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Imidazo[4,5-c]pyridines inhibit the in vitro replication of the classical swine fever virus and target the viral polymerase

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Abstract

Selective inhibitors of the replication of the classical swine fever virus (CSFV) may have the potential to control the spread of the infection in an epidemic situation. We here report that 5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-c]pyridine (BPIP) is a highly potent inhibitor of the in vitro replication of CSFV. The compound resulted in a dose-dependent antiviral effect in PK_{15} cells with a 50% effective concentration (EC_{50}) for the inhibition of CSFV $Alfort_{187}$ (subgroup 1.1) of 1.6 ± 0.4 μM and for CSFV Wingene (subgroup 2.3) 0.8 ± 0.2 μM. Drug-resistant virus was selected by serial passage of the virus in increasing drug-concentration. The BPIP-resistant virus (EC_{50}: 24 ± 4.0 μM) proved cross-resistant with VP32947 [3-[((2-dipropylamino)ethyl)thio]-5*H*-1,2,4-triazino[5,6-b]indole], an unrelated earlier reported selective inhibitor of pestivirus replication. BPIP-resistant CSFV carried a T259S mutation in NS5B, encoding the RNA-dependent RNA-polymerase (RdRp). This mutation is located near F224, a residue known to play a crucial role in the antiviral activity of BPIP against bovine viral diarrhoea virus (BVDV). The T259S mutation was introduced in a computational model of the BVDV RdRp. Molecular docking of BPIP in the BVDV polymerase suggests that T259S may have a negative impact on the stacking interaction between the imidazo[4,5-c]pyridine ring system of BPIP and F224.

Keywords: Antiviral agent; Flaviviridae; Pestivirus; Inhibition of classical swine fever virus; RNA-polymerase; Imidazopyridine

1. Introduction

Classical swine fever virus (CSFV) is, along with the bovine viral diarrhoea virus (BVDV) and the border disease virus (BDV), a member of the genus *Pestivirus* within the family of *Flaviviridae* (Van Regenmortel et al., 2000). Pestiviruses are small, enveloped, single-stranded, positive sense RNA viruses (ca. 12.5 kb). The viral genome consists of a single open reading frame (ORF) flanked by a 5′ and 3′ untranslated terminal region (UTR) (Meyers and Thiel, 1996) and is translated into a polyprotein of about 4000 amino acids. This polyprotein encodes several structural (C, Erns, E1 and E2) and non-structural (Npro, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Rice, 1996). CSFV is the causative agent of a highly contagious disease in pigs, causing major economic losses worldwide (Laevens et al., 1999; Meuwissen et al., 1999; Sharpe et al., 2001).

Although several vaccines are available against CSF, the inability to serologically differentiate vaccinated from infected pigs, accompanied by the severe economical sanctions on the affected region, resulted in the ban of prophylactic vaccination within the borders of the European Union (EU) (Anonymous, 1997; Mayer et al., 2004; van Oirschot, 2003). Recently, several marker vaccines have been developed to face this problem (De Smit et al., 2001a). However, the application is severely hampered by the reliability of the accompanying discriminatory ELISA test (Floegel-Niesmann, 2001) and the variable results in viral transmission studies (Ahrens et al., 2000; De Smit et al., 2001b; Depner et al., 2001; Uttenthal et al., 2001).

Although emergency vaccination is allowed during a CSF-outbreak according the EU council directive 80/217/EEC,

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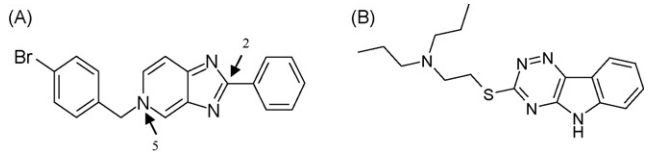

Fig. 1. Structural formulae of (A) BPIP (5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-c]pyridine) and (B) VP32947 (3-[((2-dipropylamino)ethyl)thio]-5*H*-1,2,4-triazino[5,6-b]indole).

present outbreaks are controlled by a "stamping-out" policy, i.e. the total eradication of animals in infected farms (Stegeman et al., 2000) and the pre-emptive culling of neighbouring herds. Due to the growing public criticism against the massive killing of often healthy, non-infected animals a more ethically acceptable control strategy is required (van Oirschot, 2003). The use of potent inhibitors of CSFV replication could cover the "immunity gap", which is an inherent drawback of vaccination strategies (Collett, 2005) and could be used as a first intervention in case of an outbreak. Indeed, reducing the viremia by using an antiviral may result in a reduced risk of spread.

Recently, the discovery of a novel class of compounds comprising an imidazo[4,5-c]pyridine nucleus was described that exhibited antipestivirus activity (Puerstinger et al., 2006).

The most potent compounds in this series, i.e. substituted 5-benzyl-2-phenyl-5*H*-imidazo[4,5-c]pyridines target the BVDV polymerase and inhibit the formation of BVDV replication complexes (RC) (Paeshuyse et al., 2006). We here report on the particular characteristics, resistance profile and potential mechanism of action of a representative molecule from this series against CSFV.

2. Materials and methods

2.1. Cells and viruses

A Porcine kidney cell line (PK_{15} obtained from the American Type Culture Collection, CCL-33) was maintained in Minimal Eagle Medium (MEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), antibiotics (0.25 μg/ml amphotericin, 50 μg/ml gentamicin, 1000 U/ml sodium benzylpenicillin) and 2 mM glutamine. The CSF reference-strain $Alfort_{187}$ (subgroup 1.1) was kindly provided by the European Union Reference Laboratory for Classical Swine Fever, Hannover (Germany). The field-isolate Wingene (subgroup 2.3), similar to an isolate known as "souche Lorraine" (Koenen and Lefebvre, 1995), was obtained during the Belgian CSF-outbreak in 1993–1994.

2.2. Compounds

A series of imidazo[4,5-c]pyridine derivates with different substituents at positions 2 and 5 (Fig. 1A) were synthesized as described previously (Puerstinger et al., 2006) and were used to investigate the effect of the molecular structure on their antiviral activity.

The reference compound 3-[((2-dipropylamino)ethyl)thio]-5*H*-1,2,4-triazino[5,6-b]indole (VP32947) (Fig. 1B) was synthesized by standard methods.

2.3. Antiviral assays

PK_{15} cells were seeded at a density of 5×10^3 per well in 96-well cell culture plates in MEM-supplemented with 10% heat-inactivated FCS and antibiotics (0.25 μg/ml amphotericin, 50 μg/ml gentamicin, 1000 U/ml sodium benzylpenicillin). Following 24 h incubation at 37 °C (5% CO_2) medium was removed and cultures in each well were infected with 100 $TCID_{50}$ of CSFV. After incubating for 1 h at 37 °C, cells were washed three times with PBS and fivefold serial dilutions of the compounds were added in a total volume of 100 μl. Uninfected cells, and cells receiving virus without compound, were included in each assay plate. After 3 days, medium was removed and cells were washed three times with PBS and left to dry at 37 °C. After fixation in a Pasteur oven for 1.5 h at 80 °C, cells were incubated with an in-house produced biotine conjugated polyclonal anti-CSFV serum for 1 h at room temperature (rt), plates were then washed three times with PBS + 0.05% Tween20 after which they were incubated with streptavidine-coupled peroxidase antibody (Amersham Biosciences Europe, Freiburg, Germany) for 1 h at rt. Following three wash steps, plates were incubated with detection buffer [4 mg 3-amino-9-ethylcarbazole dissolved in 1 ml *N*,*N*-dimethylformamide and 19 ml of 0.05 M acetate buffer (pH 5.2) containing 10 μl of 30% H_2O_2] for 15 min, until a dark red color appeared. The 50% effective concentration (EC_{50}) was defined as the concentration offering 50% protection of cultured cells against viral infection.

The cytotoxicity was determined by the evaluation of the cell morphology by microscopy by observing microscopically detectable alterations as loss of cell confluency, cell rounding and shrinking. Morphological changes were evaluated and CC_{50} values (= cytotoxic effects affecting 50% of cultured cells) were determined.

Both the EC_{50} and CC_{50} values were calculated using the method of Reed and Muench (1938).

2.4. Selection of BPIP-resistant CSFV $Alfort_{187}$

Two cultures of semi-confluent PK_{15} cells were infected for 1 h at 37 °C with 100 $TCID_{50}$ of the CSFV-strain $Alfort_{187}$. Following trypsinization, each of the cell cultures were seeded in 25 cm^2 flasks containing growth medium supplemented with 1 μM of BPIP were incubated for 3 days at 37 °C. This sequence was repeated with stepwise increasing the concentrations of BPIP (up to 20 μM) and until BPIP-resistant ($BPIP^r$) virus was obtained.

2.5. RNA isolation

Total RNA was extracted from cell culture supernatant using the RNeasy Mini Kit (QIAgen, Venlo, Netherlands) as described in the protocol of the TaqVet real-time PCR Kit (LSI, Lissieu,

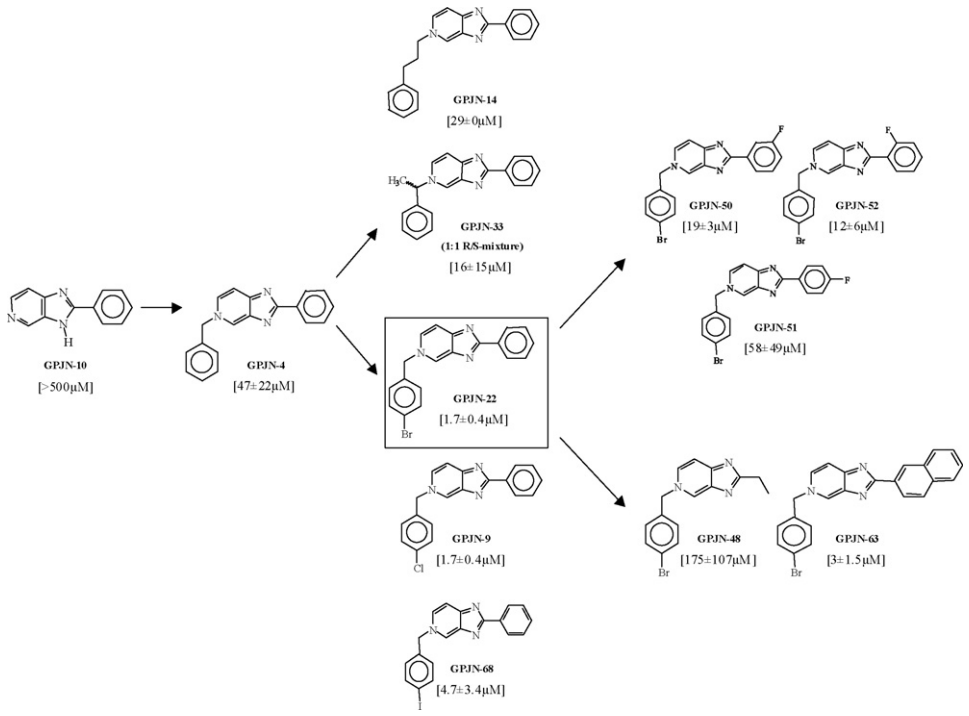

Fig. 2. Structure activity relationship of the anti-CSFV activity of a series of imidazopyridines. Compound **22** emerged as the most potent inhibitor of in vitro CSFV activity within the series; the 50% effective concentrations against CSFV Alfort$_{187}$ are given between brackets [EC$_{50}$].

France). Each extraction was accompanied by a positive and negative control sample.

2.6. Molecular cloning

For sequence determination, PCR fragments of the NS5B gene were obtained using the SuperScript™ One-Step RT-PCR System for Long Templates (Invitrogen, Merelbeke, Belgium) and were subsequently cloned into pCR-TOPO XL vectors using the TOPO® XL Cloning Kit (Invitrogen, Merelbeke, Belgium). Clones were selected using a blue/white and antibiotic resistance screening on X-gal plates containing kanamycin. The constructs were purified using the QIAprep Spin Miniprep Kit (QIAgen, Venlo, Netherlands) and fragment-insertion was verified by agarose gel electrophoresis after digestion by EcoRI (Invitrogen, Merelbeke, Belgium). Purified plasmids were used for sequencing.

2.7. Sequence analysis

The sequences of the cloned 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). The obtained sequence data were analysed using the Chromas 2.3 application (http://www.technelysium.com.au/chromas.html).

2.8. Real-time RT-PCR

RNA was extracted from infected cell cultures using the RNeasy Mini kit as described above and viral RNA levels were quantified by a CSFV-specific, probe-based real-time RT-PCR assay using the TaqVet Kit (LSI, Lissieu, France) according to the manufacturers recommendations. The assay detected a 90b sequence located in the 5′UTR, β-actin was used as an internal control. In each assay positive and negative controls were included.

2.9. Computational modelling

Because the crystal structure of the RNA-dependent RNA-polymerase (RdRp) of CSFV has not yet been determined, and the pestivirus polymerase amino acid sequences are 70–75% identical (Choi et al., 2004a; Zhang et al., 2005), the published structure of the BVDV RdRp (PDB entry 1S48) (Choi et al., 2004b) was used for the CSFV modelling calculations. Especially F224 and T259 are located in conserved sequence motifs which give us a valuable ground for taking the BVDV RdRp structure as a model for the CSFV enzyme (Zhang et al., 2005). The model of the docked complex of BPIP near F224 in BVDV RdRp as described earlier (Paeshuyse et al., 2006) was used as a starting structure for all energy calculations. These calculations were performed using the Amber 8.0 software tools (Case et al., 2005). The T259S mutation was introduced and its effect on the bound inhibitor and surrounding was investigated by energy minimisation and a simple rotation of the torsion angles of the F224 sidechain to explore the possible rotamers of F224.

3. Results

3.1. Structure activity relationship

Based upon the activity of the imidazo[4,5-c]pyridine analogues against the bovine viral diarrhoea virus (BVDV), several

2-phenyl-5*H*-imidazo[4,5-c]pyridines bearing a substituted benzyl group at the 5-position were evaluated for their ability to inhibit the in vitro replication of the classical swine fever virus (CSFV) (Fig. 2). The necessity of the presence of a substituent at the 5-position for activity is evident from the inactivity of compound **10**. By introducing a benzyl group at the 5-position, the activity of the thus obtained analogue (compound **4**) resulted in antiviral activity [EC_{50} 13 μM]. Next, the influence of substituents on the phenyl or the benzyl group was evaluated. Introduction of a halogen resulted in an improvement of the activity when a chlorine or iodine was placed at position 4 (compounds **9** and **68**, respectively). The best activity was achieved when introducing a bromine at position 4 (compound **22**). Replacement of the methylene part of 5-benzyl by 1,3-propylene seems to be tolerated (compound **14**) as well as addition of a CH_3-sidechain to this linker (compound **33**). Starting from the most active compound (compound **22**), further modifications were evaluated. Replacement of the 2-phenyl by an ethyl group resulted in a loss of activity (compound **48**). Substitution by a 2-naphthyl, however, seemed to be tolerated (compound **63**). Further introduction of fluorine in positions 2, 3 or 4 of the 2-phenyl group (compounds **52**, **50** and **51**, respectively) resulted in a reduced activity. Based on these experimental data, compound **22** (systemic name: 5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-c]pyridine) was selected for further mechanistic studies and will be further referred to as BPIP.

3.2. Antiviral activity of BPIP

BPIP inhibited the replication of $Alfort_{187}$ and Wingene in a dose-dependent manner (as was assessed by a real-time RT-PCR assay, Fig. 3). EC_{50} of, respectively 1.6 ± 0.4 and 0.8 ± 0.2 μM were calculated for $Alfort_{187}$ and Wingene (Table 1). A $CC_{50} > 275$ μM was determined resulting in a selectivity index (ratio of CC_{50} to EC_{50}) of 172 and 344 for CSFV $Alfort_{187}$ and Wingene, respectively.

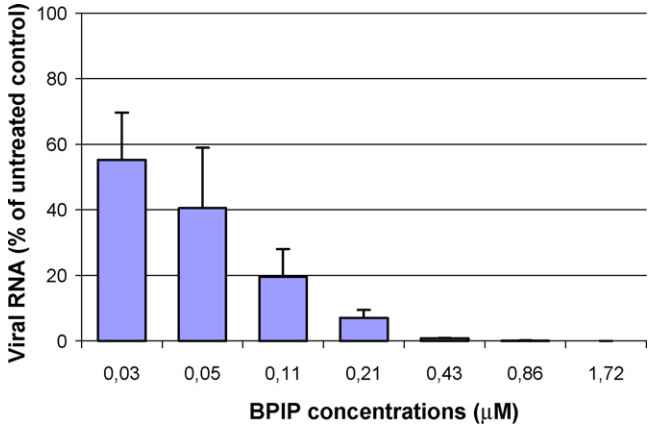

Fig. 3. Effect of BPIP on release of CSFV–Wingene in culture supernatant as determined by real-time RT-PCR 3 days after infection and treatment with BPIP.

Table 1
Susceptibility of the wild type virus $Alfort_{187}$ and Wingene and of $BPIP^r$ $Alfort_{187}$ to BPIP and VP32947

Virus	EC_{50} (μM)	
	BPIP	VP32947
WT $Alfort_{187}$	1.6 ± 0.4	2.7 ± 0.1
WT Wingene	0.8 ± 0.2	ND
$BPIP^r$ $Alfort_{187}$	24 ± 4.0	19 ± 4.6

Data are mean values ± standard deviations of at least 2 independent determinations. ND: not done.

3.3. Selection and characterization of BPIP-resistant CSFV

Two independently selected cultures of $BPIP^r$ viruses were obtained after 16 consecutive passages of the wild type virus of the strain $Alfort_{187}$ in increasing concentrations of BPIP. Both obtained drug-resistant viruses were at least 15-fold less sensitive than the wild type virus to BPIP (EC_{50} of 24 ± 4.0 μM) (Table 1). The effect of VP32947, an earlier reported non-nucleoside inhibitor of pestivirus replication, was studied on the replication of the $BPIP^r$ virus. VP32947 was sevenfold less active against the $BPIP^r$ virus than against the wild type virus and can thus be considered as cross-resistant with BPIP. Since the target of BPIP and VP32947 in BVDV replication is the RdRp, the sequence of the CSFV NS5B-gene, was determined and compared to the published sequence of the wild type CSFV-strain Alfort187 (NCBI accession number X87939). Two point mutations were identified in the $BPIP^r$ virus, of which only one caused an amino acid substitution. This A to T transversion at position 10,688, which occurred in both independently selected drug-resistant viruses, results in substitution of a threonine (T) to a serine (S) at position 259 of the NS5B protein.

3.4. Molecular modelling

The interaction of BPIP with the viral RdRp was studied by introducing the T259S mutation in a computational model based on the coordinates of the BVDV RdRp. The following potential interactions with the inhibitor have been suggested: (i) a stacking interaction between the imidazo[4,5-c]pyridine ringsystem of BPIP and F224 and (ii) hydrophobic contacts of the inhibitor with A221, A222 and F224 (Paeshuyse et al., 2006). The introduction of the T259S mutation in this model, after energy minimisation and exploration of the rotamer states of F224, results in a slight rotation of the phenyl group of the F224 residue. This rotamer state orientates the phenyl-sidechain of F224 into a non-planar position against the imidazo[4,5-c]pyridine ringsystem increasing the tilt angel up to 45° and thereby altering the distance between both aromatic systems from 3.5 Å in the wild type RdRp to 3–5 Å. Furthermore, this analysis suggests a shift of the phenyl sidechain of F224 towards the pyridine ring of BPIP. Both alterations are likely to cause a perturbation of the stacking interaction between the imidazo[4,5-c]pyridine ring system of BPIP and F224 (Fig. 4), so that a favourable stacking with a putative inhibitor becomes impossible. Furthermore, hydrophobic contacts were suggested

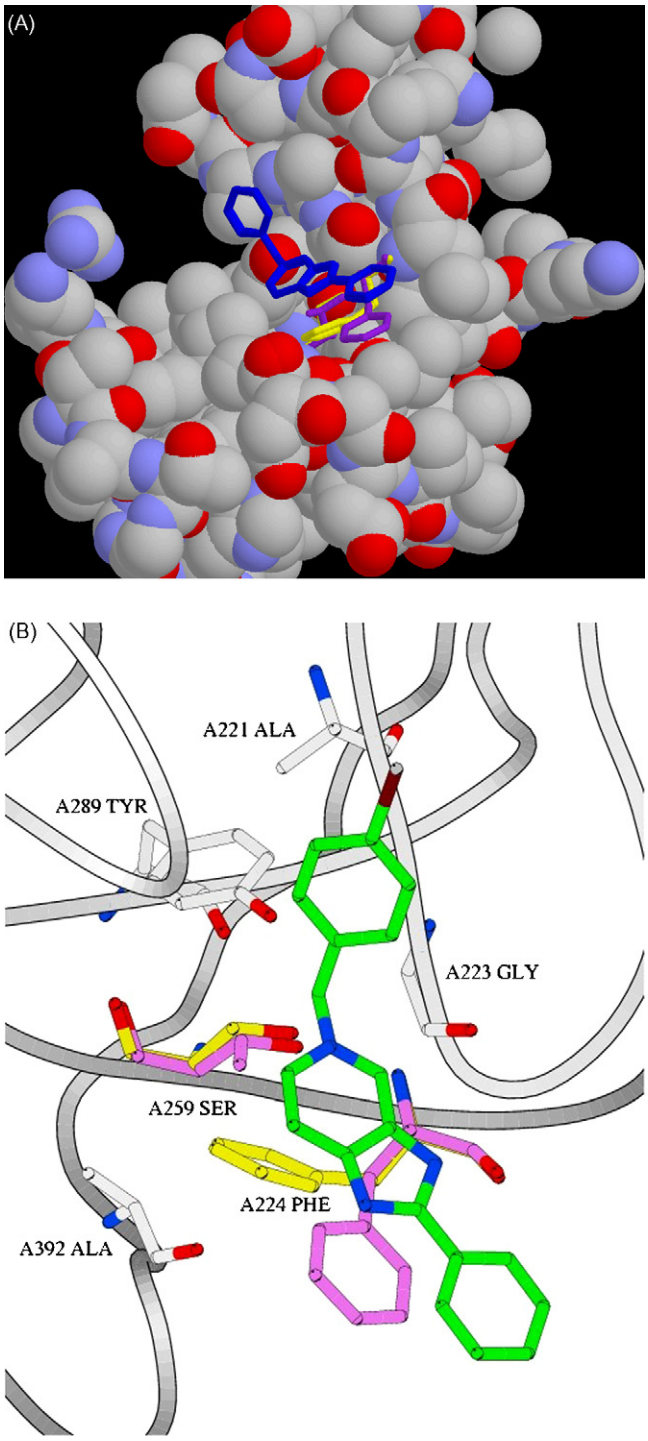

Fig. 4. Modelling of BPIP in the crystal structure of the BVDV RNA-dependent RNA-polymerase. Superposition of the structure of the RdRp of BVDV with BPIP docked in the vicinity of F224 in the wild type RdRp (purple) and in the selected BPIP^r RdRp (yellow). (A) Overview of the RdRp–BPIP interaction site. (B) Detail of the predicted interaction between the RdRp and BPIP (CPK colors). Introduction of mutation T259S results in a slight twist of the phenyl sidechain of F224 which abolishes the stacking interaction of this amino acid with BPIP. All amino acids making hydrophobic contact with BPIP are also drawn (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

between BPIP and residues A221, G223, A224, Y289 and A392.

4. Discussion

We here report the antiviral activity of imidazo[4,5-c]pyridines, a promising class of RNA-dependent RNA-polymerase inhibitors of the classical swine fever virus (CSFV). The most active compound in this series was 5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-c]pyridine (BPIP); this molecule inhibited the in vitro replication of CSFV Alfort$_{187}$ and Wingene (subgroups 1.1 and 2.3, respectively) and was selected for further mechanistic studies.

Recently, in vitro resistance of BVDV to BPIP was found to map to a single point mutation, i.e. F224 in the RNA-dependent RNA-polymerase gene (Paeshuyse et al., 2006).

To determine the mechanism of action of BPIP against CSFV, BPIP-resistant (BPIP^r) virus (strain Alfort$_{187}$) was generated. Two strains that were obtained independently proved cross-resistance with VP32947, a compound also known to specifically target the BVDV NS5B (Baginski et al., 2000). BPIP^r CSFV was found to carry the T259S mutation in the NS5B. This mutation was observed in two different clones of the selected BPIP^r viruses; since the amplification and sequencing were performed four times for each clone, PCR-based artefacts can be excluded.

To study the possible effect of this mutation on the interaction of BPIP with the CSFV polymerase, we mapped the residue in the recently published crystal structure of the BVDV NS5B (Choi et al., 2004a). Residue T259 was found to be located in close proximity to F224, the residue that was shown to be responsible for BPIP and VP32947 resistance in BVDV (Baginski et al., 2000; Paeshuyse et al., 2006) and to be situated in a β-sheet of a finger-domain, which has a similar topology among viral polymerases (Choi et al., 2004a). The latter was in agreement with a hypothetical homology model of the RdRp of CSFV (Zhang et al., 2005). After introducing the T259S mutation in the computational model of the BPIP–RdRp interaction of BVDV, a slight twist of the phenyl group of F224 was observed. This twist is most likely due to the reduced length of sidechain of residue 259 resulting from the T (–CHOHCH$_2$) to S (–CH$_2$OH) mutation. This creates an empty space close to F224, which may allow the phenyl side-chain to convert into an alternative conformational isomer, decreasing the stacking interaction with BPIP, resulting in the observed BPIP^r phenotype. We recently showed that BPIP does not inhibit the highly purified BVDV polymerase but that the compound efficiently inhibits the activity of viral replication complexes. The fact that both residue F224 and T259 are located in a fingertip, suggest that BPIP may prevent the interaction of viral and/or cellular components of the replication complex with this region of the polymerase. It may thus be assumed that the neighbourhood of residues F224 and T259 in the viral polymerase, with which the compound is believed to interact, may play a critical role in the functioning of the pestivirus replication complex.

In conclusion, BPIP is a potent in vitro inhibitor of the CSFV replication. BPIP or related analogues should also be further

evaluated for its (their) potential in curbing viral spread in an epidemic situation.

Acknowledgments

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