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In vitro and in vivo evaluation of isatin-β-thiosemicarbazone and marboran against vaccinia and cowpox virus infections

Debra C. Quenelle *, Kathy A. Keith, Earl R. Kern

University of Alabama School of Medicine, Birmingham, AL, USA

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Abstract

It has been reported previously that some thiosemicarbazone compounds have prophylactic activity against smallpox disease and therapeutic activity against vaccinia virus (VV) infections. In these studies, isatin-β-thiosemicarbazone (IBT) and marboran were administered once daily by intraperitoneal (ip) injection to mice using 30, 10 or 3 mg/kg for 5 days beginning 24, 48 or 72 h after inoculation with VV or cowpox virus (CV). Both compounds were highly effective ($p < 0.01$) at preventing mortality due to VV even when treatment was delayed up to 72 h postinfection. In CV-infected mice, neither IBT nor Marboran were effective in preventing mortality at any dosage tested when administered at 24 h postinoculation. Viral replication in liver, spleen and kidney was delayed or reduced by 100–to 10,000-fold by 10 mg/kg of marboran, but not IBT, in VV infections. Neither compound was effective against CV infection. Neither IBT nor marboran treatment of mice cutaneously infected with VV or CV reduced viral replication or clinical disease. These results suggest that this class of compound has little therapeutic potential for orthopoxvirus infections since the in vivo activity against CV, a surrogate virus for variola, is lacking.

Keywords: Orthopoxvirus; Antiviral; Animal model; IBT; Marboran

1. Introduction

While some of the thiosemicarbazones were originally found to be effective against *Mycobacterium tuberculosis*, their anti-orthopoxvirus potential was recognized as well (Domagk et al., 1946). Isatin-β-thiosemicarbazone (IBT) and *N*-methyl-isatin-β-thiosemicarbazone (marboran or methisazone) were investigated in the 1960s for efficacy against orthopoxviruses. Individuals were treated with either of these compounds in attempts to control complications following smallpox vaccination or as a prophylaxis against smallpox infection (Bauer, 1965a,c; Bauer et al., 1962, 1963, 1969; Turner et al., 1962). Later, both vaccinated and unvaccinated people who were in contact with smallpox cases were treated with marboran in a controlled clinical trial to determine efficacy (Heiner et al., 1971). In vitro (Bauer and Sadler, 1960; Bauer, 1965b), as well as, in vivo experiments (Bauer, 1955) were conducted to validate the efficacy of thiosemicarbazone derivatives against vaccinia virus, but neither compound was adequately evaluated, particularly in models of disseminated disease. About 20 years later, test compounds were evaluated against vaccinia virus in vitro and in vivo using methisazone as a positive control (Walter et al., 1981; Borysiewicz and Witalinski, 1979; Zgornaik-Nowosielska et al., 1980). In these studies, methisazone protected Swiss mice from mortality following an intracranial inoculation of VV-IHD (Borysiewicz and Witalinski, 1979).

A need exists for active compounds with differing mechanisms of action other than cidofovir (CDV) (De Clercq, 2002) in the event that smallpox is used as a bioweapon. While CDV and some of the other known anti-orthopoxvirus agents act by interfering with DNA polymerase activity (Magee et al., 2005), the mechanism of action of IBT or marboran has been suggested to be at the level of viral transcriptional termination (Prichard and Kern, 2005). Studies using an IBT-resistant VV suggested that IBT may act directly at the viral RNA polymerase complex during transcriptional elongation or act indirectly by up-regulating or down-regulating elongation factors (Prins et al., 2004).

The present studies were conducted to provide a more in depth evaluation of IBT and marboran in vitro and in VV and CV infections in mice to determine the potential of these compounds

* Corresponding author at: The University of Alabama at Birmingham, Department of Pediatrics, 128 Children's Harbor Building, 1600 6th Avenue South, Birmingham, AL 35233-1711, USA. Tel.: +1 205 934 1990; fax: +1 205 975 1992.
E-mail address: dquenell@uab.edu (D.C. Quenelle).

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or similar derivatives for treatment of orthopoxvirus infections in humans.

2. Materials and methods

2.1. Mice

Female BALB/c mice, 3–4 weeks of age and female SKH-1 mice, 4–6 weeks of age, were obtained from Charles River Laboratories (Raleigh, NC). BALB/c mice were utilized in models of systemic infection and hairless SKH-1 mice were used for cutaneous infections. Mice were housed in microisolator cages and randomly distributed at 10–15 mice per group. Mice were obtained, housed, used and euthanized according to policies of USDA and Association for Assessment and Accreditation for Laboratory Animal Care. All animal procedures were approved by University of Alabama at Birmingham, Institutional Animal Care and Use Committee prior to initiation of studies.

2.2. Cells and viruses

Cowpox virus, strain Brighton, and VV, strain Copenhagen were kindly provided by John W. Huggins, Ph.D. (Department of Viral Therapeutics, Virology Division, U.S. Army Medical Research Institute of Infectious Disease, Frederick, MD). Vaccinia virus, strain WR, IHD NYC, Elstree or IHD-W were obtained from the American Type Culture Collection (ATCC), Manassas, VA. Stock virus pools were propagated in Vero cells obtained from ATCC. In vitro plaque reduction assays in human foreskin fibroblast (HFF) cells were performed as described previously (Keith et al., 2003). Briefly, HFF cells, seeded in 6-well plates 2 days prior to use, were infected with either VV or CV by the addition of 20–30 plaque forming units (PFU) per well. After a 1-h incubation period, various concentrations of drug were added to triplicate wells, and plates were incubated at 37 °C for 3 days. After incubation, cell monolayers were stained with neutral red for approximately 5–6 h, viral plaques were enumerated and the concentration which reduced viral replication by 50% (effective concentration or EC_{50}) was determined.

2.3. Experimental inoculations

Systemic infections were initiated by intranasal (in) inoculation of BALB/c mice as described previously (Quenelle et al., 2003). Mice were anesthetized using ketamine-xylazine and infected with an approximate LD_{90} of CV-BR (9×10^5/mouse) or VV-IHD (2.5×10^4/mouse) using a micropipettor and a total volume of 40 μl per animal. To determine the extent of viral replication in tissues, 3 animals from each treated and untreated group, were euthanized on days 1, 3, 5, 7 and 10. Lung, liver, spleen, and kidney samples were removed aseptically, weighed, homogenized in MEM with 10% FBS (10% wt./vol.) and frozen at −70 °C until assayed for virus.

Samples were thawed and assayed on Vero cells using an agarose overlay plaque assay to determine VV or CV titers (Quenelle et al., 2003). Briefly, samples of organ homogenates were diluted serially and a 0.2 ml volume was placed into each of 12-well Vero cell monolayers and incubated 1 h. A 0.5% agar (SeaKem®, ME agarose, FMC BioProducts, Rockland, MD) in minimal essential medium (MEM) solution was added to each well and the cultures were incubated for 3 days. Cultures were stained with Neutral Red (Gibco, Rockland, MD) for approximately 6 h prior to enumeration of viral plaques.

Cutaneous infections were performed as described previously (Quenelle et al., 2004a). Briefly, SKH-1 mice were anesthetized, identified by implantation of electronic microchips and the skin over the snout area was abraded using a variable speed Dremmel Tool (Racine, WI) with a # 193 bit at 4500 rpm. The procedure was performed so that superficial abrasions were created without bleeding. Then CV or VV was applied to the abraded area for 10 s using a dacron swab saturated with virus solution. Clinical signs and skin lesions were scored and recorded daily. To quantify local viral replication, the site of inoculation on the snout of each animal in the group was sampled at intervals after viral inoculation using a moistened dacron swab. Swabs were placed into tubes containing 2 ml of media and frozen at −70 °C until assayed on Vero cells using a CPE endpoint dilution assay in 96-well plates to determine virus titers. The average of the 10 samples was calculated to obtain the mean $\log_{10}$ TCID50/ml for the group.

2.4. Antiviral compounds

Cidofovir (CDV, Vistide® Gilead Sciences, Foster City, CA) was diluted in sterile saline to yield the desired dosages within a 0.1 ml volume. It was administered ip once daily for 5 days. For cutaneous studies, a 3% CDV topical formulation was prepared in 1.5% carbopol gel (Noveon Inc., Cleveland, OH) as described previously (Quenelle et al., 2004b). This was applied to the orofacial area once daily for 7 days beginning 24 h postviral inoculation using approximately 0.03 ml of gel/mouse per treatment.

The chemical structures for IBT and marboran have been published previously (Pirrung et al., 2005) and were obtained through NIAID, NIH. They were weighed and suspended in 0.4% carboxymethylcellulose to yield the desired dosages of 30, 10 or 3 mg/kg within a 0.1 ml volume. Both compounds were insoluble and administered as suspensions after prolonged sonication to eliminate clumping. These compounds were administered ip for systemic infections once daily for 5 days beginning at 24, 48 or 72 h postviral inoculation. For cutaneous infections, IBT and marboran were administered at 30 mg/kg dosages once daily ip for 7 days beginning at 24 h postviral inoculation.

2.5. Statistical evaluation

Mortality rates were analyzed by Fisher's exact test and mean day of death data by Mann-Whitney U rank sum test. For cutaneous infections, mean peak lesion scores, mean peak virus titers, areas under curve virus titer-day and lesion score-day area under curve (AUC) values were compared using the Mann–Whitney U-rank sum test. A p-value of 0.05 or less was considered significant. The means represented for AUC are

Table 1
In vitro activity of thiosemicarbazones against six vaccinia virus strains and cowpox virus

Compounds	Cytotoxicity[a]	Vaccinia strains[b]						Cowpox[b]
		Copenhagen	WR	NYC	Elstree	IHD	IHD-W	Brighton
CDV	>317 ± 0	32 ± 0.5	19 ± 11	8.9 ± 3.5	22 ± 4.9	28 ± 8.1	23 ± 6.0	39 ± 5.2
IBT	>454 ± 0	14 ± 8.7	0.3 ± 0.06	1.0 ± 0.6	1.3 ± 0.9	0.31 ± 0.03	0.2 ± 0.09	33 ± 25
Marboran	>427 ± 0	3.3 ± 3.2	0.06 ± 0.03	0.3 ± 0.1	0.5 ± 0.1	0.12 ± 0.03	0.11 ± 0.02	16 ± 2.8

[a] Values represent the concentration (CC_{50}-μM) which causes cytotoxic effects (as ascertained by a 7 day neutral red uptake assay using HFF cells in 96-well plates; detailed methods, Keith et al., 2003) on 50% of the cells.
[b] Values represent the EC_{50} (μM) and are the mean of 2 or more plaque reduction assays ± S.D.

derived from the geometric mean of the Log_{10} data from 10 animals.

3. Results

IBT and marboran had lower in vitro EC_{50} values than CDV against six different strains of VV and 1 strain of CV (Table 1). IBT had EC_{50} values ranging from 14 to 0.2 μM against the VV strains and was 33 μM against CV. Marboran had EC_{50} values ranging from 3.3 to 0.6 μM against the VV strains and 16 against CV. The EC_{50} of CDV, the standard positive control, ranged from 32 to 8.9 μM against VV and was 39 μM against CV.

When IBT or marboran was administered ip using 30 or 10 mg/kg once daily for 5 days to mice infected with VV-IHD, mortality was reduced significantly ($p \leq 0.01$) when treatment was initiated at 24–48 h postviral inoculation (Tables 2 and 3). Marboran at 3 mg/kg achieved statistical significance when given 24–72 h postVV-IHD inoculation, but IBT at 3 mg/kg was only effective at 24 h postviral inoculation. CDV groups were included in all murine efficacy studies as a reference for positive control. Similar results were obtained when VV-WR was used (data not shown). Both compounds (IBT and marboran) at equivalent doses were no more effective when administered 24 h prior to VV-IHD infection or at 6 h postinoculation (data not shown). When dosages of 30 or 10 mg/kg were initiated at 72 h postVV-IHD infection, both compounds retained efficacy ($p \leq 0.01$, Tables 2 and 3). In previous studies, dosages of 100 mg/kg of marboran or IBT were administered twice daily with no evidence of morbidity, mortality or clinical toxicity (data not shown) and were highly effective in preventing mortality from VV-IHD. CDV at 15 mg/kg was effective ($p < 0.001$) against VV-IHD and WR at all time points.

When IBT or marboran was administered ip using 30, 10 or 3 mg/kg once daily for 5 days to mice infected with CV-BR, mortality was not reduced when treatment was initiated 24 h postviral inoculation (Table 4). There was an increase in the mean day to death ($p < 0.05$) at the 30 and 10 mg/kg dosages of marboran, but no significant increases were observed for IBT. CDV was highly effective ($p < 0.001$) against CV, as expected.

Viral replication in the target organs of liver, spleen and kidney, was delayed or reduced by daily ip administration of 10 mg/kg/day of marboran in mice infected with VV-IHD (Fig. 1), whereas IBT was not effective. Neither compound affected CV replication in the target organs (Fig. 2). VV or CV replication in the lung was not significantly reduced by IBT, marboran or CDV.

Viral replication in the cutaneous infection of SKH-1 mice was not reduced by either IBT or marboran (Tables 5 and 6). Neither compound at the 30 mg/kg dosage was effective in reducing the lesion score-day-area under the curve (AUC) or mean peak lesion score-day-area of VV or CV when infections were initiated by the orofacial route. In the CV infections, a lower number of vesicular lesions than expected was seen in the

Table 2
Effect of once daily intraperitoneal treatment with IBT or CDV on the mortality of BALB/c mice infected with vaccinia-IHD

Treatment[a]	Mortality		*P*-value	MDD[b]	*P*-value
	Number	Percent			
Vehicle +24 h					
0.4% CMC	15/15	100	–	7.5 ± 0.6	–
CDV +24 h					
15 mg/kg	0/15	0	<0.001	–	–
IBT +24 h					
30 mg/kg	1/15	7	<0.001	9.0 ± 0.0	.068
10 mg/kg	2/15	13	<0.001	10.5 ± 0.7	<0.05
3 mg/kg	14/15	93	NS	7.8 ± 0.4	NS[c]
Vehicle +48 h					
0.4% CMC	15/15	100	–	7.3 ± 0.9	–
CDV +48 h					
15 mg/kg	0/15	0	<0.001	–	–
IBT +48 h					
30 mg/kg	0/15	0	<0.001	–	–
10 mg/kg	3/15	20	<0.001	7.7 ± 0.6	NS
3 mg/kg	12/15	80	NS	8.0 ± 0.6	<0.05
Vehicle +72 h					
0.4% CMC	15/15	100	–	7.2 ± 0.6	–
CDV +72 h					
15 mg/kg	0/15	0	<0.001	–	–
IBT +72 h					
30 mg/kg	8/15	53	<0.01	7.8 ± 0.5	0.053
10 mg/kg	7/15	47	<0.01	8.1 ± 1.1	<0.05
3 mg/kg	15/15	100	NS	7.8 ± 0.4	<0.01

[a] IBT was prepared in 0.4% CMC and delivered ip in 0.1 ml doses. CDV was prepared in sterile saline and given ip in 0.1 ml doses. Animals were treated once daily for 5 days beginning at 24, 48 or 72 h postviral inoculation.
[b] MDD: mean day of death.
[c] NS: not significant when compared to the appropriate vehicle control.

Table 3
Effect of once daily intraperitoneal treatment with marboran or CDV on the mortality of BALB/c mice inoculated intranasally with vaccinia-IHD

Treatment[a]	Mortality		*P*-value	MDD[b]	*P*-value
	Number	Percent			
Vehicle +24 h					
0.4% CMC	15/15	100	–	7.3 ± 0.5	–
CDV +24 h					
15 mg/kg	1/15	7	<0.001	6.0 ± 0.0	NS[c]
Marboran +24 h					
30 mg/kg	0/15	0	<0.001	–	–
10 mg/kg	0/15	0	<0.001	–	–
3 mg/kg	7/15	47	<0.01	8.7 ± 1.1	.001
Vehicle +48 h					
0.4% CMC	15/15	100	–	7.5 ± 0.7	–
CDV +48 h					
15 mg/kg	1/15	7	<0.01	7.0 ± 0.0	NS
Marboran +48 h					
30 mg/kg	0/15	0	<0.001	–	–
10 mg/kg	1/15	7	<0.01	11.0 ± 0.0	.068
3 mg/kg	7/15	47	<0.01	8.7 ± 1.1	<0.01
Vehicle +72 h					
0.4% CMC	15/15	100	–	7.2 ± 0.7	–
CDV +72 h					
15 mg/kg	0/15	0	<0.001	–	–
Marboran +72 h					
30 mg/kg	3/15	20	<0.001	9.3 ± 4.0	NS
10 mg/kg	3/15	20	<0.001	10.3 ± 2.5	<0.01
3 mg/kg	9/15	60	<0.05	9.8 ± 3.5	<0.001

[a] Marboran was prepared in 0.4% CMC and delivered ip in 0.1 ml doses. CDV was prepared in sterile saline and given ip in 0.1 ml doses. Animals were treated once daily for 5 days beginning 24, 48 or 72 h postviral inoculation.
[b] MDD: mean day of death.
[c] NS: not significant when compared to the appropriate vehicle control.

Table 4
Effect of once daily intraperitoneal treatment with IBT, marboran or CDV on mortality of BALB/c mice infected with cowpox-BR

Treatment[a]	Mortality		*P*-value	MDD[b]	*P*-value
	Number	Percent			
Vehicle +24 h					
0.4% CMC	15/15	100	–	9.5 ± 0.8	–
CDV +24 h					
15 mg/kg	0/15	0	<0.001	–	–
IBT +24 h					
30 mg/kg	15/15	100	NS	8.3 ± 0.5↓	<0.001
10 mg/kg	15/15	100	NS	9.0 ± 1.0	NS[c]
3 mg/kg	15/15	100	NS	9.8 ± 1.0	NS
Marboran +24 h					
30 mg/kg	15/15	100	NS	10.4 ± 1.5	.002
10 mg/kg	15/15	100	NS	9.9 ± 1.0	<0.05
3 mg/kg	15/15	100	NS	8.9 ± 0.8	NS

[a] IBT and marboran were prepared in 0.4% CMC and delivered ip in 0.1 ml doses. CDV was prepared in sterile saline and given ip in 0.1 ml doses. Animals were treated once daily for 5 days beginning 24 h postviral inoculation.
[b] MDD: mean day of death.
[c] NS: not significant when compared to the appropriate vehicle control.

vehicle-treated groups. The IBT-and marboran-treated groups had lesion score-day AUC and peak lesion scores which were similar to our previous studies using other compounds (Quenelle et al., 2004b). The administration of IBT or Marboran did not exacerbate lesions above what is normally seen. Also, titer-day-AUC and mean peak titers were not significantly reduced by IBT or marboran. Topical CDV at 3%, the positive control, was highly effective in reducing both lesion score-day AUC ($p < 0.001$) and mean peak lesion score ($p < 0.001$), as well as virus titer'day-AUC ($p < 0.001$) and mean peak titers ($p \leq 0.001$), in both VV and CV infected mice.

4. Discussion

Thiosemicarbazones have had a lengthy history as potential prophylactic therapeutics for human disease beginning at least as early as 1946 (Domagk et al., 1946). While in vitro efficacy has been documented recently (Smee and Sidwell, 2003; Neyts and De Clercq, 2003; Pirrung et al., 2005), efficacy experiments in animal models and human clinical trials have yielded variable results (Boyle et al., 1966; Walter et al., 1981; Rao et al., 1965; Heiner et al., 1971). In mice, using the tail vein route of VV inoculation, activity of IBT or thiosemicarbazone derivatives was observed when administered parenterally within 24 h of infection (Rao et al., 1965; Boyle et al., 1966; Walter et al., 1981). Achieving statistical significance in reducing clinical disease or numbers of cases in human smallpox trials using

Table 5
Effect of once daily intraperitoneal treatment with marboran, IBT or CDV on vesicle lesion development and virus titers in a cutaneous vaccinia-WR infection of SKH-1 mice

Treatment[a]	#With lesions/ #inoculated	Lesion score-day AUC	Mean peak lesion score	#Virus positive/ #inoculated	Virus titer-day AUC	Mean peak titer score[b]
Vehicle	9/10	5.1	1.0 ± 0.5	10/10	56	6.6 ± 0.4
Marboran 30 mg/kg	3/10[d]	1.9[c]	0.3 ± 0.5[c]	10/10	47[c]	6.0 ± 1.1[c]
IBT 30 mg/kg	8/10[c]	4.6[c]	0.9 ± 0.52[c]	10/10	52[c]	6.7 ± 0.6[c]
CDV 3%	0/10[e]	0.0[e]	0.0 ± 0[e]	9/10	4.7[e]	1.2 ± 0.7[e]

[a] Treatment was initiated 24 h postinoculation once daily for seven days. Marboran, IBT, and CMC were given ip at 0.1 ml per dose and CDV was applied topically.
[b] Log_{10} TCID50/ml.
[c] NS: not statistically significant when compared to appropriate vehicle treated group.
[d] $p < 0.05$.
[e] $p < 0.001$.

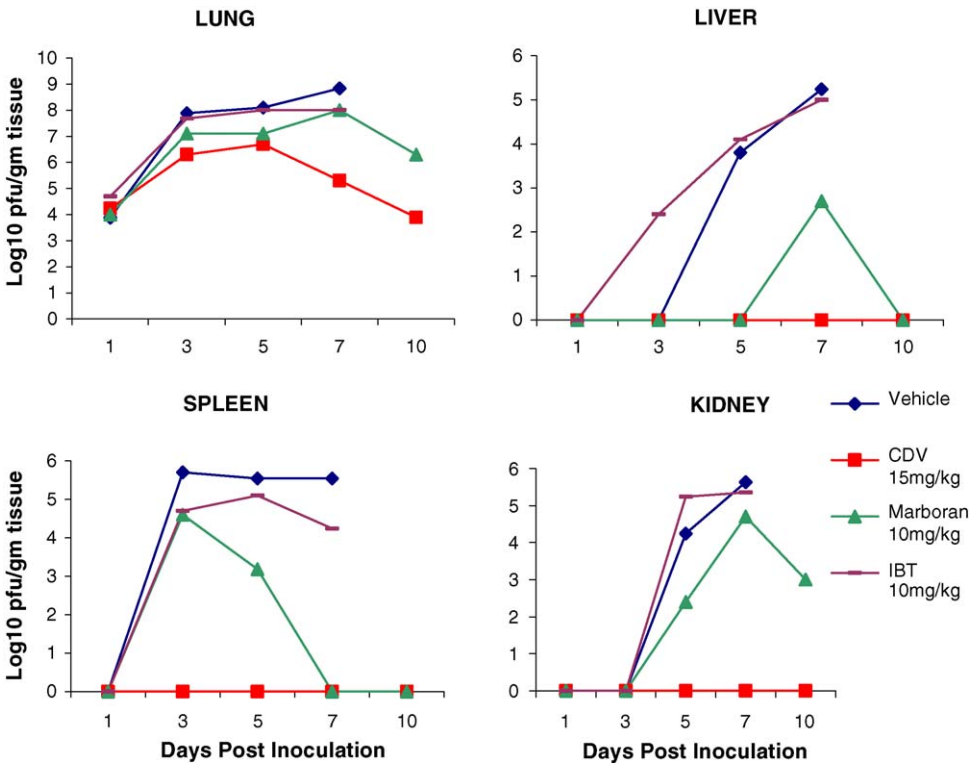

Fig. 1. Effect of once daily intraperitoneal treatment with IBT, marboran or CDV on viral replication of VV-IHD in BALB/c mice; Treatment was administered once daily for 5 consecutive days beginning at 24 h postviral inoculation. Three mice were euthanized on days 1, 3, 5, 7 and 10 for evaluation of viral replication in target organs. Each data point represents the mean value of the $\log_{10}$ pfu/gram of tissue.

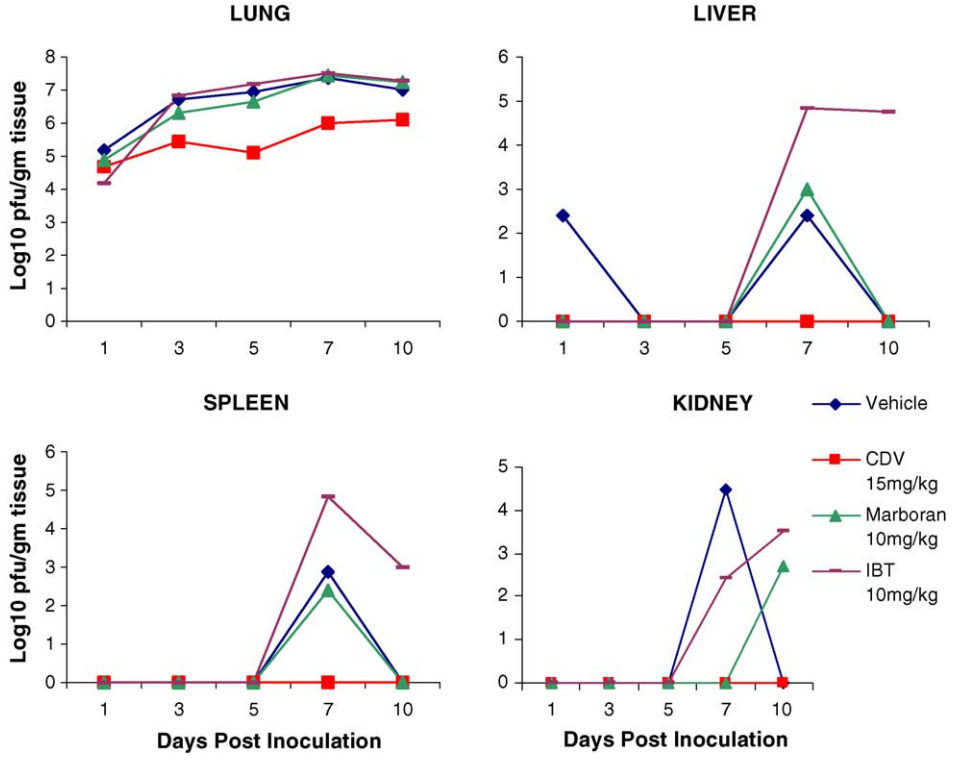

Fig. 2. Effect of once daily intraperitoneal treatment with IBT, marboran or CDV on viral replication of CV-BR in BALB/c mice; Treatment was administered once daily for 5 consecutive days beginning 24 h postviral inoculation. Three mice were euthanized on days 1, 3, 5, 7 and 10 for evaluation of viral replication in target organs. Each data point represents the mean value of the $\log_{10}$ pfu/gram of tissue.

Table 6
Effect of once daily intraperitoneal treatment with marboran, IBT or CDV on vesicle lesion development and virus titers in a cutaneous cowpox-BR infection of SKH-1 mice

Treatment[a]	#With lesions/ #inoculated	Lesion day AUC	Mean peak lesion score	#Virus positive/ #inoculated	Titer day AUC	Mean peak titer score[b]
Vehicle	2/10	1.3	0.3 ± 0.5	10/10	48	5.6 ± 1.6
Marboran 30 mg/kg	7/10	2.7[d]↑	0.6 ± 0.5[c]↑	10/10	49[c]	5.7 ± 1.6[c]
IBT 30 mg/kg	7/10	5.2[d]↑	0.7 ± 0.6[c]↑	10/10	42[c]	5.1 ± 1.7[c]
CDV 3%	1/10	0.1[e]	0.1 ± 0.2[e]	10/10	7[e]	1.9 ± 0.7[e]

[a] Treatment was initiated 24 h postinoculation once daily for seven days. Marboran, IBT, and CMC were given ip at 0.1 ml per dose and CDV was applied topically.
[b] TCID50/ml.
[c] NS: not statistically significant when compared to appropriate vehicle treated group.
[d] $p \leq 0.01$.
[e] $p < 0.001$.

marboran, however, was not accomplished (Heiner et al., 1971). Oral administration of 1–6 grams of marboran also induced vomiting, presenting complications with accuracy of drug dosing (Heiner et al., 1971).

Clearly, further evaluation of these compounds was warranted in the more recent search for anti-orthopoxvirus agents. In our studies, IBT and marboran were effective in reducing VV and CV replication in vitro, with more potent activity seen against the VV strains. In mice infected systemically with VV, treatment with IBT or marboran significantly reduced mortality with marboran having activity at lower dosages than IBT. In subsequent pathogenesis studies, VV replication in liver, spleen and kidney, but not lung, was delayed or reduced by 10 mg/kg of marboran, with little effect seen with IBT. In these VV studies, mice treated with IBT had greater than anticipated mortalities at the 10 mg/kg dosage. The lack of effect of IBT on viral replication in target organs was consistent with the mortalities observed with VV and CV infections in mice. Neither of these compounds showed activity by reducing the mortality of mice or viral replication in target organs of mice infected with CV. Increasing the dose or reducing the dosing intervals would not likely have improved results based on our previous experience. Also, no activity was seen with IBT or marboran in cutaneous infections of VV or CV. Although topical application of soluble compounds, such as CDV, has been shown to be more effective than parenteral administration (Quenelle et al., 2004b; Smee et al., 2004), insoluble compounds suspended in a gel would have an even greater likelihood of being ineffective. It has been suggested that CV has the greatest similarity compared to variola virus in its in vitro drug sensitivity (Baker et al., 2003). The failure of both IBT and marboran to alter CV infection in mice would suggest that further development of this group of compounds for treatment of smallpox infection in humans may not be warranted.

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