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The efficacy and pharmacokinetics of brincidofovir for the treatment of lethal rabbitpox virus infection: A model of smallpox disease

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ABSTRACT

Brincidofovir (BCV) has broad-spectrum in vitro activity against dsDNA viruses, including smallpox, and is being developed as a treatment for smallpox as well as infections caused by other dsDNA viruses. BCV has previously been shown to be active in multiple animal models of smallpox. Here we present the results of a randomized, blinded, placebo-controlled study of the efficacy and pharmacokinetics of a novel, "humanized" regimen of BCV for treatment of New Zealand White rabbits infected with a highly lethal inoculum of rabbitpox virus, a well characterized model of smallpox. Compared with placebo, a dose-dependent increase in survival was observed in all BCV-treatment groups. Concentrations of cidofovir diphosphate (CDV-PP), the active antiviral, in rabbit peripheral blood mononuclear cells (PBMCs) were determined for comparison to those produced in humans at the dose proposed for treatment of smallpox. CDV-PP exposure in PBMCs from rabbits given BCV scaled to human exposures at the dose proposed for treatment of smallpox, which is also currently under evaluation for other indications. The results of this study demonstrate the activity of BCV in the rabbitpox model of smallpox and the feasibility of scaling doses efficacious in the model to a proposed human dose and regimen for treatment of smallpox.

1. Introduction

Brincidofovir (BCV) is an orally-administered lipid conjugate of cidofovir (CDV, Vistide®) that was designed to avoid the renal accumulation and associated toxicity of CDV, promote oral bioavailability, and improve general distribution and intracellular delivery of the active antiviral, CDV-diphosphate (CDV-PP). BCV is active against all 5 families of dsDNA viruses that cause human disease with improved potency, by several orders of magnitude, compared with cidofovir (Lanier et al., 2010). In Phase 1 clinical trials, BCV was well tolerated and orally bioavailable with linear, dose proportional pharmacokinetics over the dose range of 0.025–2 mg/kg (Painter et al., 2012). BCV is currently in Phase 3 clinical development for the prevention of CMV (CMX001-301; NCT01769170) and treatment of adenovirus infections (CMX001-304; NCT02087306). To date, more than 1000 human subjects have been administered BCV with no evidence of cidofovir-like dose-limiting nephrotoxicity.

Intradermal inoculation of New Zealand White rabbits with a highly lethal inoculum of rabbitpox virus (RPXV) is a well characterized model of smallpox (Adams et al., 2007). The scientific community and FDA have recognized this model as appropriate for evaluation of potential smallpox therapeutics because it reproduces key human disease characteristics including high lethality with a low inoculum, an incubation period leading to disseminated disease, and clinical signs of disease including fever, viremia, and secondary skin lesions (Tran and Cargill, 2011). Preliminary studies demonstrated the antiviral activity of BCV in the RPXV model for post-exposure prophylaxis as well as treatment of active infection (Lanier et al., 2010; Rice et al., 2011a,b). When treatment was initiated 1 day prior to infection with a lethal inoculum of RPXV, all animals administered BCV at a dose of 10 mg/kg/day for 5 days survived. Similarly, BCV prevented mortality when given late in the infection cycle, after the appearance of clinical signs of the disease (fever and lesions). Rabbits administered 10 mg/kg/day BCV for 5 days beginning either 4 or 5 days after infection with a lethal inoculum of RPXV exhibited 100 and 75 percent survival,

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Table 1
Summary of study design.

Group	Number randomized[a]	BCV dose 1st/2nd/3rd (mg/kg)	Timing of dose administration		
			First dose	Second dose	Third dose
1	16 (7M/9F)	5/5/5	At or within 4 h of 1st observation of 2° lesions	At 1st observation of 2° lesions plus 48 (±4) h	At 1st observation of 2° lesions plus 96 (±4) h
2	15 (10M/5F)	20/5/5			
3	15 (8M/7F)	20/20/20			
4	16 (6M/10F)	0/0/0			
5 (PK)	9 males	20/5/5 PBMC PK sampling (hours post-dose):	Day 4 post infection 12, 24, 48	48 (±4) h after first dose N/A	96 (±4) h after first dose Predose, 24, 48, 72, 120 and 168

[a] The target minimum randomization was 6/sex/group. Randomization day was based on first observation of lesions in each animal.

respectively. Even a single 20 mg/kg dose of BCV given at the onset of lesions conferred a statistically significant survival benefit compared with placebo (Rice et al., 2011a,b).

The goals for the present study were to (1) show that performance of the intradermal RPXV model is robust and consistent by transferring it from the academic lab where it was developed to an independent contract laboratory facility; (2) identify an optimized dose for evaluation in a pivotal efficacy study; and (3) assess the efficacy and pharmacokinetic (PK) profile of a novel, "humanized" dose of BCV based on doses estimated to produce exposures in rabbits that were equivalent to or lower than exposures in humans given doses under evaluation for other indications (CMV and AdV). Accordingly, NZW rabbits were infected with a highly lethal inoculum of a purified and well characterized stock of RPXV under rigorous laboratory conditions in which all study personnel were blinded to treatment assignment and treatment was initiated in each animal individually after appearance of secondary skin lesions at a site remote from the inoculation site.

2. Materials and methods

2.1. Production, purification, and characterization of the challenge agent

Plaque purified RPXV Utrecht strain master stock was obtained from Richard W. Moyer (University of Florida College of Medicine, Gainesville, FL) and expanded in CV-1 cells to produce a single RPXV lot (Southern Research Institute, Frederick, MD). CV-1 cells were maintained in Minimal Essential Medium supplemented with non-essential amino acids, L-glutamine, penicillin/streptomycin and 10% fetal bovine serum. The RPXV virus was purified over a 36% sucrose cushion, suspended in PBS, and stored at −80 °C. The working stock was titrated by plaque assay, yielding a concentration of 5.8×10^9 plaque forming units (PFU)/mL. Specification testing was conducted including sterility, pH, endotoxin level, mycoplasma, bovine viral diarrhea virus (BVDV), and DNA sequence comparison. No growth was detected in sterility testing, pH was determined to be 6.14, endotoxin levels were determined to be 7.27 EU/mL at a 1:500 dilution, and the stock was negative for mycoplasma and BVDV. The stock material was confirmed RPXV by matching a target DNA sequence (hemagglutinin gene region; bases 167066–168003) with GenBank accession number AY484669.

2.2. Test facility, housing and study assignment of animals

The study was conducted at Battelle Biomedical Research Center (West Jefferson, OH). Seventy-one, 7 week + 3 day-old Myrtle's lineage NZW rabbits (Covance, Princeton, NJ) were single-housed in standard rabbit caging and acclimated for 7 days in the study room. Fluorescent lighting (12 h cycle), study room temperature, and humidity were maintained according to Battelle SOPs. Rabbits were fed Certified Rabbit Chow® (PMI, St. Louis, MO) and had *ad libitum* access to water. During the acclimation period animals were observed twice daily for clinical signs, body weights were recorded for randomization purposes and each animal was implanted with 2 subcutaneous temperature transponders (shoulder/back and rump/hip areas). The back of each animal was shaved to aid monitoring for secondary lesions. All animals were examined by a study veterinarian and confirmed to be in good health before release to the study. Animals were randomized by weight to select 9 rabbits for inclusion in the PK arm of the study (Group 5). The remaining 62 animals were assigned to the efficacy arm of the study in a 1:1:1:1 blinded fashion upon the presentation of secondary lesions after infection with RPXV (Groups 1–4). The overall study design is summarized in Table 1.

2.3. Inoculation, lesion monitoring prior to randomization and monitoring following randomization until termination

On Day 0, all 71 rabbits were infected with a target inoculum of 300 PFU of RPXV stock diluted in Dulbecco's phosphate buffered saline (DPBS). The concentration of stock virus dilutions used for challenge was determined within 4 h after inoculation by plaque assay to confirm the actual challenge dose (ie back titer). Inoculation was performed according to Battelle SOPs. Briefly, animals were anesthetized by intramuscular injection of ketamine/xylazine (22–50 mg/kg and 3–10 mg/kg, respectively). The challenge dose was divided in half and all animals were inoculated by bilateral intradermal injections in each thigh region (approximately 100 μL/thigh). The area of injection on both thighs was shaved, wiped with isopropanol and allowed to air dry prior to inoculation. Infected rabbits were housed in animal rooms within Biosafety Level 3 (BSL3) containment.

Beginning at inoculation, all animals were monitored 3 times daily for the presence of secondary pox lesions. Animals in the efficacy arm met randomization criteria when observed with secondary pox lesions anywhere on the body other than the primary site of inoculation. Randomization criteria required the observation of lesions to be confirmed by a second technician prior to randomization. Following randomization, lesion counts were performed once daily. The areas monitored included the ears, back, mouth, nose, eyes, footpads, and ano-genital area. Animals were also monitored for clinical signs of disease (mortality and signs of ill health including respiratory changes) twice daily until the scheduled termination of the study. Body weights, body temperatures, and respiration rates were recorded daily and compared to baseline values obtained during the acclimation period. PK animals (Group 5) were not monitored for lesions, but had clinical observations performed twice daily after inoculation.

Surviving efficacy study animals were euthanized on Day 42 after randomization. Surviving PK study animals were euthanized as scheduled 72, 120, and 168 h following administration of the last dose. The decision to euthanize an animal prior to the

scheduled termination was made independently for each animal based upon disease progression according to a protocol-specified, predetermined set of euthanasia criteria that included respiratory distress (typically open mouth breathing), general morbidity or significant changes in respiration rate, body temperature, or body weight. Following completion of the in-life phase of the study, mortality and lesion data underwent quality control, technical review, and quality assurance review.

2.4. Dose administration

Dosing kits containing BCV or placebo were prepared by Chimerix Inc. (Durham, NC). The kits, containing 3 vials labeled with the kit and dose numbers, were shipped to BBRC and stored at approximately 4 °C prior to use. Confirmatory dose formulation analysis was performed by Intertek Pharmaceutical Services (Whitehouse, NJ). Doses were administered by oral feeding of the formulation through a syringe. Rabbits were trained to accept oral administration of the dosing solution (20% w/v sucrose) on 5 occasions prior to RPXV inoculation. The total dose administered to each animal and whether there was any discernible loss was noted. The dose formulation was colored blue to aid this observation.

In the efficacy study (Groups 1–4), the first dose of each treatment was administered within 4 h of randomization. Animals were randomized and assigned dosing kits in the order in which they presented with lesions. The second and third doses of each treatment were administered at approximately 48 (±4) and 96 (±4) h after the first dose. Rabbits in Groups 1–4 received doses of 5/5/5, 20/5/5, 20/20/20, or 0/0/0 (placebo) mg/kg BCV, respectively. The 20/5/5 mg/kg dose was a humanized dose on the anticipated exposure in rabbits. It was derived using PK data from previous studies in rabbits and humans. All test facility personnel were blinded to the treatment assignment of each main study animal.

In the PK arm (Group 5), the first dose of BCV was administered to all animals on Day 4 after RPXV inoculation. Dosing was initiated in all PK animals simultaneously in order to facilitate timed blood collections. The second and third doses were administered at approximately 48 (±4) and 96 (±4) h after the first dose. Rabbits in Group 5 received only the 20/5/5 mg/kg humanized BCV treatment regimen. Test facility personnel were not blinded to the treatment assignment of the PK animals (Group 5), therefore they were not included in the primary efficacy analysis.

2.5. Viremia

Blood RPXV titers were determined for all efficacy animals (Group 1–4) by a pan-orthopox virus, hemagglutinin (HA-J7R) gene-specific qPCR assay prior to inoculation on Day 0 and 2, 4, 6, 8, 10, 15, 30, and 42 days after randomization. Rabbits were sedated with acepromazine (1 mg/kg, IM) and blood obtained from the ear vein or artery. Terminal blood samples were collected by cardiac puncture. Nucleic acids were isolated from 100 microliters of whole blood using the Specific B protocol on a NucliSens EasyMAG instrument (bioMérieux, Durham, NC) and eluted into a final volume of 40 microliters. RPXV DNA was quantified using a HA-J7R specific probe set as previously described (Kulesh et al., 2004).

2.6. Resistance monitoring

Blood for determination of genotypic resistance to BCV was collected from efficacy animals (Groups 1–4) at regular intervals beginning 6 days after randomization and continuing until the scheduled termination of the study. Positive control specimens were prepared by spiking naïve rabbit blood to a final concentration of 2000 and 20,000 PFU/mL RPXV. Nucleic acids were isolated from 100 μL of whole blood for each sample and positive control sample using the Specific B protocol on an EasyMAG instrument (bioMérieux, Durham, NC) and eluted into a final volume of 40 μL. Nucleic acid specimens were shipped to SeqWright (Houston TX) for sequencing of the RPXV polymerase gene. The virus genotype from treated and placebo control animals was compared to the genotype of the consensus sequence for challenge virus.

2.7. Assessment of immune response

The plaque reduction neutralization titer (PRNT) assay was performed in order to detect and quantify neutralizing antibodies to RPXV virus in rabbit serum (Battelle SOP Number BBRC. X-245), and determine the effect of treatment with BCV or placebo. Blood for PRNT testing was collected from efficacy animals (Groups 1–4) 6, 10, 15, 30 and 42 days after randomization until the scheduled termination of the study.

2.8. Pharmacokinetic assessments

Blood samples (8 mL) from PK animals (Group 5) were collected, PBMCs isolated, and the concentration of CDV-PP determined using a validated analytical method. Blood was collected from 3 animals at 12, 24, and 48 h after the first dose, immediately prior to the last dose (dose 3), and 24, 48, 72, 120 and 168 h after the last dose. Per IACUC limits, each rabbit was sampled three times; twice prior to termination and at termination with a minimum of 72 h between each sampling. Rabbits were sedated for blood collection.

PBMCs were isolated using Vacutainer® CPT™ cell preparation tubes with sodium citrate anticoagulant (BD, Franklin Lakes, NJ). Blood samples were mixed and centrifuged in CPT tubes at 21 °C and 1750 RCF for 20 min. The PBMC layer was extracted, washed with PBS, counted, fixed in 125 μL of 7:3 (v/v) methanol–water and stored at −70 °C prior to analysis. The concentration of CDV-PP in PBMC lysate was determined (Pyxant Labs Inc., Colorado Springs, CO). CDV-PP and its stable-labeled isotope (CDV(1,3-^{15}N-2,4,6-^{13}C-cytosine)-PP) (Chemcyte, Inc., San Diego, CA), used as an internal standard, were analyzed by LC/MS/MS utilizing electro spray ionization in the positive ionization mode and anionic exchange chromatography. Raw picogram amounts of CDV-PP were calculated and converted to pg/10^6 PBMCs using the individual cell count for each sample.

Because each rabbit was only sampled three times during the dose administration period, the PK analysis was a single composite profile based upon mean concentrations of CDV-PP. Concentrations below the lower limit of quantitation were set to 0 except where all values at a given time were BLQ, in which case the mean result was BLQ. Non-compartmental analysis was completed using WinNonlin Version 6.3 (Cetara, St. Louis, MO).

2.9. Statistical analysis

Time-to-death data combined with survival data were analyzed to identify differences in protection for the treatment groups compared with the placebo group. The Kaplan–Meier curves were plotted and the log-rank test was computed to compare each treatment group to placebo. Two-sided Fisher's exact tests were performed to compare the survival rates between each treatment group and the placebo group. *T*-tests were used to compare the change in viral load from baseline in each BCV treatment group with placebo for each post-randomization day on which samples were obtained. Fisher's exact test was used to compare survival between BCV and placebo-treated groups on each day randomized post-inoculation. Unless stated otherwise, the term "significant" is used to denote the results of pairwise comparisons that were significant at the 0.05 level.

3. Results

3.1. Model performance: inoculation, subjects meeting randomization criteria, observation and progression of clinical signs of disease

Back-titer analysis confirmed the actual inoculum was 187 PFU/mL. All animals were successfully inoculated and all 62 rabbits in the efficacy arm of the study (Groups 1–4) met randomization criteria either 3, 4 or 5 days post-infection. One rabbit randomized to 5/5/5 mg/kg was mis-dosed and subsequently removed from all analyses. Body weights were recorded at regular intervals during the study and compared with baseline (pre-inoculation) values. In general, significant decreases in mean body weight were observed 8–10 days following inoculation in BCV-treated animals with a slight trend toward greater weight loss at lower doses (data not shown). The weight loss extended through Day 14 post-inoculation in placebo treated animals. All surviving animals in all groups showed significant increases in body weight relative to baseline after Day 14 until the scheduled termination of the study. These results are consistent with previously published reports (Adams et al., 2007; Rice et al., 2011a).

Significant increases in body temperature were generally observed between Days 3 and 9 post-infection after which they gradually abated (data not shown). Fever, as defined by a 1.5°F increase in body temperature from the mean baseline value for each animal, was observed in 87.5% of rabbits on Day 3 and 100% of rabbits by Day 4. Body temperatures returned to normal in all groups by about Day 14 post-infection and remained normal for the duration of the study. These results are similar to those reported previously (Adams et al., 2007; Rice et al., 2011a). Significant changes in respiration rate relative to baseline occurred intermittently through the scheduled termination of the study. There was a trend in all animals suggestive of decreased respiration rate, most apparent 8 days post-infection before gradually increasing to Day 14 post-infection after which they were fairly constant. Mean respiration rates were generally indistinguishable between groups (data not shown).

3.2. BCV treatment increased the survival of RPXV-infected rabbits in a dose-related manner

Animals found dead or euthanized prior to the scheduled termination exhibited symptoms typical of severe RPXV disease including fever, lesions and respiratory abnormalities. The survival rates for the placebo, 5/5/5, 20/5/5, and 20/20/20 mg/kg BCV treatment groups were 25%, 47%, 73%, and 80%, respectively. Both the 20/5/5 and 20/20/20 mg/kg BCV groups had a significantly higher rate of survival compared with the placebo treated group ($p = 0.012$ and 0.004, respectively). The time-to-death and survival data are presented by Kaplan–Meier plot in Fig. 1. All deaths were attributed to RPXV disease.

3.3. BCV treatment decreased the viral load of RPXV-infected rabbits in a dose-related manner

Following inoculation, viral nucleic acid in peripheral blood was quantified by qPCR targeting a pan-orthopox virus hemagglutinin (HA-J7R) gene. Data were analyzed as the change from baseline viremia at randomization with the last observation carried forward for animals that died prior to the scheduled study termination. As expected, the majority of animals that survived to the scheduled termination exhibited viral DNA levels at or below the lower limit of quantitation of the qPCR assay regardless of treatment group. Overall, there was a dose-related decrease in mean viral load in rabbits administered BCV compared with those administered placebo (Fig. 2). The difference was apparent by Day 6 post-randomization at which time viral load was decreased across all BCV treatment groups and significantly in the 20/5/5 mg/kg and 20/20/20 mg/kg dose groups ($p < 0.05$). The BCV-responsive and dose-related decrease in viral load correlated with survival across treatment groups compared with placebo.

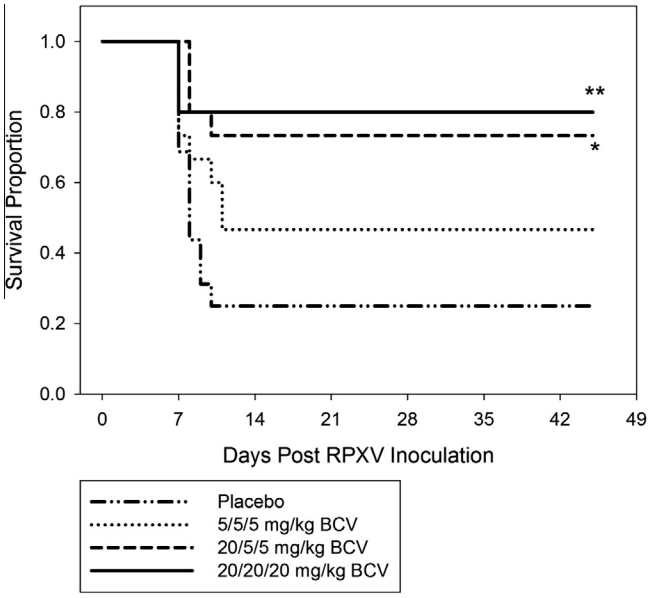

Fig. 1. Survival of RPXV-infected rabbits randomized to one of 3 different regimens of BCV or placebo. A total of 62 rabbits were infected intradermally with a target inoculum of 300 PFU RPXV on Day 0, randomized into treatment groups after the appearance of secondary lesions, and administered 3 doses of placebo or BCV with 48 h between doses. One 5/5/5 mg/kg animal was mis-dosed and excluded from the analysis. Group assignments included placebo ($N = 16$), 5/5/5 mg/kg BCV ($N = 15$), 20/5/5 mg/kg BCV ($N = 15$), and 20/20/20 mg/kg BCV ($N = 15$). Kaplan–Meier curves were generated to depict the proportion of surviving rabbits on each day of the study until the scheduled termination 42 days after randomization. BCV treatment increased survival at low, loading/maintenance, and high doses ($p = 0.270$, 0.012, and 0.004, respectively). Asterisks indicate significance at $p < 0.05$.

3.4. Lack of resistance and formation of protective immunity

A total of 63 purified nucleic acid samples (61 test samples and 2 positive controls) were shipped to SeqWright for sequencing of the RPXV polymerase gene. Consensus sequences were compared against the reference standard provided by Battelle. All samples were found to be 100% identical to the provided reference sequence within the region amplified. Following inoculation, development of neutralizing antibody to RPXV was monitored in serum by the PRNT assay. Results of ANOVA models comparing group geometric mean PRNT values for each study day are located. Neutralizing antibodies were not present for any animal prior to study start. All study groups developed neutralizing antibodies following challenge with RPXV. Titers increased by approximately 20-fold over the course of the study. However, there were no significant differences between mean PRNT values of the groups at any of the study days examined.

3.5. Earlier treatment with BCV resulted in improved survival

All 62 animals in the efficacy arm met the randomization criteria for dosing, however, the day secondary lesions were first observed varied, and therefore, animals were randomized on different days following inoculation: 24 animals on Day 3, 19 animals on Day 4, and 19 animals on Day 5 post-infection. The rate of survival in placebo treated rabbits was consistent regardless of the

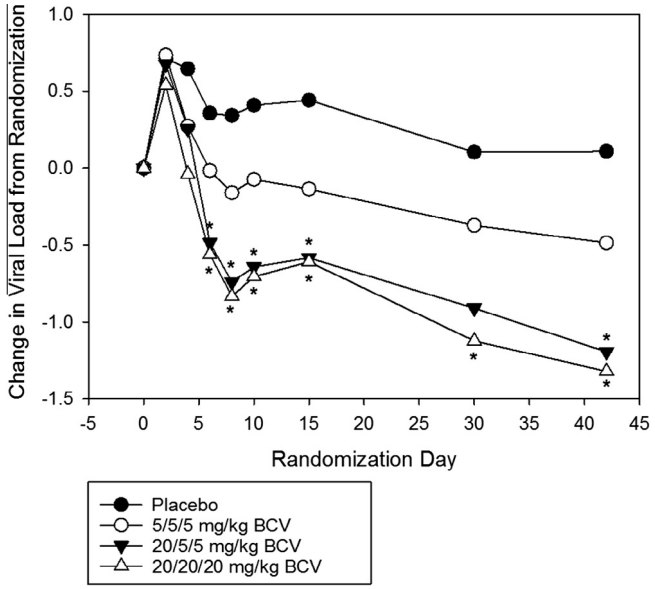

Fig. 2. Change in RPXV viral load from baseline in rabbits treated with BCV compared with placebo. Poxvirus nucleic acid in circulating blood was quantified by qPCR targeting the hemagglutinin (HA-J7R) gene in RPXV-infected rabbits treated with placebo or one of three regimens of BCV (5/5/5, 20/5/5, or 20/20/20 mg/kg each at 48 h intervals). Data are presented as the (log10) change from baseline viremia determined for each animal at randomization with the last observation carried forward for any animals that died prior to the scheduled study termination. There was a dose-related decrease in viral load in rabbits administered BCV compared with those administered placebo. The limit of detection of the qPCR assay was 6.70×10^3 gene copies/mL. Asterisks indicate significance at $p < 0.05$.

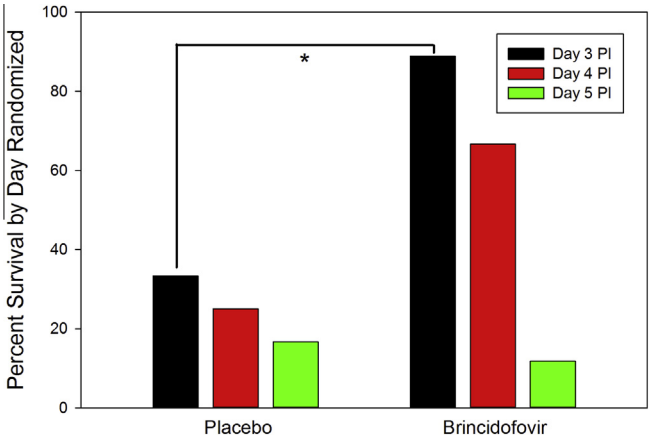

Fig. 3. Percent mortality of RPXV-infected rabbits treated with placebo or BCV by day randomized. RPXV-infected rabbits were randomized to treatment with either placebo or BCV at the first observation of secondary lesions. Approximately equal numbers of rabbits met randomization criteria on Days 3, 4 or 5 following RPXV inoculation (represented by black, red, and green bars, respectively). All 3 BCV treatment groups were pooled for this analysis. Mortality of 67%, 75%, and 83% was observed in placebo treated groups randomized on Day 3, 4, or 5. Mortality of 12%, 33%, and 62% was observed in BCV-treated groups randomized on Day 3, 4, or 5. Early randomization to BCV treatment resulted in improved survival compared with animals randomized late in the disease course. Asterisks indicate significance at $p < 0.05$.

day of randomization and overall placebo treated animals had a median time to death of 8 days post-infection (Fig. 3). By contrast, the rate of survival in BCV-treated rabbits was highly correlated with the day of randomization. Pooling animals in all 3 BCV treatment groups, 88% overall survival was observed among BCV-treated rabbits randomized on Day 3 post-infection. Survival decreased to 67% among animals randomized on Day 4 post-infection and, for animals randomized on Day 5 post-infection survival, was indistinguishable from placebo. Logistic regression analysis of survival as a function of both treatment group and day of initiation of treatment after inoculation confirmed that effects of both the dose and day of treatment initiation were significant to survival ($p = 0.0147$ and $p = 0.0296$, respectively).

3.6. Exposure to the active antiviral metabolite, CDV-PP, in PBMCs of rabbits administered an efficacious dose regimen of 20/5/5 mg/kg were equivalent to or lower than exposures in humans given the doses currently proposed for treatment of smallpox

All 9 rabbits in the PK arm of the study received a three-dose regimen of 20/5/5 mg/kg BCV initiated on Day 4 post-infection. This humanized dose regimen was derived using PK data from previous rabbit and human studies conducted by Chimerix to support regulatory submissions. The humanized dose was intended to produce exposures in rabbits equal to or lower than those produced in humans given BCV at the dose evaluated for prevention or treatment of other dsDNA virus infections. One animal in the PK arm died on Day 7 post-infection due to RPXV disease. As a result, the 48 and 168 h samples following dose 3 were not obtained from this animal. All other rabbits in the PK arm survived to the scheduled termination and all PBMC samples were collected successfully. Concentrations of CDV-PP in PBMCs following the first (20 mg/kg) and last (5 mg/kg) doses were determined and all concentration data were used in the PK analysis.

Because CDV-PP is produced by phosphorylation of cidofovir inside cells, the matrix is PBMC cells and the results are presented adjusted for cell count. The peak concentration, $C_{max,}$ was 3.99 pg/10^6 cells, occurring 24 h after the first dose, and 14.5 pg/10^6 cells, occurring 48 h after the last dose. Following the first and last doses, the extent of exposure, $AUC_{0\text{-}48}$, was 136 pg*h/10^6 cells and $AUC_{0\text{-}168}$ was 795 pg*h/10^6 cells. The half-life ($t_{1/2}$) of CDV-PP was estimated to be 30 h. Plasma concentrations of BCV, also essential for scaling to a human dose, were not determined in this study because the blood volume required for PBMC isolation and analysis prohibited the requisite blood sampling. Previous studies, however, demonstrated that plasma exposure (C_{max} and AUC) to BCV in rabbits given doses of 20 mg/kg is lower than exposure in humans given a dose of 200 mg, the dose proposed for treatment of smallpox (Lanier et al., 2010; Tippin et al., in preparation). Therefore, the focus of the PK arm of this study was limited to determination of PBMC CDV-PP concentrations.

A discussion of the pharmacokinetic results in rabbits and their use in conjunction with human pharmacokinetic data to scale to a proposed human dose for treatment of smallpox is provided as a Supplemental attachment to this research report.

4. Discussion

In the absence of the ability to conduct traditional clinical trials, the FDA has provided a path for development of antivirals for treatment of smallpox under the "animal rule" using efficacy data generated in well characterized animal models of smallpox together with human safety data. While no single animal model reproduces all clinical features of smallpox, the intradermal RPXV model in rabbits shares many key features of smallpox that make it an excellent model for use in evaluating potential therapeutics. New Zealand White rabbits are a standard laboratory species with well controlled historical lineages and bred to be specific pathogen

free. Their moderate size allows repeated blood sampling for evaluation of PK and clinical endpoints but also permits large, well-powered studies for meaningful statistical analysis.

Working in collaboration with scientists at the University of Florida, we characterized the intradermal RPXV model and performed pilot studies with BCV before transferring the model to a contract research organization for independent testing. The design of the current study was informed predominantly by 3 blinded, placebo-controlled studies performed in Florida showing increasing benefit of treatment with BCV using a 1, 2 or 3 dose regimen (Rice et al., 2011a). In those studies even a single dose of 20 mg/kg BCV provided a statistically significant survival benefit in the RPXV model when treatment was initiated in individual animals after observation of clinical signs of disease. Although a dose of 20 mg/kg is higher on a weight adjusted basis compared to the proposed human dose for treatment of smallpox (200 mg or approximately 1.5– 3 mg/kg), the favorable PK profile of BCV in humans results in higher exposures at lower doses.

In the current study, we evaluated the efficacy of three potential regimens of BCV including a "humanized" regimen consisting of an induction dose of 20 mg/kg followed by two maintenance doses of 5 mg/kg in the intradermal RPXV model. The results of this study demonstrated that this 3 dose regimen, with administrations spaced at 48 h intervals and initiated individually at the first observation of secondary lesions, resulted in a significant survival benefit compared to rabbits treated with placebo. Although a trend toward improved survival was observed at the 5/5/5 mg/kg dose, the difference was not significant compared to animals administered placebo. Further, since survival was not meaningfully improved in animals receiving the 20/20/20 mg/kg high dose regimen, the 20/5/5 mg/kg dose was concluded to be an optimized dose and therefore appropriate for evaluation in a pivotal study according to FDA guidance provided in the draft animal rule (U.S. Food and Drug Administration, 2014).

Evaluation of the efficacy of BCV based on the timing of initiation of treatment in relation to day of infection also informs its clinical utility in a smallpox outbreak. In this study, rabbits were randomized to blinded treatment after the first observation of secondary lesions which was relatively evenly distributed on Days 3, 4, and 5 post-RPXV infection. Combining all BCV-treatment groups, the mortality rate for animals that received BCV later in the disease course, beginning on Day 5 post-infection, was significantly higher than those that initiated treatment on Day 3 post-infection. Previous studies have shown a similar improvement in efficacy in this model when BCV is initiated in the absence of clinical signs of disease based solely on proximity to infection (so called post-exposure prophylaxis). These results are consistent with those of previous studies of BCV both in the RPXV model and in mouse models of smallpox, examining the effect of treatment initiated mid-way or later in the infection cycle (Parker et al., 2008; Quenelle et al., 2004; Rice et al., 2011a,b). Overall, early diagnosis and treatment with BCV is expected to correlate with improved patient outcome, and treatment initiated at or beyond the mid-point of disease may not be efficacious.

Smallpox skin lesions are a clinically relevant trigger for randomization since they were used historically to diagnose smallpox (Fenner et al., 1988). Skin lesions are also a feature of RPXV disease in rabbits. The first observation of secondary lesions in the RPXV model is variable, depending largely on the skill and training of the technician making the observation. It has been reported to occur as early as 2 days and as late as 7 days after inoculation while the median day of death is more consistent, between 8 and 10 days post-infection. The emergence of other signs of disease, specifically fever and viremia, occurs earlier, on Days 2 to 3 post-infection and more consistently (Chimerix unpublished data, Adams et al., 2007; Rice et al., 2011a,b). In the case of a smallpox outbreak, index cases may be diagnosed only after presentation of these distinctive lesions, especially due to its long absence from the clinic leading physicians to be unfamiliar with its diagnosis. For secondary cases, however, it's likely that modern diagnostic tools (i.e., analysis for smallpox virus in blood) will result in earlier diagnosis than the observation of skin lesions used historically.

In humans, the characteristics of smallpox viremia are only vaguely understood since the disease was eradicated prior to the advent of modern molecular tools. Laboratory confirmation of smallpox viremia by qPCR prior to treatment, while preferable, may be impractical in an outbreak affecting anything more than a small number of patients. Fever, even though nonspecific, would be highly relevant to diagnosis in a large outbreak when initiating treatment as early in disease as possible is a high priority. The ebola outbreak of 2014 is a relevant example of the use of fever for diagnosis, not only in the resource deficient countries where it originated, but also in the handful of cases diagnosed outside the African continent. Future analysis of viremia and fever will be used to align initiation of treatment in the RPXV model with the expected clinical diagnosis and treatment of the majority of smallpox patients.

The importance of early initiation of treatment is further emphasized by the observation that the prognosis for recovery from smallpox is dependent upon the ability of the host to quickly mount an immune response. Therefore, BCV, which acts by inhibiting viral replication, blunts peak viral load and improves survival by providing more time for the host immune response. To this end, it is important to note that data from this study indicates that BCV does not interfere with the host immune response to infection. The development of neutralizing antibodies to RPXV was monitored throughout this study and it was found that antibody titers, not present in any animal prior to infection, were present in all rabbits who survived to the scheduled termination with no difference in titers between placebo and BCV-treated animals. This observation is consistent with reports from other investigators that BCV did not interfere with formation of protective immunity following infection or vaccination in the mouse ectromelia and vaccinia models (Parker et al., 2014; Zaitseva et al., 2015). Hence patients infected with smallpox and treated with BCV would be expected to develop full protective immunity against subsequent smallpox infection.

In addition to the intradermal rabbitpox model, studies have been conducted in the intranasal ectromelia (ECTV, mousepox) model in mice, another model of smallpox that was developed in a well characterized laboratory animal species; their small size allowing the conduct of large, well-controlled, appropriately powered studies (Buller et al., 2004). In the mouse ectromelia model, death occurs about 9 days after lethal infection. As was observed in the RPXV model, a single dose of 20 mg/kg, which produces plasma exposures to BCV in mice that are lower than those produced in humans given current clinical doses of BCV, provided complete protection from lethal ECTV infection when initiated as late as 4 days post-infection. Other studies demonstrated that dose regimens employing repeated BCV administration provide complete protection when initiated as late as 5 days post-infection (Parker et al., 2008, 2009, 2010, 2012). In addition to the data supporting the efficacy of BCV in the intradermal RPXV model, these studies in a second animal model add to the weight of evidence supporting the likely efficacy of BCV for treatment of smallpox.

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Appendix A. Supplementary data

Scaling from a dose and regimen efficacious in the intradermal rabbitpox model to a dose and regimen recommended for treatment of smallpox. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2015.02.007.

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