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Buccal viral DNA as a trigger for brincidofovir therapy in the mousepox model of smallpox

Ryan Crump^{1,3}, Maria Korom^{2,3}, R. Mark Buller¹, and Scott Parker¹

¹Saint Louis University School of Medicine, 1100 S. Grand Blvd, St. Louis, MO, 63104

²The George Washington University, School of Medicine and Health Sciences, Department of Microbiology, Immunology and Tropical Medicine, 2300 Eye Street, NW Washington, DC 20037

Abstract

Orthopoxviruses continue to pose a significant threat to the population as potential agents of bioterrorism. An intentional release of natural or engineered variola virus (VARV) or monkeypox viruses would cause mortality and morbidity in the target population. To address this, antivirals have been developed and evaluated in animal models of smallpox and monkeypox. One such antiviral, brincidofovir (BCV, previously CMX001), has demonstrated high levels of efficacy against orthopoxviruses in animal models and is currently under clinical evaluation for prevention and treatment of diseases caused by cytomegaloviruses and adenoviruses. In this study we use the mousepox model of smallpox to evaluate the relationship between the magnitude of the infectious virus dose and an efficacious BCV therapy outcome when treatment is initiated concomitant with detection of ectromelia virus viral DNA (vDNA) in mouse buccal swabs. We found that vDNA could be detected in buccal swabs of some, but not all infected mice over a range of challenge doses by day 3 or 4 postexposure, when initiation of BCV treatment was efficacious, suggesting that detection of vDNA in buccal swabs could be used as a trigger to initiate BCV treatment of an entire potentially exposed population. However, buccal swabs of some mice did not become positive until 5 days postexposure, when initiation of BCV therapy failed to protect mice that received high doses of virus. And finally, the data suggest that the therapeutic window for efficacious BCV treatment decreases as the virus infectious dose increases. Extrapolating these findings to VARV, the data suggest that treatment should be initiated as soon as possible after exposure and not rely on a diagnostic tool such as the measurement of vDNA in buccal cavity swabs; however, consideration should be given to the fact that the behavior/disease-course of VARV in humans is different from that of ectromelia virus in the mouse.

Keywords

ectromelia; variola virus; antiviral; viral DNA; brincidofovir; CMX001

Correspondence to: Scott Parker.

³Authors contributed equally to this work.

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Smallpox was eradicated in 1979 following a global campaign. Therefore, variola virus (VARV) does not circulate anymore in the human population. Re-introduction of VARV would result in high-levels of mortality and morbidity, particularly because the majority of the population are un-vaccinated and those that are vaccinated (other than select laboratory workers) were likely to have been done so decades ago. There is concern that clandestine stocks of VARV exist - some of which could have been genetically modified to improve their virulence and to make them less susceptible to vaccines (3, 7). Intentional release of the virus would massively impact public health, infrastructure and the economy. To this end, the government considers it prudent to develop antiviral therapies to treat infected people. One such antiviral under development is brincidofovir (BCV, previously CMX001).

Since VARV no longer circulates in human populations, the path to licensure is governed under the FDA Animal Rule (5). There are at least two critical problems with the use of animal efficacy data for licensure. First, it is challenging to determine an efficacious course of drug treatment in humans based on pharmacokinetic data determined experimentally in another species. Second, the pathophysiology in the experimental animal model may not completely recapitulate human disease, thus making it difficult to directly compare the stages of disease.

Mousepox is arguably the best respiratory challenge rodent model for smallpox. Like smallpox, mousepox requires only a low virus inoculum of ectromelia virus (ECTV) in the upper respiratory tract to initiate severe, systemic disease. There is no obvious lung involvement during the course of early disease, although virus can be detected in respiratory gases during the pre-exanthem period; and disease presents with a characteristic rash (2, 12). Unlike ordinary smallpox, mousepox presents with major pathology in the liver and spleen, and a shorter disease course with death occurring 7 to 15 days post infection (p.i.), depending on virus dose and mouse strain. Death in smallpox occurs approximately 23 to 28 days p.i. Finally, mousepox rash development is dependent on a number of parameters including mouse strain, virus strain, route of inoculation, and virus dose (2, 12). These disease differences between mousepox and smallpox add uncertainty to selection of the appropriate trigger for dosing of antiviral therapies for pivotal efficacy studies to support licensure under the Animal Rule.

Draft FDA guidance on the selection of the trigger for therapeutic intervention in animal models under the Animal Rule supports the consideration of a “disease-defining manifestation” such as rash in the case of smallpox and/or a “biological parameter/ biomarker” (5). Except for rash and virus infectivity, there are very few disease-defining manifestations or biomarkers measured in smallpox patients that could be used in animal models as a trigger for a therapeutic intervention. In ordinary smallpox, fever onset was 12–14 days after infection with rash occurring at ~16 days p.i. (6). Virus was routinely detectable in oropharyngeal secretions a day or so prior to onset of rash (15). Thus, rash and infectivity, or viral genome equivalents in oropharyngeal secretions, could be used to stage disease in animal models. Unlike the rabbitpox model (14), onset of rash in the tested mousepox models occurs outside of the therapeutic window for antiviral testing (8), necessitating the evaluation of other biomarkers.

Previous studies have demonstrated the efficacy of BCV in mice both as a monotherapy and in combination with other regimens; however, no studies employed a disease manifestation as a trigger for initiating antiviral therapy (9, 10, 13). To do so we evaluated the onset of clinical symptoms, weight change, and viral genome equivalents in blood and saliva in 32-day old C57Bl/6 mice infected by the intranasal route (data not shown). Statistical analyses of weight change from this and a number of studies comparing the disease course in infected 8- and 16-week old C57Bl/6 mice failed to demonstrate weight change as a robust trigger to initiate treatment of infected mice within the therapeutic window of BCV. The data did suggest that viral genome copies in buccal cavity swabs or blood could provide a trigger for treatment, but an optimal relationship between virus challenge dose, the detection of genome copies, and mortality needed to be developed in adult C57Bl/6 mice.

In a previous publication we observed that vDNA in buccal swabs could be detected early on in the disease course of mousepox; however, the number of mice evaluated was small and studies that evaluated challenge dose were not performed (8). The early detection of virus is important because accurately identifying people as early as possible after exposure allows for both earlier treatment of the infected individual (possibly preventing morbidity altogether) and also will likely reduce viral transmission. Viral DNA in buccal swabs were of particular interest because vDNA appeared at a similar time as vDNA in blood, suggesting it could be a basis for a screening tool as it is far less invasive and offers a higher through-put mechanism of detection of vDNA with a reduced number of preparation steps (8). The goal of this study was to determine whether vDNA could be used as a trigger for the initiation of BCV treatment in mousepox and, by analogy, in smallpox or human monkeypox.

To this end, we conducted 6 identical animal experiments (3 with males, and 3 with females) each with a total of N=5 mice/group. Having three independent biological-replicates provided us with N=15 mice/group/sex. For each experiment, 13 groups of 5 mice were randomized to provide mouse weights in each group to within 30% of other groups (Tables 1 and 2). At day 0, group 1 mice were inoculated via the intranasal (IN) route with 5 µl of PBS/nare. At day 0, groups 2–13 were challenged via the IN route with escalating doses of ECTV (5 µl of virus inocula/nare), such that groups 2, 5, 8, and 11 received a target dose of 250 PFU; groups 3, 6, 9, and 12 received a target dose of 2500 PFU; and groups 4, 7, 10, and 13 received a target dose of 6000 PFU. The challenge virus was prepared from single use aliquots from one pool of sucrose cushion purified ECTV (4). The BCV treatment regimen consisted of 3 dosings of drug given by gastric-gavage and was based on previously published (9–11, 13) and unpublished data. The first dose was 20 mg/kg followed by 5 mg/kg given 48 hours later and another 5 mg/kg given after a further 48 hours (96 hours after first dose). Therapy was initiated on day 4 (groups 5, 6 and 7); day 5 (groups 8, 9 and 10); or, day 6 (groups 11, 12 and 13). Mice in all groups were weighed daily Monday – Friday until day 21 and then a final weight was taken at day 28. vDNA levels were measured in buccal swabs of mock-treated mice (groups 2, 3 and 4) on day 3, 4, 5, 6, and 7 p.i. No false positives were identified in the uninfected mice. Significance of mortality differences were calculated using a Fisher's exact (2-tailed) and Mantel-Cox (log-rank) tests with $P < 0.05$ considered as significant.

All groups of challenged female mice receiving drug-vehicle (groups 2, 3 and 4) experienced statistically significant mortality compared to mice inoculated with PBS (Figure 1 and Table 1). Groups receiving the higher two inocula of 2500 and 6000 PFU experienced 0% survival. We found statistically significant protection with all 3 challenge doses when BCV was initiated at day 4 p.i. (Table 1); however, when BCV was initiated at day 5 p.i., we observed significant protection only in mice challenged with the 250 and 2500 PFU doses. No significant protection was recorded when BCV was delayed until day 6 p.i. From day 3 p.i. vDNA could be detected in buccal swabs of female mice (Figure 2). At each day p.i. an increasing proportion of mice were positive for vDNA, which correlated with challenge dose, until a 100% of mice were vDNA positive by day 7 p.i. When >70% of female mice in groups 2, 3 or 4 were positive for vDNA in buccal swabs on the day of initiation of BCV treatment, only 2500 and 6000 PFU challenge groups treated on day 5 and 4, respectively, resulted in statistically significant levels of protection (Table 1; Fisher Exact Test); however, only the 2500 PFU challenge group was significant using the log-rank test ($P<0.0001$). However, the initiation of BCV treatment when all of the female mice in the 6000 PFU challenge group were positive was not efficacious. Also based on the failure of BCV treatment initiated at 6 days p.i. to significantly protect female mice challenged with 250 or 2500 PFU, it is unlikely female mice would be protected with BCV treatment starting 1 day later.

All groups of challenged male mice receiving vehicle (groups 2, 3 and 4) experienced statistically significant mortality compared to mice inoculated with PBS (Figure 3 and Table 2). Mice receiving the higher two challenge inoculums of 2500 and 6000 PFU experienced 0% survival. We found that BCV provided statistically significant protection in the 250 PFU and 2500 PFU groups when the treatment regimen began on day 4, 5 or 6. In mice challenged with the 6000 PFU dose, mice were protected only when BCV was initiated on day 4. The kinetics of detection of vDNA in buccal swabs of male mice in groups 2, 3 or 4 was similar to that observed for female mice (Figure 4). When >70% of male mice in groups 2, 3, or 4 were positive for vDNA in buccal swabs on the day of initiation of BCV treatment, a greater number of conditions showed statistically significant levels of protection (Table 2; Fisher Exact test): 2500 PFU and 6000 PFU at day 4; 2500 PFU at day 5; and 250 PFU and 2500 PFU at day 6, and all 5 conditions were significant using the log-rank test ($P<0.0002$). The initiation of BCV treatment when all of the male mice in a challenge group were positive (250 PFU, day 7; 2500 PFU day 5, and 6000 PFU, day) was efficacious only for the 2500 PFU challenge group.

Using male and female mice, we found six conditions where > 70% vDNA positivity of individuals of a group was a trigger for efficacious BCV treatment suggesting it could be used for pivotal mousepox efficacy studies for BCV under the Animal Rule; however, in another 11 conditions (61% of the total) this was not the case. So optimal conditions would need to be carefully determined and shown to be reproducible. Interestingly there appeared to be a difference between the sexes with only one and five conditions observed in female and male mice, respectively. The basis for this difference is not clear; however, sex differences in mortality following ECTV infection have been documented in other mouse strains (1).

For all challenge doses, vDNA could be detected in a small percentage of mice by day 3 p.i. well within in the first half of the disease course. In the early time points, the proportion of animals positive for vDNA increased with challenge dose. Greater than 70 % vDNA positivity for male and female was not reached until day 4, day 5, and day 6 for 6,000, 2500 and 250 PFU challenge doses, respectively. And 100 % positivity was not attained in all challenge doses for both male and female mice until day 7 p.i., late in the disease course, and outside of the published therapeutic window for BCV in the mousepox model (8, 10, 11, 13). Extrapolating these data to human monkeypox and smallpox would suggest initiation of efficacious BCV treatment based on detection of vDNA in buccal swabs would not be prudent unless the assay sensitivity could be significantly improved and assay times shortened as expected in the next generation point-of-care diagnostics. In the prairie dog model, vDNA was detected in buccal swabs before rash onset and within the therapeutic window for tecovirimat, another experimental antiviral for human orthopoxvirus infections (16); however, the appearance of vDNA was not discussed as a possible trigger for antiviral therapy.

The disease course in mice shortened as the challenge dose increased, with the mean time to death decreasing from ~10.7, 8.9 and 7.9 days for inoculations of 250, 2500 and 6000 PFU, respectively. This had a dramatic effect on the effectiveness of BCV. Following BCV treatment, the percentage of survivors decreased and therapeutic window narrowed as challenge dose increased (Table 1 and Table 2). If the observed relationship of challenge dose to disease course to efficacious BCV treatment extends to human monkeypox and VARV infections, then treatment of individuals exposed to high dose of these viruses with antivirals such as BCV should be commenced immediately without reliance of a diagnostic test.

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Highlights

- Brincidofovir effectively protected large numbers (n=15) of mice against a lethal ectromelia virus challenge.
- Buccal swabs were selected as a non-invasive mechanism to sample mice for a biomarker (vDNA) of disease.
- vDNA in buccal swabs can be detected within the therapeutic window of brincidofovir.

Figure 1.

Survival of female mice challenged with the indicated inoculum of ECTV. At day 0, mice were inoculated via the intranasal (IN) route with PBS or target doses of 250, 2500 or 6000 PFU. Oral BCV treatment consisting of 20 mg/kg followed by 5 mg/kg given 48 hours later and another 5 mg/kg given after a further 48 hours (96 hours after first dose) was initiated at day 4, day 5 or day 6 (D4, D5 or D6). Mice in all groups were monitored for weight change and vDNA levels in buccal swabs. VEH indicates mice receiving the vehicle treatment and PBS indicates mice challenged with a PBS inoculum.

Figure 2.

Percentage of female mice positive for vDNA in buccal swabs on the indicated day p.i. Mice were challenged with either PBS or a 250, 2500 or 6000 PFU inoculum of ECTV and buccal swabbed on day 3 (D3), day 4 (D4), day 5 (D5), day 6 (D6), and day 7 (D7) p.i. At days 4, 6 and 8 the 250, 2500 and 6000 treatment groups received an injection of vehicle.

Figure 3.

Survival of male mice challenged with the indicated inoculum of ECTV. At day 0, mice were inoculated via the intranasal (IN) route with PBS or target doses of 250, 2500 or 6000 PFU. Oral BCV treatment consisting of 20 mg/kg followed by 5 mg/kg given 48 hours later and another 5 mg/kg given after a further 48 hours (96 hours after first dose) was initiated at day 4, day 5 or day 6 (D4, D5 or D6). Mice in all groups were monitored for weight change and vDNA levels in buccal swabs. VEH indicates mice receiving the vehicle treatment and PBS indicates mice challenged with a PBS inoculum.

Figure 4.

Percentage of male mice positive for vDNA in buccal swabs on the indicated day p.i. Mice were challenged with either PBS or a 250, 2500 or 6000 PFU inoculum of ECTV and buccal swabbed on day 3 (D3), day 4 (D4), day 5 (D5), day 6 (D6), and day 7 (D7) p.i. At days 4, 6 and 8 the 250, 2500 and 6000 treatment groups received an injection of vehicle.

Table 1

Mortality and vDNA-detection in female mice challenged IN with ECTV and treated with BCV initiated at day 4, day 5 or day 6¹.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13
Start day of BCV treatment	N/A												
Dose (PFU)	PBS	250	2500	6000	250	2500	6000	250	2500	6000	250	2500	6000
Mice/group	15	14	15	15	15	15	15	15	14	15	15	15	15
MTD (Ave) ²	N/A	10.7	8.5	7.9	N/A	11.0	9.0	N/A	9.3	9.6	10.7	9.0	8.5
MTD (SEM) ³		0.2	0.4	0.3		0.9	0.4		0.6	0.3	0.8	0.4	0.4
# Dead ⁴	0	9	15	15	0	6	10	0	4	13	6	15	15
# Alive ⁵	15	5	0	0	15	9	5	15	10	2	9	0	0
% Survival	100	36	0	0	100	60	33	100	71	13	60	0	0
Significant ⁶		#	#	#	**	**	*	**	**	N/S	N/S	N/S	N/S
Statistics		Compared to group 1			Compared to Veh (group 2, 3 or 4)								
PCR +ve (%) ⁷	Fig.8,9	Fig.8	Fig.8	Fig.8	27	47	86	47	87	100	73	86	100

N/A, Not applicable; N/S, Not significant.

¹ At day 0, mice were inoculated via the intranasal (IN) route with PBS or target doses of 250, 2500, or 6000 PFU. Oral BCV treatment consisted of 20 mg/kg followed by 5 mg/kg given 48 hours later and another 5 mg/kg given after a further 48 hours (96 hours after first dose). Mice in all groups were monitored for weight change and vDNA levels in buccal swabs.

² Day of mean time to death (average).

³ Standard error of the mean for MTD.

⁴ Number of mice dead at the end of the study.

⁵ Number of mice alive at the end of the study.

⁶ *P<0.05, **P<0.008; Comparing infected mice receiving BCV therapy (groups 5–13) with infected mice receiving the vehicle (cages 2–4), as appropriate. #P<0.008; Comparing uninfected mice (group 1) with infected mice receiving vehicle (groups 2–4), as appropriate. Using Fisher’s exact 2-tailed test.

⁷ Percentage of animals positive for vDNA in buccal-swabs prior to initiation of BCV treatment. These data were also plotted in Figure 2. Protection includes all mice, regardless of PCR status.

⁸ See figures for % of animals that were PCR positive in the vehicle groups from day 3 to 7.

⁹ None detected.

Table 2

Mortality and vDNA-detection in male mice challenged IN with ECTV and treated with BCV initiated at day 4, day 5 or day 6¹.

Group	1	2	3	4	5	6	7	8	9	10	11	12	13
Start day of BCV treatment	N/A												
Dose (PFU)	PBS	250	2500	6000	250	2500	6000	250	2500	6000	250	2500	6000
Mice/group	15	15	15	15	15	15	15	15	15	15	15	15	15
MTD (Ave) ²	N/A	10.6	9.3	7.9	N/A	10.0	9.7	N/A	10.7	10.4	N/A	10.6	8.9
MTD (SEM) ³		0.3	0.3	0.2		0.0	0.3		0.4	0.4		0.4	0.5
# Dead ⁴	0	10	15	15	0	2	3	1	10	11	0	8	11
# Alive ⁵	15	5	0	0	15	13	12	14	5	4	15	7	4
% Survival	10	33	0	0	100	87	80	93	33	27	100	47	27
Significant ⁶		#	#	#	**	**	**	*	*	N/S	**	*	N/S
Statistics		Compared to Group 1			Compared to Veh (Groups 2, 3 or 4)								
PCR +ve (%) ⁷	Fig.8,9	Fig.8	Fig.8	Fig.8	7	73	93	40	100	93	80	100	100

N/S, Not significant.

¹ At day 0, mice were inoculated via the intranasal (IN) route with PBS or target doses of 250, 2500 or 6000 PFU. Oral BCV treatment consisted of 20 mg/kg followed by 5 mg/kg given 48 hours later and another 5 mg/kg given after a further 48 hours (96 hours after first dose). Mice in all groups were monitored for weight change and vDNA levels in buccal swabs.

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