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Efficacy of delayed brincidofovir treatment against a lethal rabbitpox virus challenge in New Zealand White rabbits

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ABSTRACT

In the event of a bioterror attack with variola virus (smallpox), exposure may only be identified following onset of fever. To determine if antiviral therapy with brincidofovir (BCV; CMX001) initiated at, or following, onset of fever could prevent severe illness and death, a lethal rabbitpox model was used. BCV is in advanced development as an antiviral for the treatment of smallpox under the US Food and Drug Administration's 'Animal Rule'. This pivotal study assessed the efficacy of immediate versus delayed treatment with BCV following onset of symptomatic disease in New Zealand White rabbits intradermally inoculated with a lethal rabbitpox virus (RPXV), strain Utrecht. Infected rabbits with confirmed fever were randomized to blinded treatment with placebo, BCV, or BCV delayed by 24, 48, or 72 h. The primary objective evaluated the survival benefit with BCV treatment. The assessment of reduction in the severity and progression of clinical events associated with RPXV were secondary objectives. Clinically and statistically significant reductions in mortality were observed when BCV was initiated up to 48 h following the onset of fever; survival rates were 100%, 93%, and 93% in the immediate treatment, 24-h, and 48-h delayed treatment groups, respectively, versus 48% in the placebo group ($p < 0.05$ for each vs. placebo). Significant improvements in clinical and virologic parameters were also observed. These findings provide a scientific rationale for therapeutic intervention with BCV in the event of a smallpox outbreak when vaccination is contraindicated or when diagnosis follows the appearance of clinical signs and symptoms.

1. Introduction

Variola virus, an orthopoxvirus and the etiologic agent of smallpox, is responsible for one of the most severe infectious diseases throughout recorded history. The mortality rate from smallpox was ~30% in endemic populations, with death typically occurring ~24–28 days following infection (Fenner et. al., 1988), and survivors are often afflicted by complications including blindness, limb deformities, and various neurologic sequelae (Fenner et al., 1988; Peterson and Damon, 2014). Following a worldwide vaccination campaign, the World Health Organization (WHO) declared smallpox eradicated in 1980 (Fenner et al., 1988).

Consequently, however, 'herd immunity' has been lost, leaving the world's population highly vulnerable to smallpox morbidity and mortality.

Prior to its eradication, smallpox was globally ubiquitous, and owing to the likely existence of undeclared stocks of variola virus retained outside of WHO-designated repository laboratories in the US and Russia (Hansen, 2012), as well as the potential for modern synthetic recreation of the virus from its genomic sequence (Henderson et al., 1999; Strikas et al., 2008), smallpox remains a significant threat due to its potential for use as a biologic weapon. Today, a single case of smallpox would be considered a national public health emergency; accordingly, the US government has advanced strategies to prepare for a possible outbreak.

A vaccine for smallpox (e.g., ACAM2000® [Sanofi Pasteur Biologics LLC], or Dryvax® [Wyeth Laboratories]) is available and considered the first line of defense in an outbreak.

However, there is only a short window (~3 days) following exposure where administration of the vaccine may be beneficial (Centers for Disease Control and Prevention, 2016; Hanna and Baxby, 2002). Although both ACAM2000 and Dryvax are

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considered safe vaccines, serious post-vaccination adverse events can occur (Sanofi Pasteur, 2016). Moreover, the vaccine is contraindicated in people identified as having a higher risk for developing post-vaccination complications and in pregnant women (Fulginiti et al., 2003; Kemper et al., 2002). Accordingly, administering the vaccine to the general population in the absence of an endemic threat is neither practical nor recommended. Therefore, therapeutic agents that can be used for the treatment of smallpox in populations where vaccination is contraindicated, as well as following the onset of symptoms in the event of a smallpox release, are needed.

Brincidofovir (BCV; CMX001) is an orally bioavailable lipid conjugate of cidofovir (CDV) that is converted intracellularly into the active antiviral, CDV-diphosphate (CDV-PP; Hostetler, 2010). The efficacy of BCV as a treatment for smallpox is being evaluated under the US Food and Drug Administration (FDA)'s 'Animal Rule'.

New Zealand White rabbits (NZW) intradermally inoculated with rabbitpox virus (RPXV), strain Utrecht, is a well-characterized animal model of lethal orthopoxvirus infection (Chapman et al., 2010; Adams et al., 2007; Rice et al., 2011b). RPXV causes a disease course in rabbits that closely resembles that of smallpox in humans. This includes an asymptomatic incubation period followed by disseminated infection characterized by fever, severe respiratory complications, secondary skin lesions, and a high mortality rate (Chapman et al., 2010; Adams et al., 2007; Rice et al., 2011b). The ~2-week course of disease in RPXV infection mirrors the 4-week course of human smallpox infection, with disease progression in rabbits approximately three times the rate of the human disease course (Fig. 1) (Chapman et al., 2010; Rice et al., 2011a,b).

Treatment with BCV upon development of secondary lesions using this model has been evaluated in proof-of-concept efficacy studies (Rice et al., 2011a,b), where it showed statistical significance in reducing mortality. BCV also demonstrated significant reductions in mortality when treatment was initiated following the detection of secondary lesions in a blinded, randomized Phase II study. The lowest effective dose regimen from that study, 20 mg/kg followed by 5 mg/kg doses at 48 and 96 h, was selected for use in the current study (Trost et al., 2015). Unlike secondary lesions, fever has been shown to be a more reliable and objective clinical indicator of RPXV infection following challenge for establishing the onset of the disease (Rice et al., 2011b; Nalca and Nichols, 2011), and was therefore designated as the randomization trigger for treatment in the current study.

The primary goal of this study was to assess the survival benefit, compared to placebo, of an efficacious dose regimen of BCV in rabbits (20/5/5 mg/kg at 48-h intervals) that produces rabbit exposures less than or equal to human exposures associated with the intended human dose regimen for smallpox. Additionally, the study was designed to identify the window of effective therapeutic intervention by means of immediate and delayed treatment following confirmation of disease onset in a lethal RPXV animal model of orthopoxvirus infection. The design of this study doubled as a Phase 3 pivotal study under the FDA Animal Rule, intended to both provide evidence of efficacy for regulatory approval and to provide additional information to guide BCV use in the event of a real world release setting. Secondary objectives included evaluation of the incidence, severity, and progression of clinical events associated with RPXV infection when immediate or delayed BCV treatment was administered compared with placebo. RPXV DNA viral load and infectious virus, as measures of potential infectivity, were also evaluated.

2. Materials and methods

The study was conducted at Battelle's Biomedical Research Center (West Jefferson, OH, USA) in compliance with the US FDA's Good Laboratory Practice guidance.

2.1. Test systems

NZW rabbits were received from Covance Research Products (Denver, PA, USA) and maintained according to Battelle's standard operating procedures. One week prior to infection with RPXV, rabbits were implanted with temperature transponder chips for monitoring body temperature (Transponder Type: IPTT-300; BioMedic Data Systems, Inc, Seaford, DE).

2.2. Study challenge

Plaque-purified RPXV, strain Utrecht, master stock was obtained from Richard W Moyer (University of Florida College of Medicine, Gainesville, FL, USA) and prepared as described by Trost et al. (2015). A target lethal inoculum of 300 plaque-forming units [PFU]) was diluted in Dulbecco's phosphate-buffered saline. RPXV inoculations were staggered over 2 days, with the actual virus concentration administered confirmed by plaque assay as 350 PFU and 316 PFU by day of inoculation. A total of 146 NZW aged 17 weeks (+ 4 and + 5 days) and weighing 2.0–2.7 kg were inoculated with the intention to randomize at least 24 animals to each treatment group. Ketamine (22–50 mg/kg) and xylazine (3–10 mg/kg) were administered intramuscularly to the epaxial muscles of the lower back to anesthetize animals prior to challenge.

Anesthesia complications resulted in the deaths of two animals during challenge. RPXV was administered intradermally as bilateral injections of equal volume (i.e., target of 150 PFU in 100 μL per injection) to the thighs of each rabbit (Trost et al., 2015).

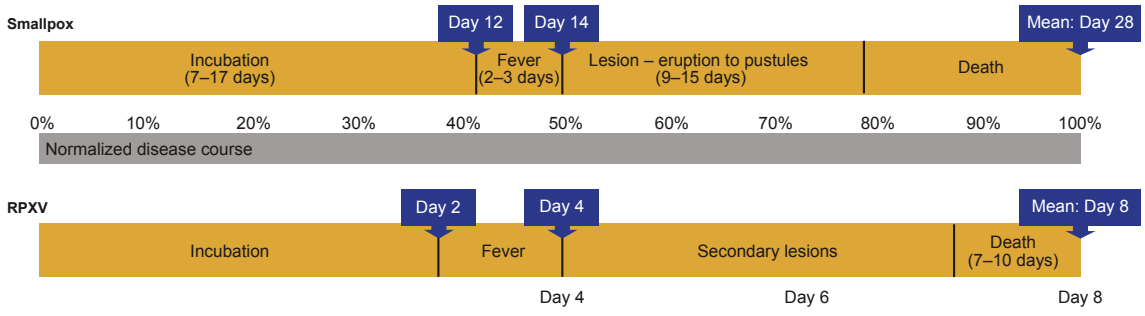

Fig. 1. Comparison of the disease course for rabbitpox and smallpox. RPXV, rabbitpox virus. Adapted from information in (Chapman et al., 2010; Rice et al., 2011a,b).

2.3. Blinding and preparation of treatment kits

A blinded randomization schedule stratified by sex, for a single center, randomized, double-blind, placebo-controlled, parallel-group study design was generated by PRA Health Sciences (Raleigh, NC, USA). Dosing formulations were prepared by Bio-Convergence (Bloomington, IN, USA) and supplied as blinded dosing kits that allowed for 8 consecutive days of dose administration beginning on the day of randomization as described in Table 1. Dosing solution analysis was performed by Intertek (Cincinnati, OH, USA) using a validated method.

2.4. Randomization of test systems

On confirmation of fever (i.e., two consecutive measurements of ≥1.5 °F increase in body temperature from baseline, 1 h ± 10 min apart), animals were sequentially assigned to treatment based on pre-numbered, blinded dosing kits, with each animal assigned to the next kit corresponding to its sex. Treatments consisted of placebo, immediate BCV (within 4 h of randomization), or BCV delayed by 24, 48, or 72 h (Table 1). All animals that presented with fever within 168 h of infection were included in the study.

2.5. Study treatment

Treatment of the animals was performed by 'oral feeding' using the appropriate blinded dosing kit formulation drawn into a 3-mL tuberculin needleless syringe.

2.6. Post-challenge study assessments

Clinical observations and morbidity/mortality assessments were each conducted three times daily (alternating 4-h windows) from confirmed onset of fever (i.e., Day 0 of randomization) through Day 8 post randomization, and then again once (clinical observations) and twice (morbidity/mortality) daily, respectively, from Day 9 post randomization through study completion. Body temperature and respiration rates were assessed every 8 h from the day of challenge to Day 8 post randomization and then once daily until study completion. Data from eight consecutive measurements (twice daily assessments) obtained prior to RPXV challenge were averaged to define the baseline body temperature and respiration rates for each animal. Body weights and lesion monitoring for the presence of secondary pox lesions (i.e., remote from the sites of inoculation) were assessed daily following challenge.

Whole blood and buccal swab samples were assessed for viral load over the course of study conduct. Blood samples (target volume 0.4 mL) were collected in tri-potassium ethylenediaminetetraacetic acid (K_3EDTA) tubes for the determination of RPXV viremia by quantitative polymerase chain reaction (qPCR) based on the pan-orthopoxvirus HA (J7R) method described by Trost et al. (2015). Buccal swabs were collected in tubes containing 1 mL of Dulbecco's phosphate-buffered saline for viral load assessments by qPCR. Blood samples (0.35 mL) were collected in K_3EDTA tubes for the determination of viral load by plaque assay (Garver et al., 2016).

Neutralizing antibodies were assessed weekly following randomization through Day 21 and again on Day 35 and at study completion by plaque reduction neutralization titer (PRNT). Blood (0.3 mL) for PRNT was collected in serum separator tubes and processed to serum. Serum titers to achieve an 80% and 50% reduction in viral plaques ($PRNT_{80}$ and $PRNT_{50}$) were assessed by the method described in Trost et al. (2015).

2.7. Statistical analysis

Assuming a survival rate of 25% in the placebo group, 24 animals per treatment group would provide 90% power to detect superior efficacy of a treatment regimen that presumed a survival rate of 75% or greater at $p < 0.05$, based on Fisher's exact tests with no adjustment for multiple group comparisons.

Following challenge, animals were monitored through Day 42 post randomization, a time point chosen to represent at least four times the median day of death in this model. The proportion of surviving animals at Day 42 post randomization was calculated for each treatment group and compared between BCV treatment groups and placebo using one-sided Fisher's exact tests with a stepdown procedure to control for multiple comparisons. A Cochran–Armitage test was performed to assess association between delay in treatment initiation and mortality. Continuous endpoints were compared using standard parametric methods, or if the normality assumption was not appropriate or verifiable, data were transformed or alternative nonparametric models were used. Time-to-event data were summarized with Kaplan–Meier methods.

Body temperature, respiration, and body weight data were analyzed using analysis of variance (ANOVA). Fixed effects included in the model for respiration, temperature, and body weight were treatment group, time point, and the interaction between them. Log-transformed and untransformed data were first modeled separately; if the ANOVA model error distribution was closer to normal for log-transformed data than for untransformed data, then log-transformed data were used in the final analysis.

For the statistical analysis of the qPCR data, the peak qPCR result (log_{10} copies/mL) for each rabbit from randomization to the end of the study was determined and group medians of the peak values were calculated. Pairwise non-parametric Wilcoxon tests were used to compare groups. A similar analysis of plaque assay data was performed with peak values reported as 'not detected' being assigned a value of 32 (the lowest obtained value, 33, −1) or 1.505 on the log_{10} scale. All statistical analyses were performed using SAS® v9.3 (SAS Institute Inc., Cary, NC, USA) or later, with all tests

Table 1
Dosing regimens by treatment group.

Treatment group (delay)	Dose (mg/kg)	BCV Dosing schedule (days following confirmation of fever)							
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1 (Immediate)	20/5/5	**BCV**	PBO	**BCV**	PBO	**BCV**	PBO	PBO	PBO
2 (+24 h)	20/5/5	PBO	**BCV**	PBO	**BCV**	PBO	**BCV**	PBO	PBO
3 (+48 h)	20/5/5	PBO	PBO	**BCV**	PBO	**BCV**	PBO	**BCV**	PBO
4 (+72 h)	20/5/5	PBO	PBO	PBO	**BCV**	PBO	**BCV**	PBO	**BCV**
5 (PBO)	0/0/0	PBO	PBO	PBO	PBO	PBO	PBO	PBO	PBO

BCV, brincidofovir; h, hours; PBO, placebo. Each animal received eight single consecutive daily administrations of a blinded dose regimen, with the first dose of each blinded treatment (BCV or placebo) administered within 4 h following the confirmation of fever. Subsequent doses were administered at ~24 ± 2-hour intervals following the first dose. All treatments were administered via oral feeding of the solution through a needleless syringe.

performed at the 0.05 significance level.

3. Results

There were no significant differences in temperatures or respiration rates among animals at baseline. All 144 rabbits intradermally inoculated with a lethal challenge of RPXV, strain Utrecht, developed fever within 72 h of inoculation and were randomized to one of the five treatment groups. Of the 144 randomized animals, 2 rabbits were randomized to treatment prior to the onset of confirmed fever (1 rabbit in the placebo group and 1 rabbit in the 24-h delayed BCV treatment group), both of which had a recorded temperature increase of 1.1 °F or more. Both these animals were included in the study analyses.

3.1. Mortality

A clinically and statistically significant reduction in mortality was demonstrated in animals that received BCV immediately, 24, or 48 h following the onset of fever with survival rates of 100%, 93%, and 93%, respectively, compared with 48% for placebo. These results are illustrated in Kaplan–Meier survival curves by treatment group (Fig. 2, $p < 0.001$ for each comparison). Survival also improved when BCV was initiated 72 h after the onset of fever, but the difference was not statistically significant compared with placebo (69% vs. 48%, respectively; $p = 0.091$). Treatment delay as a continuous variable was associated with a decrease in survival ($p < 0.001$).

3.2. Clinical signs

In general, fewer and less severe clinical signs of rabbitpox disease were observed in animals that received BCV immediately or delayed by up to 48 h after confirmed infection compared with animals that received placebo.

3.3. Body temperature

The majority of animals developed fever between 48 and 72 h following infection with RPXV. Body temperatures in all BCV treatment groups were less elevated than those in the placebo group at Day 6 post treatment. Body temperature of animals receiving immediate BCV treatment remained closest to baseline, compared with animals that received later BCV treatment or to those that received placebo (Fig. 3A). Body temperature normalized within 11 days following the onset of fever in all treatment groups, with recovery occurring more rapidly in animals treated with BCV compared with placebo, similar to observations in previous studies (Rice et al., 2011a,b).

3.4. Respiration rates

Mean respiration rates decreased in all groups on Day 1 through to Day 10 following onset of fever, as typical of RPXV infection (Trost et al., 2015), with the greatest decrease seen in the 72-h delayed BCV and placebo treatment groups. Treatment with BCV up to 48 h following onset of fever significantly lessened the decrease in respiration rates on Days 4–6 following fever (Fig. 3B). Mean respiration rates were normalized in all groups by Day 11 following onset of fever.

3.5. Body weight

Mean body weight decreases were noted in all treatment groups, as is typical in cases of RPXV infection (Chapman et al., 2010). Weight loss during peak disease (4–11 days following

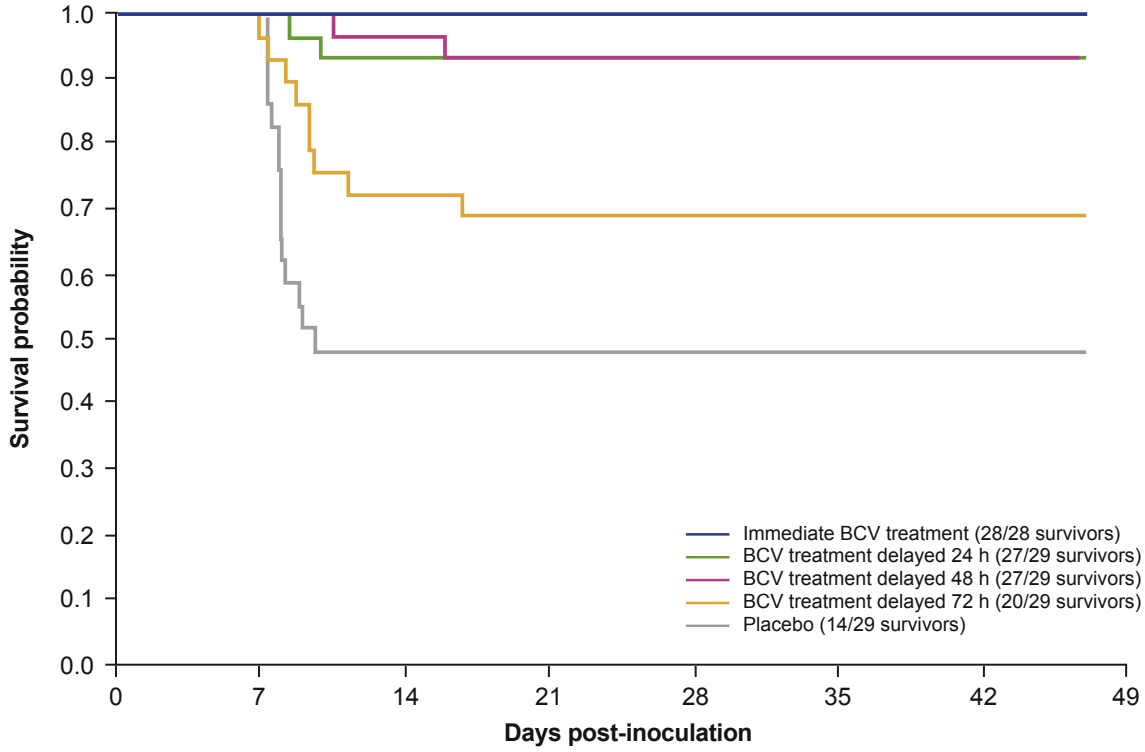

Fig. 2. Kaplan–Meier survival plot to show the effects of delayed BCV treatment on mortality by treatment group. BCV, brincidofovir; h, hours. Clinical observations and mortality were assessed at 4-h intervals from Day 0 through Day 8 post fever (randomization) and then once (clinical observations) or twice (mortality) daily through study completion.

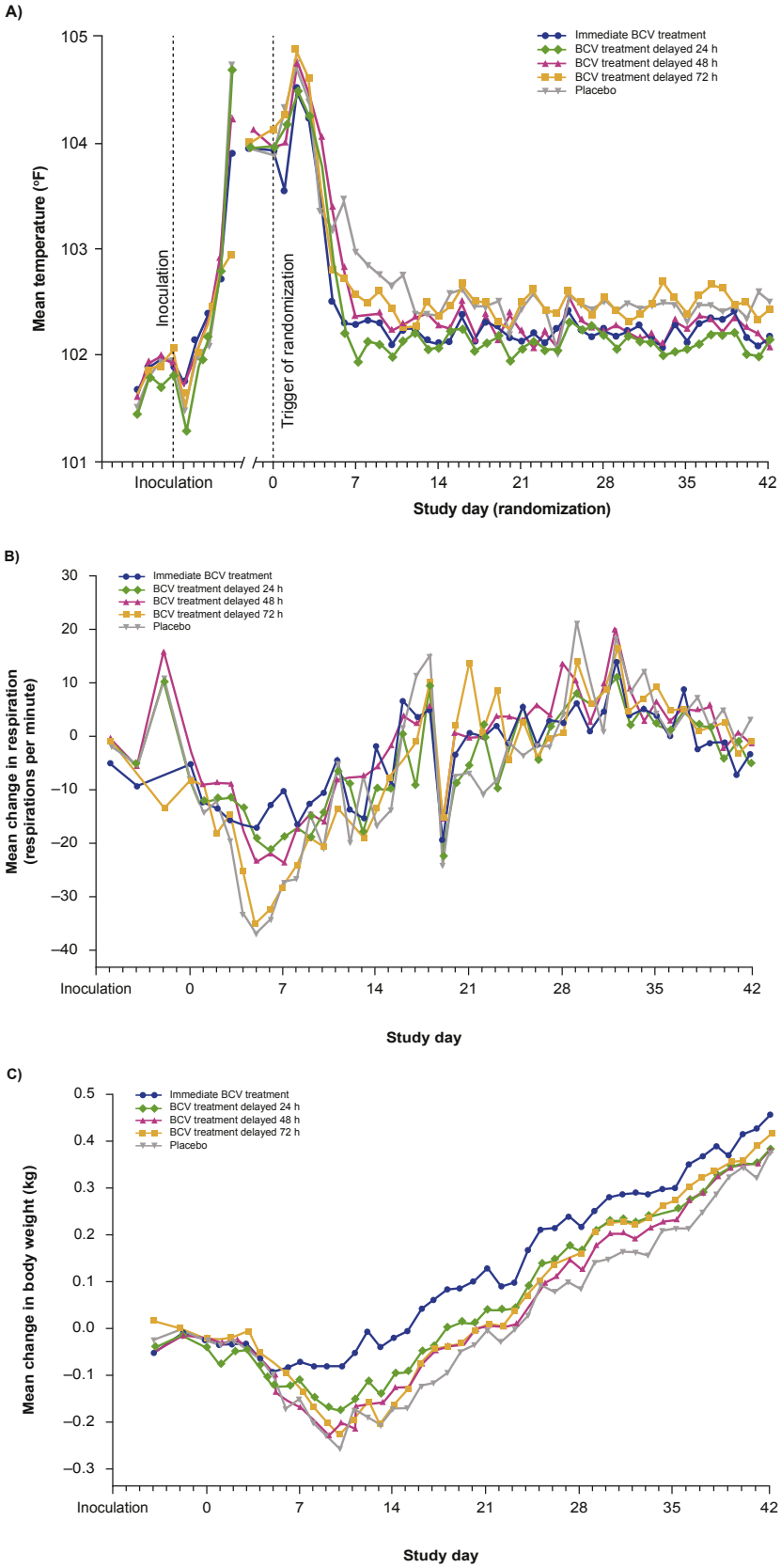

Fig. 3. Secondary clinical outcomes. A) Mean body temperature by treatment group; B) Mean change from baseline in respiration rate per minute (intention-to-treat population); C) Mean change from baseline in body weight by treatment group (intention-to-treat population). BCV, brincidofovir; h, hours. Body temperature and respiration were assessed by implanted temperature probes twice daily from Day 5 through Day 2 prior to inoculation, then three times daily from the day of inoculation with rabbitpox virus until Day 8 following randomization to treatment, then once daily until study completion. Body weights were assessed once daily. Baseline values for respiration and body weight were defined as the average value recorded between Day −5 and Day −2 prior to the day of inoculation.

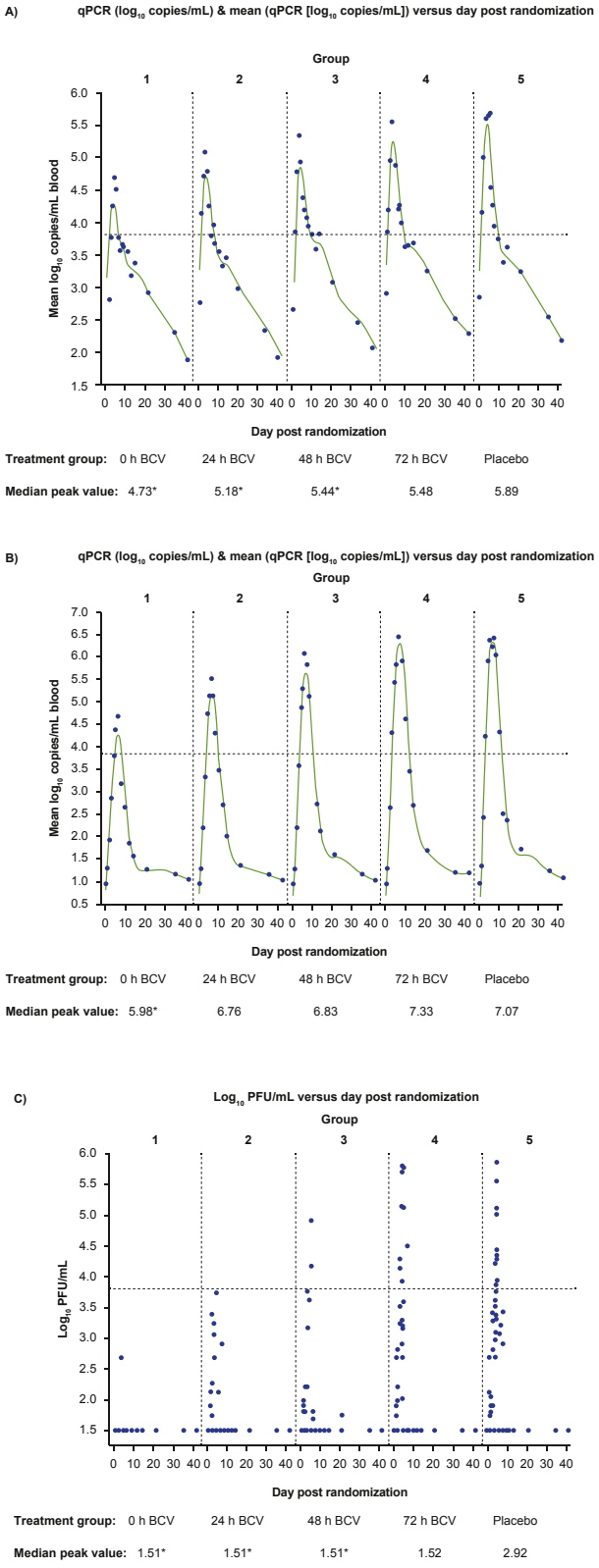

Fig. 4. Viremia from the onset of fever to end of study as assessed by A) qPCR, B) from buccal swabs, and C) plaque assay. *p < 0.05 versus placebo BCV, brincidofovir; h, hours; LLOQ, lower limit of quantitation; PFU, plaque-forming unit; qPCR, quantitative polymerase chain reaction. A) Whole blood viremia (mean log_{10} copies/mL) as determined by qPCR. The horizontal dotted line represents the validated LLOQ of the assay (6700 copies/mL or 3.83 on the log_{10} scale), although all obtained values were plotted and used in statistical tests. qPCR results of 'not detected' were assigned a value of 9 copies/mL (0.95 on the log_{10} scale). B) Buccal swab viral load (mean log_{10} copies/mL) as determined by qPCR. The horizontal dotted line represents the validated LLOQ of the assay (6850 copies/mL or 3.84 on the log_{10} scale), although all obtained values were plotted and used in statistical tests. qPCR results of 'not detected' were assigned a value of 9 copies/mL (0.95 on the log_{10} scale). C) Infectious viral titers (PFU/mL) in whole blood as determined by plaque assay. Plaque assay results of 'not detected' were assigned a value of 32 (1.505 on the log_{10} scale). The horizontal dotted line represents the validated assay LLOQ (6360 PFU/mL or 3.80 on the log_{10} scale).

challenge) was lessened in the immediate BCV treatment and 24-h delayed BCV treatment groups, compared with the placebo group. Animals that received immediate BCV treatment lost approximately 0.1 kg body weight in the first 2 weeks following onset of fever, as compared with up to 0.3 kg over the same period in animals receiving delayed BCV treatment or placebo (Fig. 3C). Mean body weight began to increase in all treatment groups by Day 11.

3.6. Secondary lesions

The majority of rabbits developed secondary pox lesions as typical of RPXV infection (Adams et al., 2007). Lesion assessment indicated that treatment with BCV immediately or 24 h following onset of fever resulted in 75% and 76% of animals exhibiting lesions, respectively. As treatment delay increased, the percentage of animals exhibiting lesions increased; 93% of animals in the 48-h treatment delay group and 100% of the animals in the 72-h treatment delay group exhibited lesions. All but two of the animals in the placebo group presented with lesions; however, both of these animals died relatively early following infection (i.e., prior to lesion development).

Further, during post-mortem inspection, lesions were noted on all but three animals that were euthanized moribund or died on study. These three animals displayed mottled lungs indicative of infection, and one animal exhibited a lesion on the lung surface.

3.7. Viral load quantification

All animals tested free of RPXV DNA in the blood by qPCR prior to inoculation. At the onset of fever, 128/144 (89%) of animals tested positive for RPXV DNA in blood. All animals tested positive for RPXV within 24 h following onset of fever. The mean peak DNA viral load for each group occurred on Day 6 following onset of fever in the immediate, 24-h, and 48-h delayed BCV treatment groups, and on Day 7 in the 72-h delayed BCV treatment and placebo groups. DNA viral loads were lowest in the immediate BCV treatment group and increased with increasing delay of treatment; peak RPXV DNA concentrations were similar in the 72-h delayed BCV treatment group and the placebo group (Fig. 4A; $p < 0.05$ for BCV within 48 h vs. placebo). The largest impact on peak DNA viral load was seen with immediate BCV treatment, which was associated with a 1.16 log_{10} decrease compared with placebo-treated animals. A similar trend was also noted for quantitative RPXV DNA in buccal swabs, with lowest RPXV DNA levels occurring in the immediate treatment group ($p < 0.05$ vs. placebo, Fig. 4B). BCV treatment initiated immediately or at 24 h or 48 h following the onset of fever was also associated with lower median peak concentration of infectious virus (PFU/mL blood) over the course of disease as measured by plaque assay (Fig. 4C; $p < 0.05$ for BCV within 48 h vs. placebo). As with other viral assessments, the most significant impact on peak infectious virus titers was seen with immediate treatment, which was associated with a 1.41 log_{10} decrease in the median peak PFU/mL compared with placebo-treated animals.

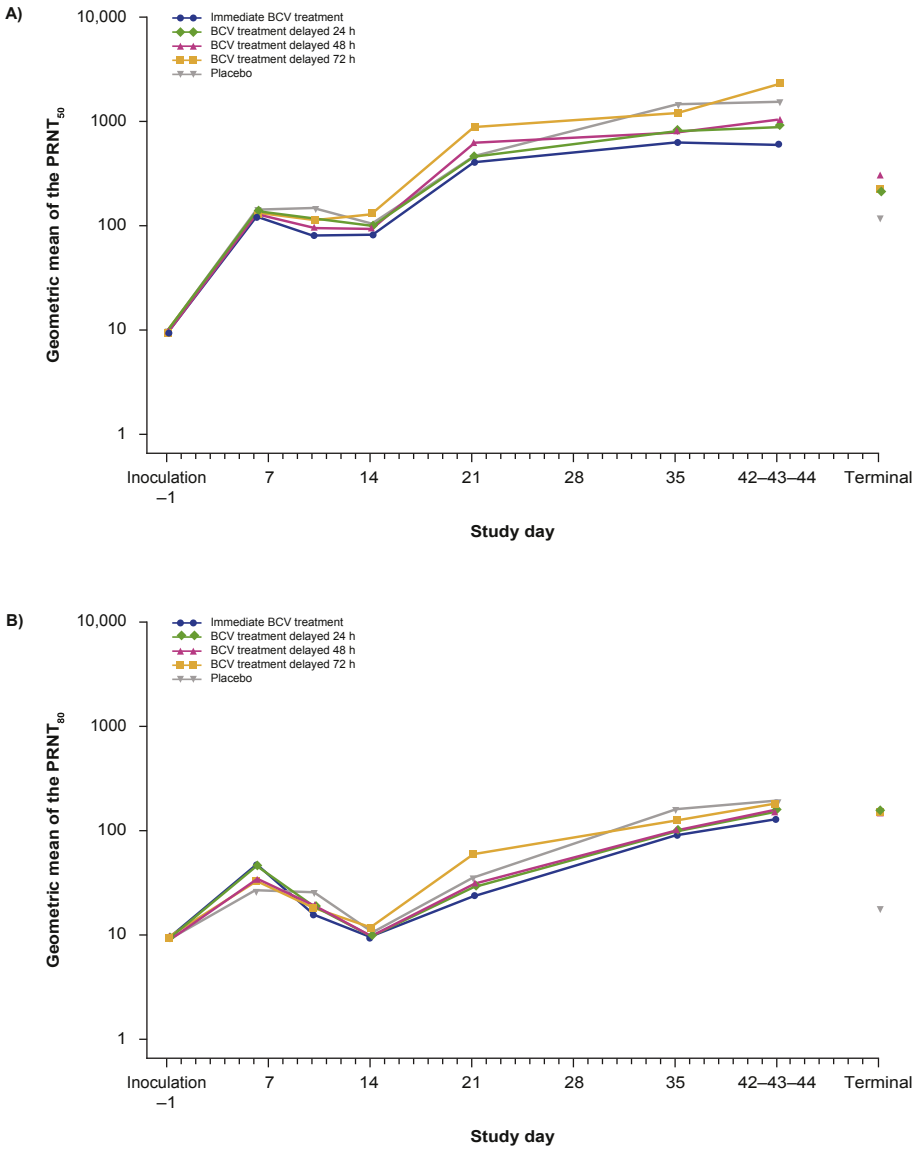

Fig. 5. Group mean $PRNT_{50}$ (A) and $PRNT_{80}$ (B). BCV, brincidofovir; h, hours; PRNT, plaque reduction neutralization titer; $PRNT_{50}$, 50% reduction in viral plaques; $PRNT_{80}$, 80% reduction in viral plaques. Serum titers to achieve an (A) 50% and (B) 80% reduction in viral plaques ($PRNT_{50}$ and $PRNT_{80}$) were assessed by the method described in (Trost et al., 2015).

3.8. Plaque reduction neutralization titer (PRNT)

The majority of animals developed neutralizing antibody titers by Day 6 following the onset of fever, and neutralizing antibodies were evident in all surviving rabbits by Day 10 following onset of fever (Fig. 5). Mean $PRNT_{50}$ and $PRNT_{80}$ neutralization titers increased rapidly following inoculation to Day 6, and decreased slightly over the early treatment period, before increasing from Day 14 in surviving animals over the remainder of the study. All animals that survived through study completion demonstrated an immune response. Mean neutralizing titers demonstrated a treatment-related response by the end of the study, where mean titers were lowest in the immediate BCV treatment group and highest in the 72-h delayed BCV treatment group or the placebo group.

4. Discussion

Initiation of BCV treatment immediately, at 24 h, and at 48 h following onset of fever resulted in clinically and statistically significant improvements in survival to a lethal RPXV challenge when compared with placebo (100%, 93% and 93%, respectively, vs. 48% for placebo; $p < 0.05$ for BCV within 48 h), meeting the primary objective of the study. In addition, BCV treatment 72 h following the onset of fever also demonstrated improvement in survival (69% vs. 48% for placebo), though this survival rate did not achieve statistical significance. Further, animals that received BCV showed a reduction in both RPXV DNA viral load as well as in infectious virus when compared with placebo.

In the RPXV model, initiation of BCV treatment 48 h following confirmation of fever occurs at approximately the midpoint of disease. This finding suggests that there is an ample therapeutic window for effective intervention with BCV in humans following smallpox exposure.

Compared with placebo, BCV resulted in reduced severity of clinical events associated with RPXV infection. Immediate BCV treatment following onset of fever resulted in lower mean changes

in body weight and respiration rate compared with placebo, and treatment with BCV initiated within 48 h of onset of fever showed a more rapid recovery in body weight compared with placebo, and significantly diminished RPXV-associated respiratory rate suppression.

Peak RPXV viral load was significantly reduced in animals that received BCV treatment within 48 h of fever onset, compared with placebo. Levels of RPXV DNA (determined by qPCR) in whole blood and buccal swabs, and infectious virus (determined by plaque assay) were time dependent, with lower median peak viremia seen with earlier BCV treatment. The reduction in infectious virus in the rabbits treated within 48 h of the onset of fever may also indicate a reduced infectivity of these animals; in the event of a smallpox outbreak, treatment resulting in reduction of infectivity could provide an additional public health benefit through mitigation of the infection rate.

All surviving rabbits, regardless of treatment group, developed neutralizing antibodies to RPXV, indicating that treatment with BCV did not prevent the development of protective immunity. The magnitude of the response was lowest in the immediate treatment BCV group, which is the expected outcome for a drug that inhibits viral replication. A similar effect was seen in a study where mice were vaccinated with the smallpox vaccine and treated with BCV (Parker et al., 2014). In that study, protective immunity, as measured by challenge with a lethal inoculum of ectromelia virus was maintained, although there was a numeric reduction in antibody titers. Overall, these results indicate that BCV treatment does not prevent the development of an immune response that facilitates recovery from the initial infection nor does it prevent establishment of an immune response that is protective against subsequent infection.

The results of this study highlight the benefits of early treatment in the event of a smallpox outbreak. Reduction in peak viral loads, inhibition of viral replication and reduced mortality was associated with earlier BCV treatment. Early BCV treatment also reduced the impact of RPXV infection on clinical manifestations of disease in this model system. Importantly, initiation of BCV through the midpoint of the disease course in this model resulted in statistically significant reductions in mortality and RPXV viral load compared with placebo (see Fig. 1).

In the aftermath of a smallpox outbreak, vaccination remains the first-line intervention but is limited by contraindications, the potential for significant adverse events, and the requirement for administration during the asymptomatic phase of infection. Thus, an effective antiviral treatment that can reduce progressive disease, infectivity, and mortality after symptoms have developed is an essential component of a medical countermeasure program. BCV could provide a survival benefit in smallpox cases even when treatment is initiated following the appearance of first clinical signs and up to the midpoint of disease when lesions have generally appeared as identified in this and other RPXV studies (Rice et al., 2011a,b; Trost et al., 2015).

Lastly, the BCV doses administered in this study resulted in plasma BCV exposures equal to or below human exposures for the proposed human BCV dose for use in the treatment of smallpox, based on comparative BCV peak plasma concentration and area under the plasma concentration–time curve. Intracellular levels of the active antiviral, CDV- PP, detected in peripheral blood mononuclear cells in rabbits were also below human exposures (Trost et al., 2015). Further, as part of the BCV development program for other indications (i.e., cytomegalovirus and adenovirus infection), the safety of BCV in humans has been studied in healthy subjects as well as in immunosuppressed or seriously ill patients with viral infections. In the latter populations where vaccination is contraindicated, an effective antiviral would be the only means of protection. Based on the safety profile for a 3- week exposure to BCV in clinical studies (Chittick et al., 2017 Short-term clinical safety profile of brincidofovir: a favorable benefit–risk proposition in the treatment of smallpox. Submitted to Antiviral Research) and the findings from the animal efficacy study presented here, a 200- mg weekly dose of BCV for a duration of 3 weeks is projected to be a safe and effective treatment for smallpox infection when administered following the appearance of clinical signs and symptoms.

These findings provide a scientific rationale for BCV as a treatment option for humans exposed to smallpox who are not immediately vaccinated in an outbreak, and in populations for whom the approved vaccine may be contraindicated.

Funding and conflict of interest

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Glossary

ANOVA	analysis of variance
BCV	brincidofovir
CDV	cidofovir
CDV-PP	cidofovir-diphosphate
K_3EDTA	tri-potassium ethylenediaminetetraacetic acid
LLOQ	lower limit of quantitation
NZW	New Zealand White rabbits
PBO	placebo
PFU	plaque-forming unit
PRNT	plaque reduction neutralization titer
qPCR	quantitative polymerase chain reaction
RPXV	rabbitpox virus

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