Proof of concept for the reduction of classical swine fever infection in pigs by a novel viral polymerase inhibitor

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INTRODUCTION

We recently identified a novel class of highly potent inhibitors of pestivirus replication (Puerstinger et al., 2006). This class of imidazo[4,5-c]pyridines specifically inhibits the replication of bovine viral diarrhea virus (BVDV) by targeting the RNA-dependent RNA polymerase (Paeshuyse et al., 2006). One of the compounds in this class, 5-[(4-bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-c]pyridine (BPIP), which was selected as the lead candidate, was also shown to efficiently inhibit the replication of other pestiviruses, i.e. border disease virus and classical swine fever virus (CSFV) (Paeshuyse et al., 2006; Vrancken et al., 2008).

CSFV is the causative agent of classical swine fever (CSF) (Le Potier et al., 2006) and can cause devastating outbreaks in pigs, with important economic impact (Sharpe et al., 2001). Epidemics in the 1990s in Belgium and The Netherlands showed the disastrous impact of CSF on the affected regions and clearly revealed the need for efficient control measures to prevent spread of the virus in epidemic situations. The inability to differentiate infected from vaccinated animals resulted in a ban on prophylactic...
vaccination within the European borders (Council Directive 80/217/EEC). Therefore, present outbreaks are controlled by complete eradication of the infected herds (stamping out) and the pre-emptive culling of neighbouring herds (Stegeman et al., 2000). This massive destruction of pigs has resulted in growing criticism by the public, with a call for ethically more acceptable strategies (van Oirschot, 2003). ‘Marker vaccines’ that allow serological differentiation between infected and vaccinated animals have been developed (Ahrens et al., 2000; Beer et al., 2007; de Smit et al., 2001a; Depner et al., 2001; Dewulf et al., 2000, 2004; Utenthal et al., 2001; Voigt et al., 2007). The use of such vaccines, however, is severely hampered by the insufficient efficacy of the accompanying discriminatory ELISA test (Dewulf et al., 2001, 2004). In addition, an inherent drawback of vaccination is the presence of an ‘immunity gap’, the time between vaccination and immunological protection against viral infection. During this ‘gap’, the virus can spread to neighbouring herds despite vaccination, thereby increasing the epidemic area. A strategy to address this problem could be the use of potent antiviral agents as additional tools in controlling the spread of infectious diseases such as CSF. In contrast to vaccines, antivirals offer the advantage of almost immediate protection; moreover, when the drug is added in feed, large numbers of animals can easily be treated in a very short time span. We have demonstrated here, for the first time, a proof of concept that selective inhibitors of in vitro CSFV replication are able to reduce (and possibly prevent) replication of CSFV in its target species.

METHODS

Compound and formulation. The synthesis of BPIP has been reported previously and was optimized for the synthesis of bulk quantities (Puerstinger et al., 2006 and unpublished data). Nuclear magnetic resonance analysis of the BPIP batch used revealed only three minor impurities of 0.27, 0.17 and 0.07%.

BPIP was formulated in feed pellets using a Labor Monoroll-type pelletizer with a horizontal ring-type die (equipped with a knife) and one roll. The die used had a width of 4 mm and a channel length of 50 mm. The feed mixture was fed to the die through a conditioner unit (horizontal mixing unit). In this experiment, no vapour was added to the mixture. After pelleting, the feed was cooled to ambient temperature using an air circulation system with dust recovery. The blank feed was pelleted first to exclude any possible contamination of the blank feed with BPIP.

Cells and virus. A porcine kidney cell line (PK15; ATCC CCL-33) was maintained in minimal Eagle’s medium supplemented with 10% BVDV-free, heat-inactivated fetal calf serum, antibiotics (0.25 μg amphotericin ml⁻¹, 50 μg gentamicin ml⁻¹ and 1000 U sodium benzylpenicillin ml⁻¹) and 2 mM glutamine. The field isolate Wingene (subgroup 2.3), similar to an isolate known as ‘souche Lorraine’ (Vanderhallen et al., 1999) and reported as moderately virulent (Dewulf et al., 2005; Floegel-Niesmann et al., 2003), was isolated during the Belgian CSFV outbreak of 1993–1994.

Antiviral assays.

Evaluation of potential BPIP resistance. The possible emergence of BPIP-resistant CSFV during antiviral treatment was evaluated by means of an in vitro biological assay. A selection of serum samples from BPIP-treated animals from which infectious virus could be isolated was evaluated for sensitivity to BPIP. The assay was carried out as described previously (Vrancken et al., 2008). Briefly, the 50% effective concentration (EC₅₀), defined as the concentration offering 50% protection of cultured cells against virus infection, was determined by addition of a fivefold dilution series of BPIP on CSFV-infected cells. An isolate was defined as BPIP resistant if a reduced sensitivity of at least 15-fold was observed against the antiviral molecule compared with the wild-type virus (Vrancken et al., 2008).

Evaluation of the effect of high concentrations of BPIP on in vitro CSFV replication. To evaluate the in vitro effect of treatment of CSFV-infected PK15 cells with a high concentration of BPIP, semi-confluent PK15 cells in six-well culture plates were infected with 10⁵ TCID₅₀ CSFV Wingene ml⁻¹. After 1 h incubation at 37 °C, the cells were washed three times with PBS after which BPIP was added at 3 μM. Cultures were further incubated for 3 days at 37 °C. After incubation, the supernatant was removed and infectious virus yield was quantified by titration.

Animals. Acute and chronic toxicity of BPIP was studied in 8-week-old conventional weaner pigs (Belgian Landrace × Piétrain) of ~20 kg in the animal facilities of the Veterinary and Agrochemical Research Centre (VAR; Belgium). Nine-week-old specific-pathogen-free Large White pigs, originating from protected breeding facilities at the Agence Francaise de Sécurité Sanitaire des Aliments (AFSSA; France), were used for evaluation of the antiviral activity of BPIP. On the day of the experimental inoculation, the mean weight of the pigs was 30 kg. All experiments were approved by the ethics committee of VAR.

Experimental design.

Acute toxicity study/oral absorption. Three weaner pigs, negative for BVDV and CSFV antigen and antibodies, were housed in a single pen. Feed was withheld for 10 h prior to administration of BPIP. BPIP was administered orally in a feed bolus at a dose of 200 mg kg⁻¹; blood samples were taken through the ear vein prior to and at 1, 2, 3, 4, 6 and 24 h after drug administration. Blood was collected in Microcollect tubes and serum was obtained after centrifugation for 15 min at 700 g. Samples were stored at –80 °C until analysis.

To evaluate toxicity, all animals were observed daily to assess their general condition. Specific parameters taken into account were: liveliness, body tension, body temperature, skin and body hair.

Chronic toxicity study. Two weaner pigs had their feed withheld for 10 h. Animals received an oral dose of BPIP at a daily concentration of 75 mg kg⁻¹ for a period of 15 consecutive days. All animals had 24 h access to the feed and were monitored daily for toxic effects. Blood was drawn at 0.5, 3 and 6 h after BPIP administration on day 10 of the treatment. The general condition of the animals was evaluated as described above. BPIP levels in the serum were analysed according to the method described below.

Protective activity of BPIP following CSFV infection. Experimental procedures and animal management were undertaken according to French legislation on animal experimentation and were carried out at the high containment facilities at AFSSA.

Eight pigs were housed individually in an isolation unit and received feed pellets containing BPIP at 75 mg kg⁻¹ day⁻¹ for a period of 15 days. One day after the first dosing, animals were infected intramuscularly with 4 ml CSFV Wingene with a median titre of 10⁶ TCID₅₀ ml⁻¹ (Dewulf et al., 2001, 2004; Tignon et al., 2008). In parallel, pigs receiving untreated feed were infected and housed in a
separate isolation unit as a positive-control group. A negative-control group was held in a separate isolation pen. All animals were observed daily for a period of 33 days. During this period, all animals had blood samples taken three [days 0–18 post-infection (p.i.)] or two (days 19–33 p.i.) times a week.

**Leukocyte count.** Leukocyte counts were performed using an MS9 haematology analyser (Schloessing Melet). Leukopenia was defined as a total leukocyte cell count less than or equal to $10^9$ cells l$^{-1}$ for two sequential blood samplings.

**Virus isolation.** An aliquot (100 μl) of whole blood, serum or homogenized organ sample was inoculated onto confluent PK13 cells seeded in a 24-well plate. After 36 h incubation at 37 °C, the supernatant was removed and the cells were washed five times with water. Subsequently, the plate was dried at 37 °C for 45 min. Next, 500 μl 2-propanol was added to the wells and the cells were left to incubate for 15 min at 5 °C. After removal of the 2-propanol, the plates were dried at 37 °C for 15 min. Finally, 400 μl fluorescein isothiocyanate-labelled polyclonal antibodies was added to the wells and incubated for 1 h at 37 °C. The plates were washed three times with water, dried and examined using a UV microscope.

**Virus neutralization assay.** The presence of CSFV-specific antibodies was evaluated using a virus neutralization assay (Holm-Jensen, 1981). Briefly, a twofold dilution series of tested sera was incubated with a CSFV Alfort187 solution of 100 TCID50 in 50 μl for 1 h at 37 °C. Next, a PK13 cell suspension was added and further incubated for 60–72 h at 37 °C. After incubation, the medium was removed and the cells fixed (1 h at 80 °C in a Pasteur oven) and incubated with an in-house biotin-conjugated polyclonal anti-CSFV serum. The plates were then incubated with streptavidin-conjugated horseradish peroxidase antibody and a dark colouration revealing the presence of CSFV was obtained after incubation with detection buffer containing 3-amino-9-ethylcarbazole, N,N-dimethylformamide, acetate buffer and H2O2. Antibody titres were defined based on the first of magnitude) and data were normalized using an internal standard.

**Determination of serum BPIP levels.** Serum levels of BPIP were determined by high-performance liquid chromatography to show that the pigs were receiving therapeutic levels of BPIP. Serum samples were combined with equal volumes of ethyl acetate and vortexed three times for 30 s. The aqueous phase was snap frozen in an ethanol/dry ice mixture. The organic phase was transferred to a separating funnel and washed three times with water. The aqueous phase was snap frozen in an ethanol/dry ice mixture. The organic phase was transferred to a new centrifuge tube and the ethyl acetate was evaporated in a vacuum centrifuge at 40 °C in a Pasteur oven) and incubated with an in-house biotin-conjugated polyclonal anti-CSFV serum. The plates were then incubated with streptavidin-conjugated horseradish peroxidase antibody and a dark colouration revealing the presence of CSFV was obtained after incubation with detection buffer containing 3-amino-9-ethylcarbazole, N,N-dimethylformamide, acetate buffer and H2O2. Antibody titres were defined based on the first dilution where CSFV could be detected.

**Sequence analysis.** The statistical significance of differences between mean virus load (EGC and virus titres) of different animal groups was evaluated by using a two-tailed Student’s t-test with unequal variances with a 95% confidence interval.

**RESULTS**

**Oral absorption of BPIP**

Three animals each received a feed bolus containing BPIP (dose 200 mg kg$^{-1}$). All animals readily consumed the BPIP bolus (within ~3 min). No adverse effects were observed in any of the treated pigs. Fig. 1 depicts the plasma drug concentration against time following administration. One hour after administration, a mean concentration of 9.31 ± 5.11 μM was reached in the plasma. At 2 h after dosing, the plasma concentration was 5.73 ± 3.17 μM. An apparent second plasma peak appeared with a maximum concentration of 8.86 ± 2.58 μM (6 h) followed by a continuous decrease to a plasma concentration of 4.56 ± 3.25 μM at 24 h after dosing. The mean peak concentration ($C_{\text{max}}$) was determined as 10.2 ± 4.32 μM and the time to peak concentration ($T_{\text{max}}$) as 3.3 ± 2.5 h. No metabolites were detected.

**A dose regimen of 75 mg kg$^{-1}$ day$^{-1}$ for 15 days does not result in toxicity**

Two animals received feed containing BPIP (75 mg kg$^{-1}$ day$^{-1}$). The animals consumed the BPIP-containing
feed daily for 15 consecutive days. No adverse effects were observed. Analysis of serum samples taken at 0.5, 3 and 6 h after BPIP administration on day 10, following the onset of a steady-state plateau, revealed BPIP levels of between 0.950 and 3.495 μM (Fig. 1).

**BPIP significantly reduces viraemia and viral load in CSFV-infected pigs**

Eight animals received feed containing BPIP (mean daily dose of 75 mg kg⁻¹ day⁻¹) and were inoculated intramuscularly with CSFV Wingene 24 h later. A group of four animals (P1–P4) who had received feed that did not contain BPIP was also infected. Treatment was continued for 14 consecutive days; the presence of infectious virus and viral genome was monitored for a period of 33 days. Two animals of the untreated control group (animals P3 and P4) reached a moribund state showing distinct ataxia and skin haemorrhages and were euthanized at 22 days p.i.

The untreated control pigs invariably tested positive from 5 days p.i. to euthanasia (animals P3 and P4 at 22 days p.i.; animal P2 at 33 days p.i.) or recovery (animal P1 at 26 days p.i.) by means of virus isolation (Fig. 2a). Seven of eight BPIP-treated animals developed a short transient viraemia between 5 and 12 days p.i. with a peak viraemia at 7 days p.i. Three animals were positive for virus isolation only for 2 days, one animal for 4 days, two animals for 5 days and one animal for 7 days. One animal (animal 8) remained negative for virus isolation for the entire period of observation (Fig. 2a). Virus titration at 7 days p.i. revealed a mean virus load of 1.75 ± 1.04 log₁₀ TCID₅₀ ml⁻¹ in BPIP-treated animals and 4.75 ± 0.5 log₁₀ TCID₅₀ ml⁻¹ in the untreated control group. Thus, on average, virus titres were 1000-fold lower in treated than in untreated animals, resulting in a highly significant difference in viral load between the groups (P=0.00005) (Fig. 2b).

No infectious virus could be isolated from the blood of any of the BPIP-treated animals at the end of the experiment (33 days p.i.). However, infectious virus was isolated at the time of euthanasia at 22 days p.i. (animals P3 and P4) and 33 days p.i. (animal P2) from three of four animals from the untreated control group; the remaining animal tested negative for virus isolation at the end of the experiment (33 days p.i., animal P1). All samples where no infectious virus could be isolated remained negative when passaged six times in cell culture.

Real-time RT-PCR analysis on whole blood revealed that BPIP-treated animals had detectable viral RNA between 2 and 19 days p.i. Only one of eight treated animals tested
positive at 2 days p.i. (animal 5; EGC not quantifiable). Viral RNA was detected as early as 2 days p.i. in all animals of the untreated control group and these animals remained positive until they were euthanized on day 22 or at the end of the experiment (day 33). At each time point of the observation period, viral RNA levels were markedly and significantly lower \((P \leq 0.001)\) in treated than in untreated animals (Fig. 3).

The tonsils of both treated and untreated animals were analysed (at day 22 for euthanized animals or at day 33 for the other animals) for the presence of CSFV (by means of real-time RT-PCR and virus isolation) (Table 1). Very low levels of viral RNA were detected in the tonsils of treated animals \((\text{mean } \log_{10}\text{ EGC}=2.18 \pm 0.78)\) and no infectious virus was detected in the tonsils of any of the eight treated animals. By contrast, \(\log_{10}\text{ EGC}\) values in untreated animals ranged from 4.57 to 7.31 and infectious virus was detected in the tonsils of three of four of these animals.

### Leukocyte count

All untreated animals were leukopenic from 2 days p.i. until the end of the experiment (33 days p.i.). The evolution of mean leukocyte counts is depicted in Fig. 4. Three BPIP-treated animals had normal leukocyte counts for the entire period of observation. In five of eight BPIP-treated animals, leukopenia was observed between days 5 and 7. Two of these animals remained in a leukopenic state until day 9 p.i.

### Virus neutralization

Three of eight BPIP-treated animals seroconverted at 14 days p.i. with antibody titres between 1:10 and 1:15. Four other BPIP-treated animals had seroconverted at the next blood sampling, i.e. at day 16 p.i. (titres 1:10 to 1:15); the remaining animal seroconverted at 19 days p.i. (titre 1:20). One animal of the positive-control group seroconverted as late as 29 days p.i. with an antibody titre of 1:30.

### Sequence analysis

Sequence analysis of the NS5B gene was carried out on viral RNA isolated from the tonsils of the BPIP-treated (isolated at 33 days p.i.) and untreated group (isolated at 22 days p.i. for euthanized animals and 33 days p.i. for the remaining animals). No amino acid mutations were determined when compared with the inoculated virus.

### Antiviral assays

It was assessed whether virus isolated from BPIP-treated animals at 7 days p.i. [the only blood sampling where infectious virus could be isolated from all (viraemic) treated animals] had reduced sensitivity to BPIP. Therefore, the \(\text{in vitro}\) sensitivity to BPIP of the isolates from each animal (treated and untreated) was determined in cell culture. No significant difference in sensitivity was noted compared with the wild-type virus (see Table 2).

The effect of a high concentration of BPIP (3 \(\mu\text{M}\)) on \(\text{in vitro}\) CSFV replication was then evaluated. At this concentration, BPIP resulted in a complete inhibition of production of infectious virus, whereas the untreated control yielded an infectious titre of \(10^5\) infectious virus particles per ml.

### Table 1. Virus isolation and real-time RT-PCR results (expressed as \(\log_{10}\text{ EGC}\)) of tonsils of BPIP-treated and untreated pigs at 33 days p.i.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal</th>
<th>Virus isolation</th>
<th>(\log_{10}) EGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPIP-treated</td>
<td>1</td>
<td>−</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>−</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>−</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>−</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>−</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>−</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>−</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>−</td>
<td>0.81</td>
</tr>
<tr>
<td>Mean</td>
<td>NA</td>
<td></td>
<td>2.18 ± 0.78</td>
</tr>
<tr>
<td>Untreated</td>
<td>P1</td>
<td>−</td>
<td>4.57</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>+</td>
<td>7.31</td>
</tr>
<tr>
<td></td>
<td>P3*</td>
<td>+</td>
<td>6.97</td>
</tr>
<tr>
<td></td>
<td>P4*</td>
<td>+</td>
<td>6.97</td>
</tr>
<tr>
<td>Mean†</td>
<td>NA</td>
<td></td>
<td>6.46 ± 1.27</td>
</tr>
</tbody>
</table>

*Pigs euthanized at 22 days p.i.
†Based on the two surviving animals.
DISCUSSION

We recently identified various imidazopyridines as selective inhibitors of the in vitro replication of BVDV and CSFV (Paeshuyse et al., 2006; Puerstinger et al., 2006; Vrancken et al., 2008). Here, we studied whether the lead compound within this class, BPIP, was able to reduce virus replication in CSFV-infected pigs.

BPIP was formulated in feed for oral administration as a rapid and convenient way to deliver the drug, particularly if this strategy was employed for large herds. Administration of 75 mg kg\(^{-2}\) day\(^{-1}\) of BPIP in feed to healthy animals for 15 consecutive days did not result in detectable adverse effects. Plasma levels of approximately 0.9–3 \(\mu\)M were obtained, which is close to or above the in vitro EC\(_{50}\) value.

Treatment at this dose regimen of intramuscularly CSFV-infected pigs resulted in a highly significant reduction (\(P<0.00005\)) of infectious virus titres at peak viraemia at 7 days p.i. [the only blood sampling in which infectious virus could be isolated from all (viraemic) treated animals]. Not only were infectious virus titres reduced, but real-time RT-PCR also revealed a marked and significant lower genome load in the BPIP-treated group compared with the untreated group (\(P<0.001\)). The latter observation also makes it highly unlikely that a possible carry-over of BPIP in the tested blood samples acted as a major contributor to the observed reduction of the infectious virus titres. The fact that infectious virus could not be detected in the blood of BPIP-treated animals at time points where viral RNA was still detected can be attributed to the higher sensitivity of the real-time RT-PCR assay (Le Dimna et al., 2008). The marked reduction, in both duration and titre of virus in BPIP-treated animals, was corroborated by a mild and transient leukopenia. These observations support the hypothesis that the progressive decrease in the viral genome load may be the result of the concerted action of a direct antiviral effect and cell-mediated immunity (Suradhat et al., 2001), followed by virus clearance from the blood of treated animals as a result of a humoral response.

Even though the absence of viral genome in the blood of BPIP-treated animals at 33 days p.i. indicated complete clearance of infectious virus, the viral genome could still be detected in tonsils at the end of the experiment (33 days p.i.). This long-term persistence of the viral genome in tonsils is consistent with previous observations in vaccinated pigs (Koenig et al., 2007). In the current study, no infectious virus could be isolated (even after six cell culture passages) from tonsils (positive by real-time RT-PCR) of

### Table 2. Susceptibility of CSFV Wingene expressed as EC\(_{50}\) (\(\mu\)M) isolated from BPIP-treated pigs (animals 1–7) at 7 days p.i.

<table>
<thead>
<tr>
<th>BPIP-treated animal</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7 Group mean</td>
<td></td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>(P) value</td>
<td>0.57</td>
</tr>
</tbody>
</table>
BPIP-treated animals, indicating that a persistent infection of CSFV is unlikely. The viral genome integrity was confirmed using a previously reported RT-PCR panel (Haegeman et al., 2006), indicating that the fact that a positive real-time RT-PCR signal in tonsils in which no infectious virus was detected was not the result of the difference in assay sensitivity (Le Dimna et al., 2008) or genome fragmentation (Haegeman et al., 2006), but most probably due to the presence of neutralizing antibodies (Koenig et al., 2007).

It should be emphasized that BPIP is a lead compound and that optimization of this class of molecules will be needed to obtain analogues with improved in vitro antiviral activity and high bioavailability. Fully optimized molecules should result in increased, if not complete, suppression of virus replication in pigs.

Although BPIP exerted good in vitro activity against CSFV, this compound is markedly more potent against BVDV in vitro (Paeshuyse et al., 2006; Vrancken et al., 2008). Within the same class of imidazopyridines, inhibitors of hepatitis C virus replication have been developed (Puerstinger et al., 2007a) and further optimized, resulting in compounds with high (1 nM) activity (Puerstinger et al., 2007b; Vliegen et al., 2009). A similar lead optimization strategy for CSFV may therefore be expected to result in more potent inhibitors of CSFV replication. This class of compounds is relatively easy to synthesize and is stable (J. Neyts, unpublished data), which would be a prerequisite when stockpiling such compounds to contain future outbreaks of CSFV.

The present proof of concept study is the first to report that a reduction or inhibition of CSFV replication in the target species can be achieved by using selective antiviral agents and provides an entirely novel approach towards the eradication of epizootic animal diseases. An antiviral treatment could be used as a primary control measure against virus spread in case of an outbreak. Indeed, the reduction in virus shedding could curb virus spread if an antiviral therapy was applied prophylactically as a `ring treatment’ around the source of the infection. Stabilizing the epidemic area with the help of an antiviral agent may give the authorities time to organize the appropriate countermeasures and to reduce the number of animals needing to be slaughtered, avoiding a shortage of rendering capacity. Furthermore, use of an antiviral drug may allow bridging of the ‘immunity gap’ when combined with a subunit vaccine (Grubman, 2005).

Further studies are planned to evaluate the effect of BPIP (or analogues) on transmission of CSFV (to treated or untreated sentinels) to explore the full potential of an antiviral treatment as a primary control measure in cases of CSF outbreaks.

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