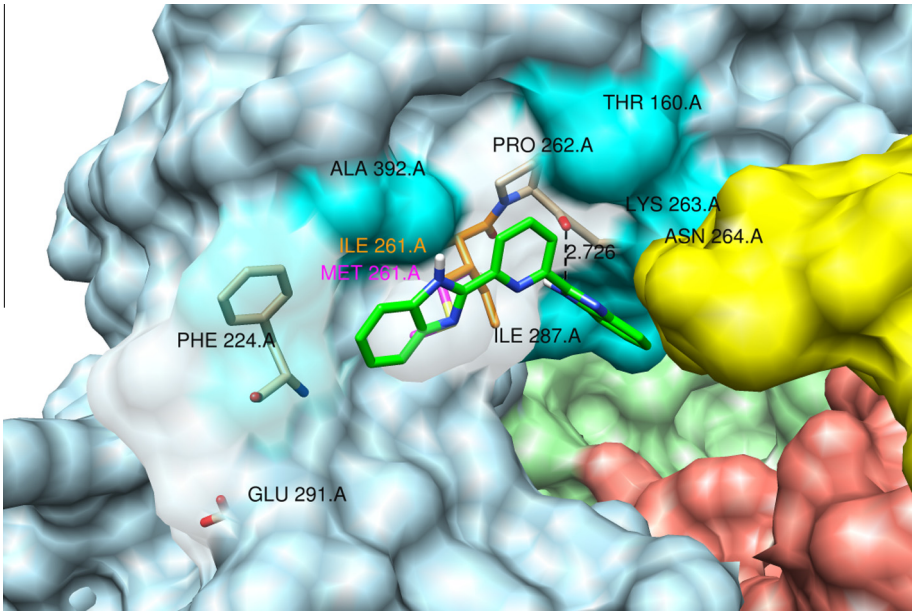


Fig. 5. Modeling of BBP near the position I261M in the RNA-dependent RNA polymerase. The different domains of the BVDV polymerase are colored as follows: the N-terminal domain is in yellow (residues 92–138), the finger domain is in blue (residues 139–313 and residues 351–410), the palm domain is in green (residues 314–350 and residues 411–500), and the thumb domain in red (thumb, residues 501–679). Carbons of residue I261l are colored orange and the I261M mutation has magenta carbons. Residues F224 and E291 are also made visible in stick representation. The ligand carbon atoms are colored green. Hydrophobic interactions are visualized by a cyan color. BBP has a hydrogen bond from an N in the 5 membered ring to the main chain O atom in P262.

inhibited the BVDV RdRp activity. Likewise, BPIP (Paeshuyse et al., 2006), AG110 (Paeshuyse et al., 2007) and LZ37 (Paeshuyse et al., 2009) also had no inhibitory effect on the highly purified RdRp. However, in the context of the host cell, the Pestivirus NS5B interacts with other viral and cellular proteins and together with the RNA genome, assembles in a membrane-associated replication complex that is involved in viral replication (Uttenthal et al., 2005). The replication activity of the latter was shown to be inhibited by BPIP (Paeshuyse et al., 2006), AG110 (Paeshuyse et al., 2007) and LZ37 (Paeshuyse et al., 2009). We therefore evaluated the effect of BBP on RCs isolated from MDBK cells that had been infected with either the wild-type virus or the BBP^{res} BVDV. BBP inhibited the function of the wild-type RC 4-fold more than that of the mutant strain. This may be explained by (*i*) a specific conformation of the viral RdRp in the RC that is needed for a productive interaction with the RNA template, or (*ii*) a protein–protein interaction between NS5B and the others viral non-structural proteins and host protein blocking compound binding sites on the viral polymerase finger domain (Paeshuyse et al., 2007). The observation that BBP inhibits the function of RC but not that of the purified RdRp may be explained by the fact that, following binding to the NS5B, the compound might disturb the proper formation of a functional replication complex. We hypothesize that BBP may inhibit the protein–protein interactions and stability between the different non-structural proteins that compose the replication complex, interfering with its correct functioning.

The previously reported BVDV inhibitors BPIP, AG110, and LZ37 proved to be cross-resistant with BBP-resistant BVDV. BPIP^{res} and LZ37^{res} BVDV were shown earlier to carry the F224S and F224Y mutation (Paeshuyse et al., 2006, 2009), respectively in their polymerase; while AG110^{res} BVDV carry the E291G mutation (Paeshuyse et al., 2007). The cross-resistance of these different classes of inhibitors might be explained by the proximity (7 Å) of E291, F224 and I261 in the RdRp structure. Furthermore, it was shown that a singular mutation, namely F224S, was sufficient to cause a ~6-fold reduction in activity of BBP (see Table 4). The I261M mutation might influence the structure of RdRp in the close vicinity of residues 291 and 224, causing conformational changes in this cavity of the polymerase, thus explaining the cross-resistant to these different inhibitors.

The findings made for BVDV were paralleled by observations made with CSFV. Although BPIP belongs to a structurally different class of compounds, BPIP^{res}-virus proved also insensitive to BBP (EC_{50} > 100 μM). Despite the obvious cross-resistance of BPIP and BBP, the mutations in the CSFV RdRp (i.e. I261N and P262A) are different from the previously reported mutation (i.e. T259S) that confers resistance to the imidazopyridine BPIP. However, residues I261 and P262 are located in close proximity to both T259 and F224 (Paeshuyse et al., 2006; Vrancken et al., 2008). Considering the clear cross-resistance and very close vicinity of the residues I261 and P262 to T259 and F224, it is reasonable to assume that BBP may interact with NS5B of CSFV at a site very close to (if not identically to) the binding-site of BPIP. Combining these observations suggests that the cross-resistance among several structurally unrelated classes of pestivirus inhibitors are the result of mutations that lead to conformational changes in this "cavity" of the viral polymerase.

In conclusion, we identified a highly potent and selective inhibitor of pestiviruses replication. Structural analogues of this molecule also exhibit anti-pestivirus activity. BBP possibly interacts with the fingertip of the BVDV-RdRp region that is a "hot spot" for inhibition of pestivirus replication.

Acknowledgements

We thank Carolien De Keyzer, Stijn Delmotte and Tom Bellon for excellent technical assistance, Suzanne Kaptein for performing the YFV and DENV assays and Leentje Persoons for performing the screen against unrelated DNA and RNA viruses. This work was supported by a postdoctoral fellowship from the Research Foundation Flanders-FWO to Jan Paeshuyse, the IWT-SBO project #100042, KU Leuven Grant (GOA/10/014) and by Grant G.0728.09N of the Research Foundation Flanders-FWO.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.03.010.

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