Antiviral Research 80 (2008) 309–315

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Treatment of Venezuelan equine encephalitis virus infection with (−)-carbodine

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ARTICLE INFO

Article history:
Received 3 April 2008
Received in revised form 2 July 2008
Accepted 8 July 2008

Keywords:
Venezuelan equine encephalitis
Virus
Carbodine
TC-83
C3H/HeN mouse
Antiviral

ABSTRACT

Venezuelan equine encephalitis virus (VEEV) may cause encephalitis in humans, for which no FDA-approved antiviral treatment is available. Carbocyclic cytosine (carbodine) has broad-spectrum activity but toxicity has limited its utility. It was anticipated that one of the enantiomers of carbodine would show enhanced activity and reduced toxicity. The activity of the D-(−) enantiomer of carbodine [(−)-carbodine] was evaluated by infectious cell culture assay and was found to have a 50% effective concentration (EC_{50}) of 0.2 μg/ml against the TC-83 vaccine strain of VEEV in Vero cells, while the L-(+) enantiomer had no activity. Virus titer inhibition correlated with intracellular cytidine triphosphate reduction after treatment with (−)-carbodine, as determined by HPLC analysis. Pre-treatment with 200 mg/(kg d) resulted in significant improvement in survival, virus load in the brain, weight change, and mean day-to-death in a mouse model of TC-83 VEEV disease. A single dose of (−)-carbodine resulted in a slight extension of mean time to death in mice infected with wild-type VEEV. Post-virus exposure treatment with (−)-carbodine was effective in significantly improving disease parameters in mice infected with TC-83 VEEV when treatment was initiated as late as 4 days post-virus installation (dpi). It is remarkable that (−)-carbodine is effective when initiated after the establishment of brain infection.

1. Introduction

Venezuelan equine encephalitis virus (VEEV) is a New World alphavirus that causes periodic outbreaks of disease in equines and humans. VEEV is present in various host species in sylvatic transmission cycles in areas of South and Central America. Adaptive mutations during replication of enzootic VEEV are required for conversion to an epizootic strain of VEEV associated with epidemic potential (Weaver, 2005). Humans are not efficient hosts for productive VEEV transmission, and tangential infections in man generally follow equine outbreaks, as equines serve as efficient amplification hosts (Calisher, 1994). Equine virulence is known to occur only in viruses of VEEV subtype 1, including mainly 1AB and 1C viruses, as well as 1E VEEV varieties (Weaver et al., 2004). Outbreaks with considerable morbidity and mortality, including encephalitis, occur sporadically, exemplified recently by the 1995 outbreak that began in Venezuela and involved approximately 75,000–100,000 people (Weaver et al., 1996). Aside from natural infection risk in endemic areas, the potential exists for the use of modified virus as a biological weapon (Hawley and Eitzen, 2001), which underlies the importance of developing therapies for use in the case of natural outbreaks, as well as for the treatment of disease after intentional release (Sidwell and Smee, 2003).

The Trinidad donkey (TrD) strain has been well characterized in vitro and in vivo, but work with TrD, currently listed as a select agent, is highly restricted. TC-83 is an attenuated vaccine strain of VEEV, exempt from the select agent status, which was derived from TrD for use in human vaccination (Berge et al., 1961). TC-83 differs from the parental strain in 12 nucleotide positions (Johnson et al., 1986; Kinney et al., 1993), 2 of which (1 in the 5′-noncoding region and 1 in the E2 envelope glycoprotein) have been shown to be associated with the attenuated phenotype (Kinney et al., 1993). In general, attenuated VEEV strains will replicate to high titers in the brain of different mouse strains, but there is usually no morbidity or mortality associated with infection (Ludwig et al., 2001; Schoneboom et al., 2000). However, intranasal (i.n.) installation of TC-83 in the C3H/HeN mouse strain results in morbidity and mortality, likely because of a reduced mucosal IgA response (Hart et al., 1997; Ludwig et al., 2001). The TC-83 infection of C3H/HeN mice has been characterized, serving as a useful and relatively safe model for

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doi:10.1016/j.antiviral.2008.07.002

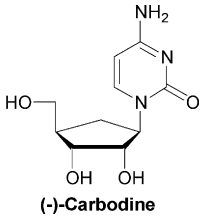

Fig. 1. Structure of (−)-carbodine.

antiviral studies (Julander et al., 2008). Peak virus titers are present in the brains of C3H/HeN mice 4 days post-virus i.n. installation (dpi), and neurological pathology is observed 6 dpi (Julander et al., 2008).

The carbocyclic analog of cytidine (cyclopentylcytosine or carbodine) inhibits cellular cytidine triphosphate (CTP) synthetase, which converts UTP to CTP, resulting in an indirect inhibition of virus replication through a reduction of CTP pools (De Clercq et al., 1990). Carbodine has been shown to be a broad-spectrum antiviral in cell culture, with activity against several unrelated viruses (Andrei and De Clercq, 1990; De Clercq et al., 1990; Neyts et al., 1996). Addition of exogenous cytidine (cyd) or uridine results in a reversal of antiviral activity of carbodine in various cell lines (De Clercq et al., 1990). The natural nucleosides are dextrorotatory (D), but both D- and levorotatory (L)-analogs have been shown to inhibit metabolic enzymes (Gumina et al., 2002). It is unknown if the activity of the racemic carbodine is due to the one or the other enantiomer. The purpose of this study is to determine the activity of the D-(−)- and L-(+)-enantiomers of carbodine against TC-83 VEEV in cell culture to determine their activity and mode of action. Another objective of this study is to determine the effect of carbodine treatment on disease parameters in the C3H/HeN mouse model of TC-83 VEEV disease as well as in a TrD mouse model.

2. Materials and methods

2.1. Venezuelan equine encephalitis virus

The TC-83 vaccine strain of VEE was obtained from USAMRIID (Fort Detrick, MD) and was passaged in Vero cells before use. Virus was prepared in MEM containing no FBS for mouse inoculations. Animals were anesthetized with ketamine + xylazine (100 and 10 mg/kg, respectively) and challenged by i.n. installation with 0.05 ml of the diluted virus containing $10^{6.4}$ 50% $\log_{10}$ cell culture infectious doses ($CCID_{50}$) of virus. Virus was previously titrated in mice to determine an optimal infectious dose.

Experiments with the TrD strain of VEEV were conducted at Colorado State University. Virus was obtained from infected Vero cells and titrated by plaque assay. Fifty 50% lethal doses (LD_{50}), consisting of approximately 4×10^5 plaque-forming units, were nebulized into a volume 5 ft^3 containing mice over a 15-min period using a Glas-Col aerosol inhalation exposure system.

2.2. Test articles

Carbocyclic cytidine (carbodine) racemic mixture and enantiomerically pure (+)- and (−)-carbodine were synthesized using an intermediate, chiral cyclopentanol, which was synthesized as shown in Fig. 1 (manuscript in preparation). The synthesized compounds were diluted in 10% DMSO in physiological saline and stored at 4 °C until use. Ampligen, a double-stranded RNA that is used as a positive control, was obtained as a viscous solution from Hemispherx Biopharma (Philadelphia, PA) and was used undiluted at 12 milligrams compound per kilogram of body weight administered daily (mg/(kg d)). Compound was stored at −20 °C and was thawed and heated up to 50 °C prior to use to ensure a homogenous mixture. Interferon alfacon-1 (infergen), a consensus interferon, was obtained from Lawrence Blatt (InterMune, Brisbane, CA).

2.3. Cell culture efficacy studies

The antiviral activity of carbodine and its enantiomers were evaluated in vitro by cytopathic effect (CPE) inhibition assays determined by visual (microscopic) examination of the cells, increase of neutral red (NR) dye uptake into cells, and virus yield reduction (VYR) (Sidwell and Huffman, 1971). Eight half-$\log_{10}$ concentrations of each compound were evaluated against the TC-83 strain of VEEV, at a concentration of 10 $CCID_{50}$ per well, in 96-well flat-bottomed microplates plated with Vero cells (ATCC CCL-81). Plates were read after incubation at 37 °C for 3 days. For NR uptake, dye (0.034% in medium) was added to plates for 2 h, after which the dye was eluted from the cells, and absorbed dye was quantified.

A virus yield reduction assay was used to determine the 90% effective concentration (EC_{90}), or the amount of (−)-carbodine necessary to reduce the virus titer by 1 $\log_{10}$ in Vero cells. Samples obtained from initial efficacy studies were titered at each dosage level tested by serial dilution of the frozen/thawed cell/supernatant mixture onto monolayers of Vero cells. Titration was determined by observance of visual CPE. A selective index (SI_{90}) for this assay was determined by dividing the CC_{50} determined by visual inspection by the EC_{90} determined by VYR.

2.4. Reversal of antiviral effect of carbodine

Increasing half-log concentrations of cytidine (Sigma–Aldrich Inc., St. Louis, MO) from 0.32 to 100 μg/ml were added to infected cells treated with 100 μg/ml (−)-carbodine. The EC_{50} and CC_{50} were recorded, and the SI was calculated.

2.5. HPLC analysis of CTP reduction

Vero cells were grown to near confluency in Corning T-25 or T-75 flasks in MEM supplemented with 10% FBS and 50 μg/ml gentamicin. Cells were then treated with various concentrations of the compounds (32, 10, 3.2, 1.0, 0.3, 0.1, 0.03 and 0.01 μg/ml or MEM control) in MEM with 2% FBS and 50 μg/ml gentamicin. After incubation at 37 °C for 24 ± 1 h, nucleotides were extracted from the cell monolayers, with replicate flasks being trypsinized and used for comparative cell counts in parallel. Nucleotides were extracted by adding 3.5% perchloric acid, rocking, and holding at 4 °C for 5–10 min. Supernatant fluid was then neutralized with 1N KOH/1 M imidazole, collected, and frozen at −20 °C. Precipitates were removed by centrifugation and/or filtration before injection into the high pressure liquid chromatography (HPLC) system.

HPLC was performed on a Millipore Waters™ model 510 system at RT, equipped with a SAX partisil 10, 25 cm column (Phenomenex, Torrance, CA), a 5 cm guard column, as well as the Phenomenex SecurityGuard™ system. Detection was with the Waters™ model Lambda-Max 541 LC spectrophotometer. The area of the nucleotide peaks was determined using the Shimadzu CR601 Chromatopac™ integrator (generally set as follows: slope = 500 default or daily by slope test, drift = 1000, width = 10). Buffers were 0.01 M ammonium phosphate pH 7.4 and buffer 1.0 M ammonium phosphate pH 3.5. HPLC was run as follows: low concentration buffer prewash (~8 mL), 40 min gradient from 0 to 100% of 1 M buffer at 1 mL/min, 1 M buffer postwash (10 mL), 0.01 M buffer postwash (10 mL). A 300-μL aliquot of sample crude was injected per run. Synthetic CTP controls were run frequently to account for any variation in HPLC peak separation or detection. Controls for peak location were

the 5′-triphosphate sodium salts of cytidine, uridine, adenosine, and guanosine (Sigma–Aldrich Inc., St. Louis, MO). Untreated controls consisted of Vero cell crudes grown without carbodine in parallel with each replicate test series. CTP peak sizes were normalized based on the ATP peak from the respective cell preparation to account for differences in extraction efficiency or non-specific metabolic inhibition or cytostatic effects.

2.6. Animals

Female C3H/HeN mice (16–18 g) or female ICR mice (18–20 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were randomly assigned to cages and individually marked with eartags. Mice were fed standard mouse chow and tap water ad libitum.

2.7. Facilities

Experiments with VEEV TC-83 were conducted in the BSL-2/3 animal suite at Utah State University Laboratory Animal Research Center, and work with VEEV TrD was conducted at Colorado State University.

2.8. Titration of virus titer by infectious cell culture assay

The virus titers in tissues or plasma were assayed using an infectious cell culture assay wherein a specific volume of either tissue homogenate or plasma was added to the first tube of a series of dilution tubes. Serial dilutions were made and added to Vero cells. Three days later, CPE was used to identify the end-point of infection. Four replicates were used to calculate the infectious doses per milliliter of plasma or gram of tissues (Reed and Muench, 1938).

2.9. Experimental design of animal studies

Mice were challenged i.n. with $10^{6.4}$ $CCID_{50}$ of TC-83 VEEV diluted in MEM containing no FBS. Groups were treated by intraperitoneal (i.p.) injection with 100 or 200 mg/(kg d) (−)-carbodine, given bid for 8 days, beginning 4 h prior to virus challenge. Ampligen was used as a positive control compound and was administered i.p. −4 h and 2 dpi at a dose of 12 mg/kg per treatment. Placebo control mice were treated with saline on the same schedule as the ampligen treatment. Mortality was checked daily for 21 days. Mice were weighed on 0, 4, and 7 dpi. Toxicity controls (uninfected mice) were treated in parallel, and weight changes were noted. Brain samples were taken 4 dpi from mice treated with carbodine, ampligen, or placebo to determine the effect of treatment on virus titer in these organs by infectious cell culture assay.

For studies with TrD, mice were treated a single time at −4 h or 3 dpi with (−)-carbodine in relation to aerosol challenge with virus. Mice were treated as above by i.p. injection. Mice were observed daily and survival was recorded.

A post-virus exposure treatment experiment was conducted to determine if therapeutic treatment with (−)-carbodine at 200 mg/(kg d) with the same treatment schedule as above would be efficacious. Mice were challenged i.n. with an LD_{90} dose of TC-83 VEEV virus. Treatment was initiated −4 h, 1, 2, 4, and 6 dpi in three separate experiments. Mice were weighed, and mortality was checked as in the previous experiment.

2.10. Statistical analysis

Survival data were analyzed using the Wilcoxon log-rank survival analysis (JMP™ Software, The Statistical Discovery Software, SAS Institute, Inc.). All other statistical analysis was done using one-tailed Student's *t*-test.

Table 1
Activity of carbodine and its enantiomers in Vero cells infected with TC-83 VEEV

Compound	EC_{50} (vis)[a]	EC_{50} (NR)[b]	EC_{90}[c]	IC_{50}[d]	SI_{90}[e]
(−)-Carbodine	0.3 ± 0.2[f]	0.2 ± 0.0	0.4 ± 0.2	>100	>250
(+)-Carbodine	>100	>100	>100	>100	1
(±)-Carbodine	0.68	0.70	1.7	>100	>59
Interferon alfacon-1	0.06	0.05	0.06	>10	>167

[a] Concentration (μg/ml) of drug in which 50% of cells are protected from viral cytopathic effect (CPE) as determined by visual (vis) inspection.
[b] Concentration (μg/ml) of drug in which 50% of cells are protected from viral CPE as determined by neutral red (NR) dye uptake assay.
[c] Concentration (μg/ml) of drug that reduces virus titer by 1 $\log_{10}$.
[d] Concentration (μg/ml) of drug in which 50% of uninfected cells in a confluent monolayer are damaged by the compound as determined by NR dye uptake.
[e] Selective index, determined by dividing the IC_{50} by the EC_{90}.
[f] Average (μg/ml) ± standard deviation taken from two to three separate experiments.

3. Results

3.1. Effect of the enantiomers of carbodine on TC-83 VEEV in cell culture

Analysis of the racemic mixture (±)-carbodine and the D-(−)- and L-(+)-enantiomers in the treatment of TC-83 VEEV in Vero cells was performed. The D-(−)-carbodine enantiomer was found to have activity at nanogram concentrations, while the L-(+)-enantiomer was not active at the highest concentration tested (EC_{50} of >100 μg/ml) and the racemic mixture showed intermediate activity (Table 1). Toxicity was not observed by visual inspection or by neutral red uptake assay in uninfected cells treated with the highest concentration of any of the carbodine preparations tested.

The activity of (−)-carbodine was confirmed by a virus yield reduction assay, which determined the EC_{90}, or the concentration of compound required to reduce virus by approximately 1 $\log_{10}$. The average EC_{90} was 0.4 ± 0.2 μg/ml, which was similar to the EC_{50} concentration determined previously. The SI_{90} values, as determined in Vero cells (by dividing the CC_{50} by the EC_{90}), were >250, 1, and >167 for (−)-carbodine, (+)-carbodine, and (±)-carbodine racemic mixture, respectively (Table 1).

3.2. Effect of cytidine co-treatment on the activity of (−)-carbodine

A reversal study was conducted in which increasing amounts of cytidine were added to infected cells treated with half-log dilutions of (−)-carbodine. The activity observed for (−)-carbodine was reversed by the addition of cytidine in a dose-dependent manner (Table 2). Activity of (−)-carbodine was completely abolished after treatment with 10–32 μg/ml of cytidine (Table 2). No toxicity was observed at any of the cytidine or (−)-carbodine concentrations tested, as determined by visual inspection or neutral red uptake assay.

3.3. Reduction of intracellular CTP after (−)-carbodine treatment

Previous studies have shown that racemic carbodine is an effective inhibitor of CTP synthetase in various cell lines. It was anticipated that (−)-carbodine would also reduce intracellular CTP levels. Vero cell monolayers were treated with various concentrations of (−)-carbodine to determine the effect of this compound on the reduction of intracellular CTP pools in Vero cells. CTP was reduced in a dose-dependent manner in Vero cells treated with (−)-

Table 2
Reversal of (−)-carbodine action by co-treatment with cytidine

Treatment	Treatment conc.[a]	Cytidine conc.[b]	Visual inspection results			Neutral red uptake assay results		
			EC_{50}[c]	IC_{50}[d]	SI[e]	EC_{50}	IC_{50}	SI
(−)-Carbodine	100	100.0	>100	>100	1.0	>100	>100	1.0
(−)-Carbodine	100	32.0	>100	>100	1.0	>100	>100	1.0
(−)-Carbodine	100	10.0	>100	>100	1.0	86	>100	>1.2
(−)-Carbodine	100	3.2	22	>100	>4.5	21	>100	>4.8
(−)-Carbodine	100	1.0	4.4	>100	>22.7	1.7	>100	>58.8
(−)-Carbodine	100	0.32	0.22	>100	>454.5	0.19	>100	>526.3
(−)-Carbodine	100	–	0.18	>100	>555.5	0.17	>100	>588.2
Interferon alfacon-1	10	–	0.06	>10	>166.7	0.056	>10	>178.6
None	–	100.0	>100	>100	1.0	>100	>100	1.0

[a] Concentration of (−)-carbodine or infergen given in μg/ml.
[b] Concentration of cytidine added to infected cells in μg/ml.
[c] 50% effective concentration (μg/ml) of treatments alone or in combination with cytidine.
[d] 50% inhibitory concentration (μg/ml) of treatments alone or in combination with cytidine.
[e] Selective index determined by dividing the IC_{50} by the EC_{50}.

carbodine (Fig. 2). Complete reduction to undetectable levels was observed after treatment with 0.32 μg/ml and above, but no appreciable reduction in virus yield was seen at lower concentrations, suggesting that CTP had to be reduced to below detectable levels in order to achieve an antiviral effect. Virus titer reduction, as determined by virus yield reduction assay, correlated with the reduction of CTP in Vero cells (Fig. 2). Intracellular CTP was not reduced after treatment with concentrations of (+)-carbodine as high as 10 μg/ml, which was around 1000-fold higher than the concentration of (−)-carbodine that resulted in a 65% reduction in intracellular CTP (data not shown).

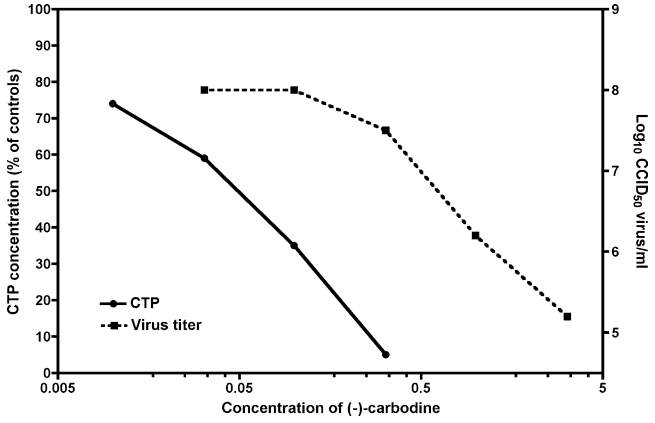

Fig. 2. Effect of (−)-carbodine on intracellular CTP pools and virus titer in Vero cells. A dose–response in intracellular CTP pool reduction (left *Y*-axis) is observed with increasing amounts of (−)-carbodine. Virus titer reduction shows a dose-responsive decline as CTP concentrations approach the limit of detection (right *Y*-axis).

3.4. Effect of (−)-carbodine on TC-83 VEEV infection in a mouse model

To determine the efficacy and toxicity of (−)-carbodine, animal studies were conducted in which infected C3H/HeN mice were treated i.p., with 100, 10, or 1 mg/(kg d) twice daily (bid) for 8 days. A significant improvement in survival was seen after treatment with 100 mg/(kg d), although a 50% mortality rate was observed (Table 3). Weight change and mean day-to-death were also significantly improved after treatment with 100 mg/(kg d) of (−)-carbodine. No activity, however, was observed with 10 or 1 mg/(kg d) treatment (Table 3).

In a subsequent study, the dose was increased, and groups of mice were treated with either 200 or 100 mg/(kg d). A significant increase in survival, weight change, and mean day-to-death was also observed with both doses given according to the same schedule as above (Table 3). In this initial dose escalation study, toxicity, as indicated by weight loss between 0 and 7 dpi, was observed in sham-infected mice treated with 200 mg/(kg d) (−)-carbodine. In subsequent experiments, however, toxicity control mice treated with this dose gained weight similar to untreated normal control

Table 3
Effect of i.p. (−)-carbodine treatment[a] on survival and weight change of C3H/HeN mice infected with Venezuelan equine encephalitis virus[b]

Exp.	Treatment	Dosage	Toxicity controls		Infected, treated		
			Alive/total	Mean wt. change (g)[c]	Alive/total	Mean wt. change (g)[c]	MDD ± S.D.[d]
1	(−)-Carbodine	100 mg/(kg d)	3/3	0.4 ± 0.8	5/10***	−0.2 ± 0.9**	13.2 ± 1.6**
	(−)-Carbodine	10 mg/(kg d)	–	–	1/8	−2.1 ± 1.3	10.0 ± 0.0
	(−)-Carbodine	1 mg/(kg d)	–	–	0/8	−3.0 ± 1.6	9.6 ± 0.7
	Placebo	–	–	–	1/10	−2.2 ± 1.5	9.7 ± 0.5
	Normal controls	–	2/2	0.9 ± 0.6	–	–	–
2	(−)-Carbodine	200 mg/(kg d)	3/3	−1.5 ± 0.5*	6/10***	−2.5 ± 0.8**	11.5 ± 2.6**
	(−)-Carbodine	100 mg/(kg d)	–	–	2/10**	−1.9 ± 0.6**	12.8 ± 1.3***
	Ampligen	12 mg/treatment	–	–	3/10*	−2.2 ± 2.8	10.4 ± 1.0*
	Placebo	–	3/3	−0.1 ± 0.7	2/15	−3.9 ± 1.9	8.6 ± 2.0
	Normal controls	–	3/3	1.1 ± 0.5	–	–	–

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ as compared with placebo-treated controls.
[a] Carbodine was administered bid for 8 days beginning 4 h prior to virus challenge.
[b] Anesthetized 16–18 g female mice were infected intranasally with $10^{6.4}$ $CCID_{50}$ TC-83 vaccine strain VEEV.
[c] Weight change was calculated using the weights of mouse groups on days 0 and 7.
[d] Mean day-to-death of mice dying prior to 21 dpi.

Table 4
Effect of post-virus exposure i.p. (−)-carbodine treatment on survival and weight change of C3H/HeN mice infected with Venezuelan equine encephalitis virus[a]

Exp.	Treatment	Dosage	Time of treat. initiation[b]	Toxicity controls		Infected, treated		
				Alive/total	Mean wt. change[c] (g)	Alive/total	Mean wt. change[c] (g)	MDD[d] ± S.D.
1	(−)-Carbodine	200 mg/(kg d)	−4 h	–	–	8/10***	0.1 ± 0.6***	11.0 ± 2.8
	(−)-Carbodine	200 mg/(kg d)	24 h	–	–	10/10***	0.0 ± 0.4***	>21.0 ± 0.0***
	Placebo	–	−4 h	–	–	0/20	−1.7 ± 0.8	9.1 ± 1.5
2	(−)-Carbodine	200 mg/(kg d)	2 dpi	3/3	1.1 ± 0.2	9/10***	−1.0 ± 0.7***	12.0 ± 0.0***
	(−)-Carbodine	200 mg/(kg d)	4 dpi	–	–	9/10***	−0.3 ± 0.9***	11.0 ± 0.0***
	Placebo	–	4 dpi	–	–	0/10	−2.8 ± 1.0	9.1 ± 0.6
3	(−)-Carbodine	200 mg/(kg d)	4 dpi	2/2	0.1 ± 0.5	8/8***	−0.9 ± 1.4	>21 ± 0.0
	(−)-Carbodine	200 mg/(kg d)	6 dpi	–	–	4/10	−2.1 ± 1.4	9.8 ± 0.4
	Ampligen	12 mg	−4 h	–	–	9/10**	−0.2 ± 1.5**	11.0 ± 0.0
	Placebo	–	−4 h	–	–	4/15	−2.2 ± 1.7	10.3 ± 0.5
	Normal controls	–	–	5/5	0.1 ± 0.5	–	–	–

**$P < 0.01$, ***$P < 0.001$, as compared with placebo-treated controls.
[a] Anesthetized 16–18 g female mice were infected intranasally with $10^{6.4}$ $CCID_{50}$ TC-83 vaccine strain VEEV.
[b] Mice were treated bid for 8 days beginning on the hour or day indicated. Ampligen treatment was given −4 h and 2 dpi.
[c] Weight change was calculated using the weights of mouse groups on days 0 and 7.
[d] Mean day-to-death of mice dying prior to 21 dpi.

mice (Table 4). Because of the highly significant increase in survival (as compared with placebo treatment), the 200 mg/(kg d) dose was used in subsequent studies.

Brain samples taken from infected mice on 4 dpi were evaluated by infectious cell culture assay to determine if treatment with 200 mg/(kg d) of (−)-carbodine would result in reduced virus in this target organ. A significant decrease in virus titer was observed in the brains of infected mice. Peak brain titers are observed on 4 dpi in C3H/HeN mice infected with TC-83 VEEV. Brain virus titer on 4 dpi was reduced by an average of 10-fold in (−)-carbodine-treated mice as compared with virus titer in the brains of placebo-treated mice (Fig. 3).

3.5. Effect of (−)-carbodine on TrD VEEV infection in a mouse model

An experiment was conducted with (−)-carbodine treatment of mice infected with TrD VEEV. Animals were treated just prior to or 3 days post-virus aerosol exposure. A slight, but significant, extension in mean time to death was observed in animals receiving prophylactic (−)-carbodine treatment as compared with placebo treatment (Fig. 4). Despite the extension of survival, all animals died from the infection.

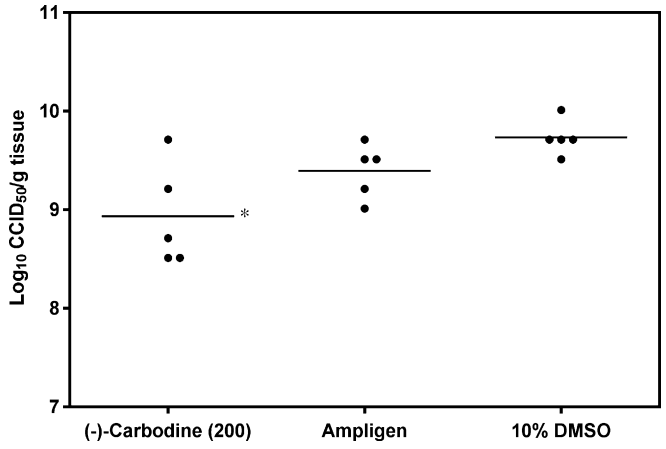

Fig. 3. TC-83 VEEV brain titers of C3H/HeN mice infected with Venezuelan equine encephalitis virus (TC-83) and treated i.p. with (−)-carbodine or ampligen (*$P \leq 0.05$ as compared to placebo).

3.6. Post-virus challenge treatment studies

Ideally, an antiviral compound should be effective when administered after the onset of clinical signs. To determine how long after virus challenge the initiation of (−)-carbodine treatment could be delayed, mice were treated beginning −4 h, 2, 4, or 6 dpi. A significant improvement in mortality, weight change, and mean day-to-death were observed when treatment of mice was initiated as late as 4 days after virus infection (Table 4, Experiment 2). These data were repeated in a subsequent experiment, confirming the efficacy of (−)-carbodine in significantly improving survival and mean day-to-death as compared with placebo treatment, although weigh change was not significantly improved (Table 4, Experiment 3). Despite the lack of significance, a trend towards improved weight change was observed. Significant improvement of disease parameters was also observed with −4 h, 24 h, and 2 dpi (Table 4). No improvement was observed when treatment began 6 days after infection.

Ampligen was used as a positive control compound in antiviral studies. Variable rates of survival were seen with ampligen treatment, although significant improvement in survival was observed in each experiment wherein ampligen was used (Tables 3 and 4).

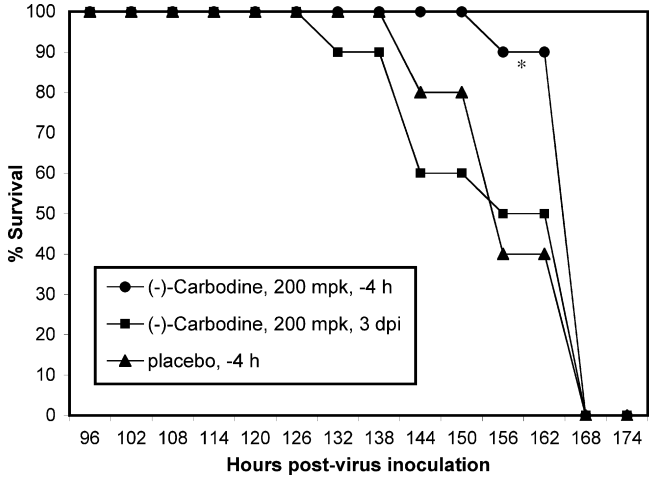

Fig. 4. Effect of (−)-carbodine on survival and mean time to death in ICR mice infected by aerosol exposure with TrD VEEV. Groups of mice were treated either −4 h or 3 dpi (*$P < 0.05$, as compared with placebo treatment).

Significant improvement in weight change was also observed in one study, but no significant improvement in mean day-to-death was seen, although there was a trend towards improvement (Table 4). Virus titers in the brains of infected mice 4 days after virus challenge and ampligen treatment initiation were reduced approximately 0.5 log, which was a significant reduction when compared with placebo treatment (Fig. 3).

4. Discussion

Carbodine was shown to be active against TC-83 VEEV in Vero cells. The activity of carbodine was also demonstrated to be dependent on the D-enantiomer, as the L-enantiomer showed no activity in cell culture. The natural substrate of the cellular CTP synthetase is D-cytidine, so it stands to reason that the D-enantiomer of carbodine would be the active constituent of the racemic mixture. Many L-nucleosides, however, have been shown to inhibit cellular and viral enzymes as well as or better than the natural D-enantiomer configuration (Gumina et al., 2002; Mathe and Gosselin, 2006). HBV was shown to be susceptible to the (+)-enantiomer of carbocyclic 5′-norguanosine, while the (−)-enantiomer was active against cytomegalovirus, vaccinia virus, and measles, demonstrating enantioselectivity of different viruses (Rajappan et al., 2002). The lack of efficacy of L-(+)-carbodine suggests a strong enantioselectivity of the host CTP synthetase enzyme, rather than virus enantioselectivity, which has been observed for various degradation enzymes that favor D-enantiomers (Maury, 2000).

In previous studies, carbodine was shown to inhibit cellular CTP synthetase in a wide variety of cell lines, which results in decreased CTP available for viral replication (De Clercq et al., 1990). In the present study, the addition of increasing concentrations of cytidine to infected cells treated with (−)-carbodine showed a reversal of antiviral activity, similar to previous reversal data by De Clerq and colleagues. This supports the previous hypothesis regarding the mode of activity of carbodine in inhibiting CTP synthetase, demonstrating the activity of the D-enantiomer is reversed by the addition of exogenous cytidine. Levels of intracellular CTP, measured in Vero cells after treatment with the enantiomers of carbodine, were reduced with increasing amounts of (−)-carbodine. This reduction of CTP pools correlated with decreasing virus concentration in Vero cells, further supporting the inhibition of CTP synthetase as a primary mode of action. Reduction of CTP pools was only observed after treatment with (−)-carbodine and not with the (+)-enantiomer. Virus reduction was first observed only at concentrations that reduced intracellular CTP pools to below the limits of detection, which also corresponded with some slight cytotoxicity as seen by a decrease in cell count as compared with untreated cells. It appears that the (−)-enantiomer of carbodine is responsible for the activity observed with the treatment of racemic carbodine.

When infected C3H/HeN mice were treated with (−)-carbodine, significant improvement in various disease parameters was observed. It was anticipated that treatment of animals with the (−)-carbodine enantiomer might reduce previous toxicity reportedly associated with carbodine treatment. However, some toxicity, seen as weight loss, was observed in the first experiment evaluating the 200 mg/(kg d) dose. Mice treated with 200 mg/(kg d) in subsequent experiments gained similar weight as uninfected, placebo-treated control mice, and it is unclear what differences between the initial and later experiments could have contributed to this difference in toxicity. Toxicity after treatment with racemic carbodine has also been observed in a mouse model of influenza disease (Shannon et al., 1981). Cyclopentenyl cytosine, similar to carbodine but with an additional double bond in the carbocyclic ring, has been shown to cause hypotension and death in human patients with solid tumors after 24 h continuous i.v. infusion every 3 weeks with a dose of 4.7 mg/m^2 (Politi et al., 1995). It is possible that removal of the (+)-carbodine enantiomer might reduce the toxicity observed after treatment with racemic carbodine, but this is yet to be determined. Overall, (−)-carbodine treatment was well tolerated in C3H/HeN mice infected with TC-83 VEEV.

For a compound to exhibit clinical utility, it is important therapeutic activity must be achieved when treatment is initiated after the onset of clinical symptoms. Patients will generally not seek medical attention until disease symptoms are manifest, therefore, an antiviral agent should be efficacious later in the course of disease. Efficacy of (−)-carbodine was also observed when treatment was initiated as late as 4 dpi. In the C3H/HeN mouse model of VEEV disease, virus is detected in the brain as early as 2 dpi. At 4 dpi, virus in the brain has reached peak titers around 9.5 $CCID_{50}$, which correlate with perivascular cuffing and meningitis in the brains of infected mice (Julander et al., 2008). These results show that (−)-carbodine is effective despite peak virus levels and observable pathologic changes in the brain. It is remarkable for a compound to exhibit protective activity after the establishment of high viral titers in the brain.

An initial, limited experiment to determine the effect of (−)-carbodine against TrD VEEV showed a slight indication of efficacy in a delay in the mean time to death when mice were treated with the drug just prior to virus challenge. This gave some indication that this drug might be having an effect against the virus, although all the mice eventually died. A positive control compound was not used as a comparison, so it is unknown if the virus challenge was overwhelming and a significant antiviral effect may be more apparent with a lower challenge dose.

While these results are encouraging, further studies should be conducted to better characterize the efficacy of this compound against TrD VEEV disease, as the TC-83 VEEV vaccine strain has been shown to respond differently to interferon treatment (Spotts et al., 1998; White et al., 2001). Funding for (−)-carbodine synthesis has expired, so no further experiments are possible in the near future, although it would be important to conduct further experiments against TrD VEEV if a source of (−)-carbodine becomes available. Further testing of (−)-carbodine against TrD infection would give a better indication of the therapeutic utility of this agent in the treatment of natural VEEV infection.

Acknowledgements

We thank Luci Wandersee and Jeremy Strange for their work with treatments and data collection. We also thank Jacob Bingham for his work with HPLC data collection. This work was supported by Contracts NO1-AI-15435 and NO1-AI-30048 from the Virology Branch, NIAID, NIH and by UO19 AI056540, NIAID, NIH.

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