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Treatment of lethal Ebola virus infection in mice with a single dose of an *S*-adenosyl-L-homocysteine hydrolase inhibitor☆

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Abstract

Ebola Zaire virus causes lethal hemorrhagic fever in humans, for which there is no effective treatment. A variety of adenosine analogues inhibit the replication of Ebola virus in vitro, probably by blocking the cellular enzyme, *S*-adenosyl-L-homocysteine hydrolase, thereby indirectly limiting methylation of the 5′ cap of viral messenger RNA. We previously observed that adult, immunocompetent mice treated thrice daily for 9 days with 2.2–20 mg/kg of an adenosine analogue, carbocyclic 3-deazaadenosine, were protected against lethal Ebola virus challenge. We now report that a single inoculation of 80 mg/kg or less of the same substance, or of 1 mg/kg or less of another analogue, 3-deazaneplanocin A, provides equal or better protection, without causing acute toxicity. One dose of drug given on the first or second day after virus infection reduced peak viremia more than 1000-fold, compared with mock-treated controls, and resulted in survival of most or all animals. Therapy was less effective when administered on the day of challenge, or on the third day postinfection. Single or multiple doses of the same medications suppressed Ebola replication in severe combined immunodeficient mice, but even daily treatment for 15 consecutive days did not eliminate the infection. Published by Elsevier Science B.V.

Keywords: Filovirus; Ebola virus; Antiviral therapy; *S*-Adenosyl-L-homocysteine hydrolase

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1. Introduction

Ebola Zaire virus (EBO-Z), a member of the family *Filoviridae*, causes the most virulent viral hemorrhagic fever of humans (Peters et al., 1996). The case-fatality rate approached 90% in two large hospital-associated outbreaks in Zaire (the present Democratic Republic of the Congo) in 1976 and 1995 (Johnson et al., 1977; Sanchez et al., 1995; Peters and LeDuc, 1999). The virus also causes lethal illness in wild nonhuman primates, but its true natural reservoir has not been identified. Human-to-human transmission occurs by means of body fluids during close contact. There is no vaccine or effective therapy (Bray and Huggins, 1998).

Wild-type EBO-Z can lethally infect newborn mice, but does not produce illness in adult, immunocompetent mice (Van der Groen et al., 1979; McCormick et al., 1983). The need for a convenient small animal model of EBO hemorrhagic fever, to permit in vivo evaluation of experimental antiviral drugs and to test candidate vaccines, spurred the recent successful adaptation of EBO-Z to adult mice, through sequential passage in progressively older suckling mice (Bray et al., 1998). Intraperitoneal (i.p.) inoculation of as little as one virion of doubly plaque-purified ninth-mouse-passage EBO-Z ('mouse-adapted virus') is lethal for adult, immunocompetent mice. Rapid viral replication in the liver, spleen, and other tissues, with accompanying cell lysis, results in death 4–7 days after infection. The target cells and tissues in mice are apparently identical to those of EBO-Z-infected guinea pigs, nonhuman primates and humans (Jaax et al., 1996; Ryabchikova and Netesov, 1998; Zaki, 1998; Connolly et al., 1999). This model is therefore suitable for initial in vivo antiviral drug evaluation, vaccine testing, and studies of EBO pathogenesis.

A number of adenosine analogs, including the compounds carbocyclic 3-deazaadenosine (C-c^{3}Ado) and 3-deazaneplanocin A (c^3-Npc A), possess strong antiviral activity, both in vitro and in vivo (Montgomery et al., 1982; Glazer et al., 1986; Tseng et al., 1989). Their proposed mechanism of action, through inhibition of a cellular enzyme, *S*-adenosyl-L-homocysteine (SAH) hydrolase, is discussed later. These compounds are especially active against negative-sense RNA viruses, including paramyxoviruses and rhabdoviruses (De Clercq and Montgomery, 1983; De Clercq, 1987; De Clercq et al., 1989; De Clercq, 1998). Both C-c^{3}Ado and c^3-Npc A inhibit the replication of filoviruses in vitro, including EBO-Z, EBO Sudan, and Marburg virus, with 50% inhibitory concentrations (IC_{50}) against EBO-Z of 30 and 2 μM, respectively (Huggins et al., 1998). Their low in vitro toxicity and resultant high therapeutic indices (188 and >850) make them attractive candidates for in vivo evaluation.

Initial testing in a model of EBO-Z infection in severe combined immunodeficient (SCID) mice showed that thrice-daily (TID) treatment with either compound significantly reduced the levels of virus in tissues and prolonged survival, but did not prevent death (Huggins et al., 1995). Further evaluation in adult, immunocompetent mice, using mouse-adapted EBO-Z, revealed that TID treatment for 9 days with C-c^{3}Ado protected nearly all animals against lethal challenge, providing treatment was begun on day -1, 0 or 1 with respect to infection (Huggins et al., 1999). Partial survival was obtained if therapy was begun on day 2 or 3.

These and other adenosine analogs have also shown strong activity against other viruses in small animal models. Wyde et al. (1990) found that cotton rats challenged intranasally with respiratory syncytial virus and treated with daily doses of 1–10 mg/kg C-c^{3}Ado on days 1–3 after infection developed significantly lower pulmonary virus titers than untreated controls. Single or multiple doses of C-c^{3}Ado or c^3-Npc A significantly reduced tailpox formation in weanling mice after intravenous vaccinia virus challenge (De Clercq et al., 1984; Tseng et al., 1989). Similar treatment strategies, with single or multiple injections of drug, protected newborn mice against lethal vesicular stomatitis virus infection (De Clercq and Montgomery, 1983; De Clercq et al., 1989). Our attempts to simplify and improve the therapy of experimental EBO-Z infection led to the present series of experiments, in which the effect of treatment with a single dose of C-c^{3}Ado or c^3-Npc A is assessed.

2. Materials and methods

2.1. Viruses, cells and antiviral compounds

The adaptation of EBO-Z to adult, immunocompetent mice has been described previously (Bray et al., 1998). Briefly, a stock of EBO-Z '76 (Mayinga) virus, previously passaged three times intracerebrally in suckling mice and twice in Vero cells (Geisbert and Jahrling, 1995), was passaged nine more times by subcutaneous (s.c.) or i.p. inoculation in progressively older suckling BALB/c mice. Virus recovered from the liver of a moribund ninth-passage mouse was plaque-purified twice, amplified and frozen down in aliquots. This 'mouse-adapted virus' is lethal for adult BALB/c and other mouse strains when inoculated i.p. The 50% lethal dose (LD_{50}) is 0.03 plaque-forming units (pfu), or approximately one virion.

Vero clone E6 monkey kidney cells (Vero C1008, ATCC CRL 1586) were propagated in Eagle's minimal essential medium with Earle's salts (EMEM), nonessential amino acids, 10% fetal bovine serum (FBS), glutamine, penicillin, and streptomycin at 37°C in a 5% CO_2 atmosphere. To determine the titer of EBO-Z preparations, samples were serially diluted in EMEM containing 2% FBS, adsorbed onto confluent Vero E6 cells in 12-well dishes, incubated for 1 h at 37°C, covered with an agarose overlay, and returned to the incubator (Moe et al., 1981). A 1:5000 dilution of neutral red dye in buffered saline solution was added 6 days later, and plaques were counted the following day.

Carbocyclic 3-deazaadenosine (3-deazaaristeromycin, C-c^3Ado) was synthesized as described previously (Montgomery et al., 1982) by Southern Research Institute (Birmingham, AL), and its purity was confirmed. This compound is stable indefinitely at ambient temperature (J Secrist, personal communication). 3-Deazaneplanocin A ((−)-9-[trans-2′,trans-3′-dihydroxy-4′-hydroxymethyl-cyclopent-4′-enyl]-3-deazaadenine) (c^3-Npc A) was synthesized as described previously (Tseng et al., 1989) by Starks Associates (Buffalo, NY) and stored frozen; spectrographic and chromatographic analysis showed >99% purity (J. Driscoll, personal communication). The compounds were dissolved in phosphate-buffered saline (PBS) at an appropriate concentration for inoculation in a 0.1 ml volume.

2.2. Antiviral therapy and toxicity studies

Infectious material and animals were handled in maximum-containment biological safety level 4 facilities at the United States Army Medical Research Institute of Infectious Diseases, Frederick, MD. Laboratory personnel wore positive-pressure protective suits (ILC; Dover, Frederica, DE) equipped with high-efficiency particulate air filters and supplied with umbilical-fed air.

Adult, female immunocompetent BALB/c mice and SCID mice on a BALB/c background were obtained from the National Cancer Institute, Frederick, MD, housed in filtertop microisolator cages and given commercial mouse chow and water ad libitum. Cages, chow and water for SCID mice were sterilized by autoclaving before use.

All drug treatment experiments with immunocompetent mice were performed in a similar manner. In each, a cohort of 8- to 16-week-old female BALB/c mice was challenged by i.p. inoculation of 10 pfu (300 LD_{50}) mouse-adapted virus suspended in EMEM, routinely given as a divided dose in both sides of the lower abdomen, to ensure that virus entered the peritoneal cavity in every case. For drug treatment, C-c^3Ado or c^3-Npc A dissolved in PBS was inoculated beneath the skin of the upper back. One group of infected mice in each experiment was mock-treated with PBS (placebo group) and held for daily observation of weight loss, illness, and death; other groups were treated at various time points, with respect to virus challenge, and observed in the same manner. In some experiments, two mice from each treatment or control group were anesthetized and exsanguinated each day; or, every other day postinfection, serum was collected for virus titration, as already described.

Drug testing in SCID mice was performed essentially as above, using 6- to 7-week-old female animals, except that most experiments compared the protective efficacy of single versus repeated doses of C-c^3Ado or c^3-Npc A. In one experiment,

mice were infected i.p. with 10 pfu virus and treated on day 1 only, or every second, third or fourth day, beginning on day 1, with 1 mg/kg c^3-Npc A. In other experiments, mice were treated on day 1, or on days 1–3, 1–4, or 1–7, with 1 mg/kg c^3-Npc A. In a final trial, a cohort of infected mice was treated for up to 15 days, beginning on day 1 postchallenge, with daily doses of 1 mg/kg c^3-Npc A. Therapy was discontinued for subgroups of mice after day 1, 5, 9 or 15, and body weight and survival were then followed for each subgroup. Within each subgroup, two mice were terminally exsanguinated on each even-numbered day for determination of serum viral titers. Uninfected control groups were either left untreated, or were treated daily with 1 mg/kg c^3-Npc A from days 1 to 15, to assess possible drug toxicity.

Acute toxicity was also assessed by treating uninfected immunocompetent BALB/c mice with a single s.c. injection of up to 320 mg/kg C-c^3Ado, or up to 10 mg/kg c^3-Npc A, and observing them daily for illness, weight loss, or death. In one experiment, groups of mice were inoculated once with 1, 5 or 10 mg/kg c^3-Npc A, and two of them were anesthetized and exsanguinated on days 1, 3, 6, and 9 thereafter. Serum levels of urea nitrogen (BUN) and creatinine (Cr), and of the liver-associated enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were determined using a Kodak 250 automated chemistry analyzer (Eastman Kodak, Rochester, NY). The results in drug-treated animals were compared with those of five untreated mice.

3. Results

3.1. Dose-ranging studies

The effects of various doses of C-c^3Ado or c^3-Npc A, inoculated s.c. on day 1 postinfection, on the survival of EBO-infected mice are shown in Table 1. All mock-treated or untreated control mice died. A single dose of 20 mg/kg or more of C-c^3Ado, or of 0.25 mg/kg or more of c^3-Npc A, was sufficient to protect most or all animals against death. In this and other experiments, those treated mice that died tended to live longer than mock-treated mice, consistent with a partial degree of antiviral protection. Similar results were obtained in two other dose-ranging experiments (not shown).

3.2. Toxicity testing

Inoculating single doses of up to 320 mg/kg C-c^3Ado or up to 10 mg/kg c^3-Npc A (quantities 4- to 10-fold greater than the protective doses cited in Section 3.1) in uninfected adult mice did not result in weight loss, behavioral change or other visible signs of toxicity over the subsequent

Table 1
Survival of adult, immunocompetent BALB/c mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and treated with various amounts of Ca-c^3Ado or c^3-Npc A on day 1 postinfection

Drug	Dose (mg/kg)	Survivors/total	Percent surviving	Significance vs. placebo[a]	Mean days to death ± S.D.[b]
Ca-c^3Ado	80	5/5	100	<0.01	–
	40	4/5	80	<0.01	8.0
	20	4/5	80	<0.01	8.0
	10	0/5	0	–	7.2 ± 0.89
c^3-Npc A	1	5/5	100	<0.01	–
	0.5	5/5	100	<0.01	–
	0.25	5/5	100	<0.01	–
	0.125	2/5	40	–	7.7 ± 0.58
Placebo	–	0/5	0	–	7.0
None	–	0/5	0	–	6.6 ± 0.55

[a] Value of p by Fisher's exact test.
[b] For those animals that died.

Table 2
Survival of adult, immunocompetent BALB/c mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and treated with one dose of Ca-c^{3}Ado on the indicated day

Experiment number	Treatment day	Dose (mg/kg)	Survivors/total	Percent surviving	Significance vs. placebo[a]	Mean days to death ± S.D.[b]
1	0	80	5/6	83	<0.01	9.0
	1	80	5/6	83	<0.01	9.0
	2	80	6/6	100	<0.01	–
	3	80	1/6	17	–	7.8 ± 0.84
	4	80	0/6	0	–	7.5 ± 0.55
	Placebo	–	0/6	0	–	6.3 ± 0.52
2	0	80	2/6	33	–	7.5 ± 0.58
	1	80	6/6	33	<0.01	–
	2	80	6/6	100	<0.01	–
	Placebo	–	0/6	0	–	6.3 ± 0.52
3	3	160	0/5	0	–	7.61.1
	3	240	0/5	0	–	7.21.5
	Placebo	–	0/5	0	–	6.60.55

[a] Value of p by Fisher's exact test.
[b] For those animals that died.

2 weeks. Serum samples collected 1, 3, 6, and 9 days after inoculation of 1, 5 or 10 mg/kg c^3-Npc A revealed no evidence of hepatic injury (increase in circulating AST or ALT) or renal impairment (increased BUN or Cr), compared with mock-treated controls.

3.3. Treatment of EBO-Z infection with C-c^{3}Ado

On the basis of the presented results, we chose to employ a single s.c. inoculation of 80 mg/kg C-c^{3}Ado as a standard dose for studies of EBO-Z therapy. Table 2 presents the outcome of three experiments in which this dose was administered to groups of five or six mice at various time points with respect to EBO-Z challenge. All mock-treated mice succumbed to infection; most were found dead on day 6, with a mean time to death (MTD) of 6.3–6.6 days. When mice were treated with 80 mg/kg C-c^{3}Ado within 1 h after infection ('day 0'), all became ill on day 4–5 after challenge, but two out of six recovered and survived in one experiment, and five out of six in another. Treatment on day 1 or 2 appeared to be more effective: nearly all animals treated on day 1, and all mice treated on day 2, remained active and survived challenge, showing a significant increase in survival with respect to controls. Mice treated on day 3 failed to survive, even when the dose of C-c^{3}Ado was increased to 160 or 240 mg/kg, but a slight prolongation of the MTD was observed. No mice treated on day 4 survived infection, in this or other experiments.

Fig. 1 shows additional data from the second experiment in Table 2. Mice were treated with C-c^{3}Ado on day 0, 1, or 2, or were mock-treated with PBS on day 0. All animals in the latter group were dead by day 7 postinfection (Fig. 1A). All mice treated on day 0 became ill by day 5–6, losing a maximum of 17% of their mean prechallenge body weight. Four of the six mice in this group died, but their deaths were delayed with respect to mock-treated controls. All mice treated on day 1 or 2 remained active and survived. However, therapy did not completely prevent illness, as shown by a decline in body weight beginning 4–6 days after infection (Fig. 1B). Mice treated on day 1 lost 4% and those treated on day 2 lost 12% of their body weight, before beginning to recover. The peak geometric mean titer of circulating virus in the serum of mice treated on day 0 with C-c^{3}Ado (4×10^6 pfu/ml on day 4 postinfection) was almost 100-fold lower than that in mock-treated mice (2.5×10^8 pfu/ml) (Fig.

1C). Peak mean titers of mice treated on day 1 or day 2 (1.4×10^5 and 5.2×10^4, respectively), were lower than those of mice treated on day 0, correlating with the better observed health and lesser degree of weight loss observed in the former two groups. Circulating virus was no longer detectable by day 9 in surviving animals.

3.4. Treatment of EBO-Z infection with c^3-Npc A

Data from two experiments are presented in Table 3. All mice treated with a single dose of 1 mg/kg c^3-Npc A on day 0, 1 or 2, and most animals treated on day 3, survived infection. Fig. 2 shows data on body weight from the first of these experiments, in which all treated mice survived challenge. Mice treated on day 0 became ill, with ruffled fur and slowed activity, beginning on day 4–5, and underwent a period of weight loss, then began to regain weight on day 10. By contrast, mice treated on day 1 or 2 appeared healthy throughout the experiment and continued to gain weight, except for a brief arrest of weight gain on days 5–6. Therapy on day 3 resulted in the onset of weight loss on day 4, which reversed abruptly

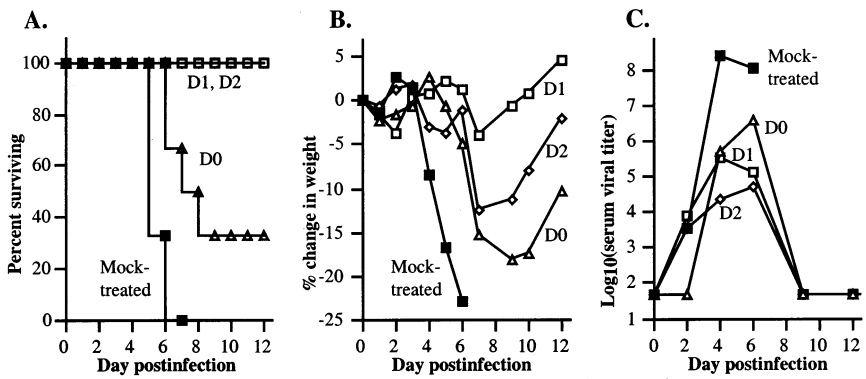

Fig. 1. (A) Survival of groups of six adult BALB/c mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and inoculated once, on day 0, 1, or 2 with respect to infection, with 80 mg/kg Ca-c^3Ado, or mock-treated with PBS on day 0. (B) Mean percent change in body weight of mice in the same experiment. (C) Geometric mean serum viral titers of two mice/day from the same groups, terminally exsanguinated on days 2, 4, 6 or 8 postinfection.

Table 3
Survival of adult, immunocompetent BALB/c mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and treated with 1 mg/kg c^3-Npc A on the indicated day

Experiment number	Treatment day	Survivors/total	Percent surviving	Significance vs. placebo[a]	Mean days to death ± S.D.[b]
1	0	5/5	100	<0.01	–
	1	5/5	100	<0.01	–
	2	5/5	100	<0.01	–
	3	4/4	100	<0.05	–
	Placebo	0/5	0	–	7.4 ± 0.89
2	1	8/8	100	<0.001	–
	2	8/8	100	<0.001	–
	3	5/8	63	<0.05	8.0
	4	0/8	0	–	7.9 ± 0.64
	Placebo	1/8	13	–	7.1 ± 0.69

[a] Value of p by Fisher's exact test.
[b] For those animals that died.

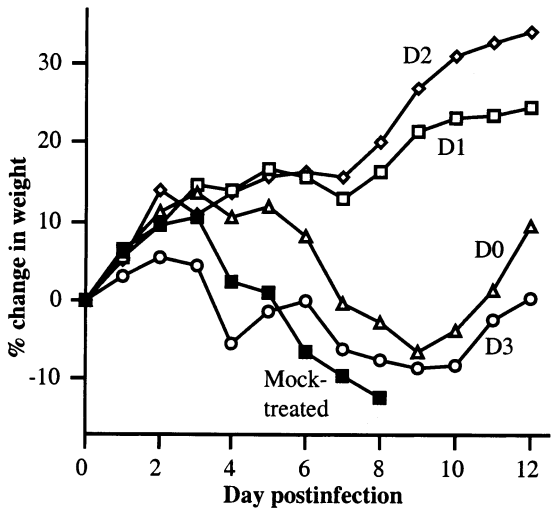

Fig. 2. Mean percent change in body weight of groups of five adult BALB/c mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and inoculated, on day 0, 1, 2, or 3 with respect to infection, with 1 mg/kg c^3-Npc A, or mock-treated with PBS on day 0.

on day 5, then resumed on day 7, resulting in eventual weight loss almost as severe as that of mock-treated controls.

A further experiment was performed to compare the effect of single or repeated doses of c^3-Npc A on weight loss and viremia. Mice were treated with 1 mg/kg drug on day 1 alone, on days 1 and 2, or on days 1, 2 and 3 postinfection, or were mock-treated on day 1. All animals in the latter group died, with a MTD of 5.8 days, while all treated mice survived infection. As shown in Fig. 3A, all three treated groups underwent a brief period of weight loss, commencing on day 3–5 postinfection, and reached their minimum postinfection weight on day 6. There was an inverse correlation between the number of doses and loss of body weight, since the change in mean body weight, relative to the day of challenge, of those treated with one, two or three doses of drug was -4, 0 and $+1\%$, respectively. All groups regained weight on day 7. Therapy on day 1 alone reduced the maximum viral titer by more than 10 000-fold, from a mean of 5.5×10^8 pfu/ml in mock-treated controls to 1.2×10^4 pfu/ml (Fig. 3B). Additional doses of c^3-Npc A resulted in still lower viremia on days 4–5 (see Fig. 3). Serum viral titers in all groups increased on days 5–6 to a range of 3.5×10^3 to 1.7×10^4 pfu/ml, coinciding with the point of maximum weight loss shown in Fig. 3A. No virus was detectable on day 8.

3.5. Treatment of EBO-Z infection in SCID mice

Adult SCID mice infected with the same quantity (10 pfu) of mouse-adapted EBO-Z used to challenge immunocompetent mice developed a

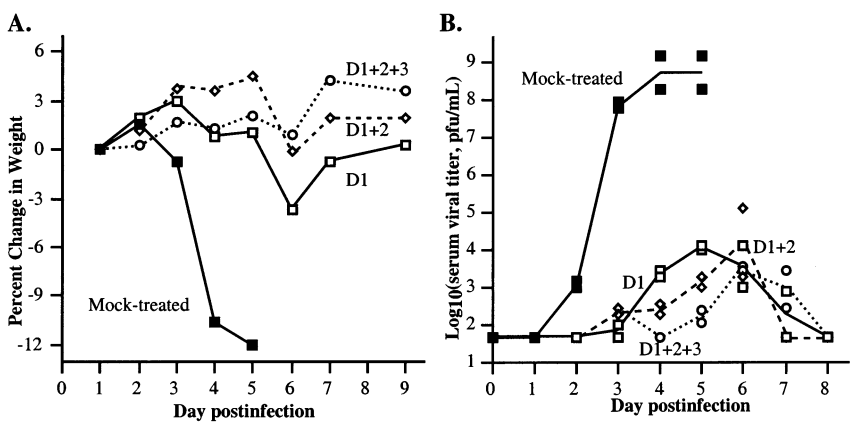

Fig. 3. (A) Mean percent change in body weight of groups of six adult BALB/c mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and inoculated on day 1; or on day 1 and 2, or on day 1, 2 and 3, with respect to infection, with 1 mg/kg c^3-Npc A, or mock-treated with PBS on day 0. (B) Individual and geometric mean serum viral titers of two mice/day from the same groups, terminally exsanguinated on days 1–8.

Table 4
Survival of 6- to 7-week-old SCID mice infected with 10 pfu (300 LD_{50}) mouse- adapted EBO-Z and treated with a single dose of Ca-c^3Ado or c^3-Npc A on the indicated day

Drug	Dose (mg/kg)	Treatment day	Survivors/total	Mean days to death ± S.D.	Significance vs. placebo[a]
Ca-c^3Ado	80	0	0/5	7.6 ± 0.55	<0.05
		1	0/5	8.2 ± 0.84	<0.05
		2	0/5	8.0	<0.05
		3	0/5	6.8 ± 0.45	–
c^3-Npc A	1	0	0/5	7.6 ± 0.55	<0.05
		1	0/5	7.8 ± 0.45	<0.05
		2	0/5	7.8 ± 0.45	<0.05
		3	0/5	6.6 ± 1.5	–
Placebo		0	0/5	6.0 ± 1.2	–

[a] Value of p by two-way t-test.

Table 5
Survival of adult SCID mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and treated, beginning on day 1 postinfection, with one dose or with repeated doses of 1 mg/kg c^3-Npc A

Experiment number	Treatment days	Survivors/total	Mean days to death ± S.D.[a]	Significance vs. placebo[b]	Significance vs. day 1 only[b]
1	1	0/6	8.7 ± 0.52	<0.001	–
	1,5,9	0/6	10.0 ± 0.63	<0.001	<0.1
	1,4,7,10	0/6	10.2 ± 0.41	<0.001	<0.001
	1,3,5,7,9	0/6	11.8 ± 1.6	<0.001	<0.01
	Placebo	0/6	6.3 ± 0.52	–	–
2	1	0/5	7.6 ± 0.55	<0.001	–
	1–4	0/5	9.6 ± 0.55	<0.001	<0.001
	Placebo	0/5	5.6 ± 0.55	–	–
3	1	0/10	9.0 ± 0.47	<0.0001	–
	1–3	0/10	11.0 ± 0.47	<0.0001	<0.0001
	1–7	0/10	14.5 ± 0.71	<0.0001	<0.0001
	Placebo	0/10	6.7 ± 0.95	–	

[a] For those animals that died.
[b] Value of p by two-way t-test.

pattern of illness resembling that of normal animals, with onset of visible illness and weight loss at 3–4 days, and death at 5–7 days postinfection. However, despite this similarity in the disease course, the same drug treatment regimens that were highly protective for normal mice failed to prevent the death of SCID mice. Treatment with one dose of 80 mg/kg C-c^3Ado, or with 1 mg/kg c^3-Npc A, on day 0, 1 or 2 increased the MTD of SCID mice by 1–2 days, compared with mock-treated controls, but did not prevent death (Table 4). Treatment on day 3 had little effect.

In a further series of experiments, multiple doses of drug were administered, in an attempt to eliminate the virus. As shown in Table 5, repeated inoculations of c^3-Npc A, begun on day 1 postchallenge, and given either on a daily basis or as regular, but less frequent doses, further increased the MTD, compared with animals treated only on day 1. However, even mice that were treated daily from days 1 to 5, 7, 9 or 15 eventually succumbed to infection (Table 5 and Fig. 4).

Examination of sequential viral titers showed that, in contrast to immunocompetent mice treated with c^3-Npc A, in which serum viral titers rose to a maximum value of 10^5 pfu/ml or less

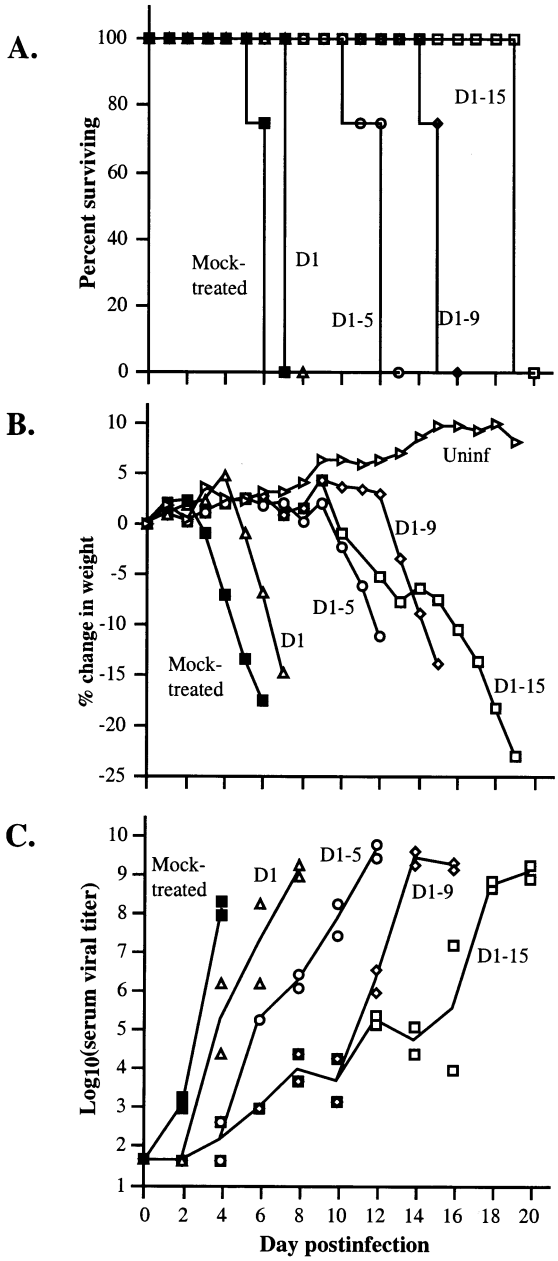

Fig. 4. (A) Survival of groups of four SCID mice infected with 10 pfu (300 LD_{50}) mouse-adapted EBO-Z and treated on day 1; or on days 1–5; 1–9; or 1–15, with respect to infection, with 1 mg/kg c^3-Npc A, or mock-treated with PBS on day 1. (B) Percent change in mean body weight of the same groups of mice, plus a group of four uninfected mice treated with the same dose of drug from days 1 to 15. (C) Individual and geometric mean serum viral titers of two mice/day from groups of animals infected and treated in parallel with the groups in (A) and (B).

before declining and disappearing, serum viral titers in SCID mice did not rise, then fall. Instead, circulating viral concentrations rose slowly during treatment, but after cessation of therapy, titers increased rapidly to values exceeding 10^9 pfu/ml (Fig. 4C). In mice treated with c^3-Npc A for 15 days, serum viral titers gradually increased, from a mean of approximately 100 pfu/ml on day 4, to about 10^3 pfu/ml on day 6, 10^4 pfu/ml on day 8 and 10^5 pfu/ml on day 12. Discontinuation of treatment after day 1, 5, 9 or 15 resulted in a rapid rise to lethal levels.

For each regimen, discontinuation of therapy was followed by the onset of weight loss and visible illness in 2–3 days, and death in 3–5 days, consistent with the rapid increase in viral titers observed after cessation of treatment (Fig. 4A,B). These experiments revealed no obvious evidence of drug toxicity from repeated dosing with c^3-Npc A, since those EBO-infected animals that received the largest number of drug inoculations always lived the longest. Interestingly, however, although mice treated on days 1–5 or 1–9 continued to gain weight while receiving therapy, the group that was treated from days 1–15 began to lose weight on day 10, a few days before the onset of weight loss in animals treated only through day 9 (Fig. 4). This effect does not appear to be the result of error, rather it suggests that, after day 10, the combination of the progressively increasing viral load and continuing drug treatment may have somewhat worsened the overall clinical illness, compared with stopping the drug on day 9.

4. Discussion

SAH hydrolase inhibitors are the most active antiviral compounds yet identified for the treatment of EBO-Z infection in mice. As shown in the present study, a single inoculation of one of these agents is sufficient to cure this rapidly progressive and uniformly lethal illness. Some mice can even be rescued if treated on the third day after a 300 LD_{50} viral challenge, a time when the animals are already visibly ill and losing weight, and viral titers in the liver and spleen exceed 10^9 pfu/g (Bray et al., 1998). A series of experiments (in-

cluding some not presented here) have consistently shown that treatment on day 1 or 2 postinfection is more protective than treatment on the day of challenge. These compounds thus appear to be most effective after active viral replication has begun, but before widespread dissemination of infection and extensive tissue damage have occurred. Interestingly, the dose of c^3-Npc A required to protect mice against EBO-Z (one dose of 1 mg/kg or less) was much lower than the amounts found to be needed to protect against vaccinia (8 mg/kg) or vesicular stomatitis virus (10 mg/kg) (De Clercq et al., 1984; Tseng et al., 1989).

As already mentioned, earlier studies of EBO-Z therapy employed wild-type (nonmouse-adapted) EBO-Z and immunodeficient (SCID) mice (Huggins et al., 1995). EBO infection in these animals progresses much more slowly than in other animal models or in humans: the mice become ill 20–25 days or more after viral challenge, and die on day 25–30. TID treatment with C-c^3Ado or c^3-Npc A significantly reduced viral titers in the serum and tissues of these animals, and increased the MTD, but did not prevent death. However, this partial success paved the way for an initial evaluation of c^3-Npc A therapy in nonhuman primates, in which thrice-daily treatment for 9 days with 0.03 or 0.1 mg/kg, beginning on the day before infection, significantly prolonged the survival of African green monkeys infected with EBO Sudan virus, but again did not prevent death (Huggins et al., 1995).

Further evaluation of adenosine analogs for EBO-Z therapy awaited the adaptation of EBO-Z to adult, immunocompetent mice (Bray et al., 1998). Initial treatment strategies in this new animal model were based on the results of pharmacokinetic studies of C-c^3Ado and c^3-Npc A in mice, which showed that C-c^3Ado had a serum half-life of approximately 23 min after intravenous inoculation, while that of c^3-Npc A was 13 min (Coulombe et al., 1993, 1995). Tissue concentrations peaked within 2 h, and little or no drug was detectable at 24 h. Based on its longer half-life, C-c^3Ado was chosen for further therapeutic evaluation in immunocompetent mice. When treatment was begun the day before challenge, as little as 0.7 mg/kg C-c^3Ado given TID for 9 days sufficed to protect all animals from death (Huggins et al., 1998). A dose of 20 mg/kg also resulted in complete survival, but was associated with increased weight loss postinfection, suggesting drug toxicity. When therapy was begun on day 1 or 2, TID doses of 2.2 or 6.7 mg/kg were highly protective, but 20 mg/kg resulted in a lower rate of survival, once more suggesting that the higher drug dose was toxic in the presence of viral infection. When treatment was begun on day 3, most animals died; 20 mg/kg was the least protective dose.

Adenosine analogues are believed to exert their antiviral activity by bringing about a reduction in methylation of the 5′ cap of viral mRNA. These compounds inhibit a host cell enzyme, SAH hydrolase (Wolfe and Borchardt, 1991; De Clercq, 1998). SAH is a product of cellular methylation reactions, which use *S*-adenosylmethionine (SAM) as the methyl group donor. The continual removal of SAH, through its hydrolysis to adenosine and homocysteine, is necessary for methylation reactions to proceed in a forward direction. Inhibition of SAH hydrolase causes an increase in the intracellular SAH/SAM ratio, with feedback inhibition of both cellular and viral transmethylation reactions. Diminished methylation of the 5′ cap of viral messenger RNA by the viral (guanine-7-)methyltransferase results in inefficient translation of viral transcripts. The correlation between the antiviral potency of adenosine analogues and their ability to reduce SAH hydrolase activity supports this proposed mechanism of action (De Clercq, 1998). Because viral transmethylases tend to be inhibited by lower SAH/SAM ratios than their cellular counterparts, inhibition of SAH hydrolase is a reasonable antiviral strategy (Liu et al., 1992).

If these compounds act only through inhibition of viral cap methylation, then the potency of a single inoculation of C-c^3Ado or c^3-Npc A seems quite remarkable, considering the brevity of their serum half-life. Such a profound and lasting effect from one dose of drug may imply that the compound transiently achieves a high concentration within virus-infected cells, disrupting and irreversibly crippling an essential step in viral replica-

tion. Alternatively, the drug, or a modified metabolite, may accumulate within cells in a 'depot' form, from which it is gradually released, exerting a prolonged antiviral effect. This occurs in the case of cidofovir and other nucleoside analogues containing phosphonyl groups, the diphosphate forms of which bind reversibly to choline, essentially creating an intracellular drug reservoir (Hitchcock et al., 1996). However, no such 'depot' has been described for adenosine analogues. The fact that the drugs are less efficacious when administered on day 0, compared with day 1 or 2, argues against a prolonged antiviral effect, since an intracellular depot of drug would presumably be highly effective in snuffing out early viral replication.

Another possible basis for the strong antiviral activity of adenosine analogues involves an increase in the resistance of the virus-infected host cell, or a bolstering of the host immune system. Since EBO-Z initially infects cells of the monocyte/macrophage system, viral replication within these cells might be especially susceptible to drugs that alter macrophage function. Two adenosine analogues, MDL 28842 and MDL 201112, have in fact been reported to block some important functions of murine macrophages, including the release of tumor necrosis factor-alpha in response to inoculation of lipopolysaccharide (LPS) (Lambert et al., 1995). A single injection of either drug rescues mice after lethal LPS challenge (Parmely et al., 1993). Similarly, the SAH hydrolase inhibitor 3-deazaadenosine blocks the release of interleukin-1 from LPS-treated human monocytes (Schmidt et al., 1990). It is thus conceivable that some of the protective effect of C-c^{3}Ado or c^3-Npc A results from a modification of the metabolism or antiviral activity of EBO-Z-infected macrophages, making them less hospitable hosts for viral replication. To begin to assess this question, we have initiated studies of the effect of drug inoculation on circulating levels of various cytokines in uninfected mice, as an index of macrophage activity. Adenosine analogues have also been shown to inhibit T-cell activation (Wolos et al., 1993a,b), but it is less obvious how this effect would be of benefit to the virus-infected host.

Comparative studies in immunocompetent and SCID mice demonstrated that an intact immune system is required to obtain the full benefit of therapy with C-c^{3}Ado or c^3-Npc. In immunocompetent animals, it appears that two separate events bring about a cure of EBO-Z infection. First, inhibition of viral replication by drug treatment keeps peak serum viral titers below a 'lethal threshold' of approximately 10^6 pfu/ml. Second, the onset of a protective immune response further suppresses viral replication and eventually eliminates the virus. The latter event can apparently be recognized by the return of weight gain, which roughly coincides with a downturn in the serum viral titer (Figs. 1 and 3). To test this hypothesis, we have begun a study to measure the time course of development of anti-Ebola antibodies (immunoglobulin (Ig)M and IgG) in mice that survive infection because of treatment with SAH hydrolase inhibitors, to determine whether the appearance of antibody correlates with the disappearance of circulating virus. The task of antiviral therapy in immunocompetent animals is therefore to keep the viral burden below the lethal threshold, preventing irreversible tissue damage and supporting the host until its own immune response becomes master of the situation. In immunodeficient animals, on the other hand, the suppression of viral replication that follows drug administration is succeeded by rapid proliferation to lethal levels. Repeated daily treatment slows the rate of accumulation of virus and delays the onset of illness and weight loss, but is ultimately incapable of preventing the development of disease and death (Fig. 4C).

Other types of therapy are effective to varying degrees against EBO-Z infection in a variety of animal models (Bray and Huggins, 1998). The recombinant B/D chimeric form of human interferon-alpha (IFN-α B/D), for example, has proven to be highly protective in mice (M Bray, unpublished data). A single injection of IFN-α B/D given on the day of challenge only delayed death, but a 5–7-day course of the same dose, begun on day 0, 1 or 2 postinfection, was highly protective. However, a recent trial of IFN-α 2b therapy in EBO-Z-infected cynomolgus monkeys resulted only in a delay in the onset of viremia,

fever and illness; all animals succumbed to infection (Jahrling et al., 1999). Antiserum to EBO-Z has also provided varying degrees of protection, depending upon the animal model. Purified IgG with strong virus-neutralizing activity, recovered from the serum of horses hyperimmunized with EBO-Z, prevented the death of EBO-Z-infected baboons, if administered within 2 h after viral challenge (Mikhailov et al., 1994; Kudoyarova-Zubavichene et al., 1999). The same material protected guinea pigs against lethal infection, but only delayed the death of EBO-Z-infected mice and cynomolgus monkeys (Jahrling et al., 1996; Jahrling, et al., 1999).

We are continuing our evaluation of the efficacy of these and other adenosine analogues for the treatment of EBO-Z infection. Based on our experience with treating EBO-Z infection in mice, we will attempt to achieve survival in nonhuman primates by treating early in infection with high but nontoxic doses of drug, in order to hold viral replication below the lethal threshold until the host immune system eliminates the infection. However, if it should prove impossible to control EBO-Z replication in primates with these compounds alone, we may ultimately find that a combination of an adenosine analogue, antiserum and IFN constitutes the most successful treatment regimen.

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