

3-Deazaneplanocin A induces massively increased interferon- α production in Ebola virus-infected mice[☆]

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Abstract

3-Deazaneplanocin A, an analog of adenosine, is a potent inhibitor of Ebola virus replication. A single dose early in infection prevents illness and death in Ebola virus-infected mice. The ability of this and similar compounds to block both RNA and DNA viruses has been attributed to the inhibition of a cellular enzyme, *S*-adenosylhomocysteine hydrolase (SAH), indirectly resulting in reduced methylation of the 5' cap of viral messenger RNA. However, we found that the protective effect of the drug resulted from massively increased production of interferon- α in Ebola-infected, but not uninfected mice. Peak interferon levels increased with the extent of disease at the time of treatment, indicating that production was boosted only in virus-infected cells. Ebola virus has been shown to suppress innate antiviral mechanisms of the type I interferon response. 3-Deazaneplanocin A appears to reverse such suppression, restricting viral dissemination. Further development should focus on identifying adenosine analogues that produce a similar effect in Ebola virus-infected primates. Published by Elsevier Science B.V.

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1. Introduction

Ebola Zaire virus (EBO-Z), a nonsegmented negative-strand RNA virus in the family Filoviridae, causes the most virulent hemorrhagic fever of humans. The case fatality rate approached 90% in two large hospital-associated outbreaks in Africa in 1976 and 1995 (Sanchez et al., 2001; Bray, 2002). EBO-Z infects and destroys cells of the mononuclear phagocytic system (MPS cells) in the spleen, liver and other tissues throughout the body, and spreads from these cells to hepatocytes, fibroblasts, adrenal cortical cells and many other cell types (Sanchez et al., 2001). Progressive tissue destruction, massive cytokine release, endothelial cell dysfunction, fluid shifts and disseminated intravascular coagulation lead to shock and death.

No licensed antiviral medications are active against EBO-Z or other filoviruses. However, a number of synthetic analogues of adenosine that have broad-spectrum antiviral activity against both RNA and DNA viruses are potent inhibitors of EBO-Z replication (Huggins et al., 1995, 1999; Bray and Paragas, 2002). A single dose of one such compound, 3-deazaneplanocin A (c^3 -Npc A), prevented illness and death in mice infected with a mouse-adapted variant of EBO-Z (Bray et al., 2000). c^3 -Npc A and related substances inhibit a host cell enzyme, *S*-adenosylhomocysteine (SAH) hydrolase, which breaks down the SAH produced from *S*-adenosylmethionine (SAM) in the course of cellular methylation reactions (De Clercq, 1987, 1998; De Clercq et al., 1989). Inhibition of SAH hydrolase increases the intracellular SAH/SAM ratio, resulting in feedback inhibition of methylation (Cools and De Clercq, 1990). The antiviral activity of SAH hydrolase inhibitors has been attributed to diminished methylation of the 5' cap of viral messenger RNA by the viral (guanine-7-methyltransferase, which impairs translation of viral transcripts (Oxenrider et al., 1993).

The remarkable ability of a single dose of c^3 -Npc A to arrest a rapidly progressive, uniformly lethal Ebola virus infection in mice suggested to us that additional mechanisms of action might be contributing to the drug's protective effect. In support of this notion, we found that the efficacy of early c^3 -Npc A treatment could be eliminated

by co-administering antibodies to murine interferon alpha/beta (IFN- α/β), indicating that the drug was producing its effect by way of the type I IFN response (Bray, 2001). The synthesis and secretion of type I IFN by virus-infected cells and its induction of an 'antiviral state' in neighboring uninfected cells provide a barrier to early viral dissemination (Sen, 2001). To further investigate the relationship of c^3 -Npc A treatment to the type I IFN response, we measured the production of IFN- α in Ebola virus-infected mice treated with c^3 -Npc A or with placebo; we compared production of IFN- α to that of an unrelated chemokine marker of EBO-Z infection, and examined pathologic changes in the tissues of drug- and placebo-treated mice.

2. Materials and methods

2.1. Virus and antiviral compound

The adaptation of an isolate of EBO-Z from the 1976 outbreak to lethal virulence for adult, immunocompetent mice ('mouse-adapted EBO-Z') and the method of virus titration have been described (Bray et al., 1998). As reported in that paper, the virus is much more efficient at initiating infection in mice than in cell culture; its LD₅₀ when inoculated i.p. in mice is approximately 0.03 plaque-forming units (pfu), or approximately 1 virion. 3-deazaneplanocin A [(-)-9-[trans-2',trans-3'-dihydroxy-4'-hydroxymethyl-cyclopent-4'-enyl]-3-deazaadenine] (c^3 -Npc A) was provided by Dr John Driscoll, National Cancer Institute (NCI), Bethesda, MD. The compound was dissolved in phosphate-buffered saline (PBS) for injection.

2.2. Infection and antiviral therapy in mice.

All experiments were performed in biological safety level 4 facilities at USAMRIID. Adult female BALB/c mice were obtained from the NCI. The results of five experiments (representative of a series of 12) are presented here. In all of them, a cohort of 8–12-week-old mice was inoculated i.p. with 1000 pfu (30 000 LD₅₀) of mouse-adapted EBO-Z. Groups of infected mice were injected

once s.c. with 2 mg/kg of c³-Npc A, either 3, 2 or 1 day before infection (days -3, -2 or -1), or within 30 min after infection (day 0) or 1 or 2 days after infection (day 1 or 2). Negative control groups were treated with a placebo (PBS) on day 0. Uninfected control groups were injected with either the same dose of c³-Npc A on day 0, 1 or 2 or with PBS on day 0. Within each of these groups, a subgroup of 5 mice was held for daily observation of weight loss, illness, and death, while on each day postinfection, a group of 3–5 of the remaining mice were lethally exsanguinated. Their serum was collected and frozen at –70 °C until tested. Serum cytokine concentrations were determined for individual mice. Serum viral titers were determined for individual mice in all experiments except for those shown in Fig. 2, for which titrations were performed on pooled sera.

2.3. Cytokine assays

Serum cytokine concentrations were measured using commercial kits (for IFN- α : PBL Biomedical Laboratories, New Brunswick, NJ; for macrophage chemotactic protein-1 (MCP-1), interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α): Biosource International, Camarillo, CA). All samples were tested in duplicate. High-titer samples were diluted for repeat testing. Concentrations in pg/ml were determined from linear curve fits of the optical densities measured using serially diluted cytokine standards provided with the kits. For presentation on logarithmic plots, all readings of zero were re-assigned a value of 1.0 pg/ml.

2.4. Immunohistochemistry (IHC) and electron microscopy (EM)

Portions of liver and spleen were immersion-fixed for 30 days in 10% neutral-buffered formalin, then embedded in paraffin and sectioned onto glass slides. Sections were pretreated with Proteinase K (DAKO, Carpinteria, CA), blocked with commercially prepared peroxidase block and serum-free protein block (DAKO), then immunolabelled using rabbit anti-Ebola serum in a com-

mmercial immunoperoxidase system (DAKO EnVision + TM). Liver and spleen from uninfected mice, and the substitution of normal rabbit serum for rabbit anti-Ebola serum, served as negative controls. Small fragments of tissue were processed for EM by immersion-fixing for 1 h in 2% glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.4), rinsing in buffer, and post-fixing for 1–2 h in 1% osmium tetroxide in buffer. The material was then rinsed, stained with 0.5% uranyl acetate in ethanol, dehydrated in ethanol and propylene oxide, and embedded in PolyBed 812 resin for sectioning.

3. Results

3.1. Efficacy of pre- and postchallenge treatment

We previously reported that one s.c. injection of 1 mg/kg of c³-Npc A on day 0, 1 or 2 postinfection prevented death in mice infected with 10 pfu (300 LD₅₀) of mouse-adapted EBO-Z (Bray et al., 2000). No toxicity was observed with single doses of up to 10 mg/kg. In the present series of experiments, we inoculated mice with a 100-fold larger dose of virus (1000 pfu, 30 000 LD₅₀) and treated them with 2 mg/kg of c³-Npc A or with PBS as placebo. Again, no toxicity was observed; uninfected mice treated with this dose of drug gained weight as rapidly as naïve mice (not shown). We broadened our study to include single-dose treatment on day -3, -2 or -1; one of three such experiments is shown in Fig. 1. In all experiments, all infected, placebo-treated mice became ill (weight loss, ruffled fur, diminished activity) on day 3 and died by day 6 (Fig. 1A, Fig. 1B). Mice treated on day -3 or -2 showed a slightly slower rate of weight loss than placebo controls, and a few mice in these groups survived infection. Mice treated on day -1 underwent a period of weight loss, beginning on day 3–4, but nearly all survived. Treatment on day 0 or 1 prevented visible signs of illness, weight loss and death. In other experiments, mice treated on day 2 also went through a period of weight loss beginning on day 3, but most survived infection (see below).

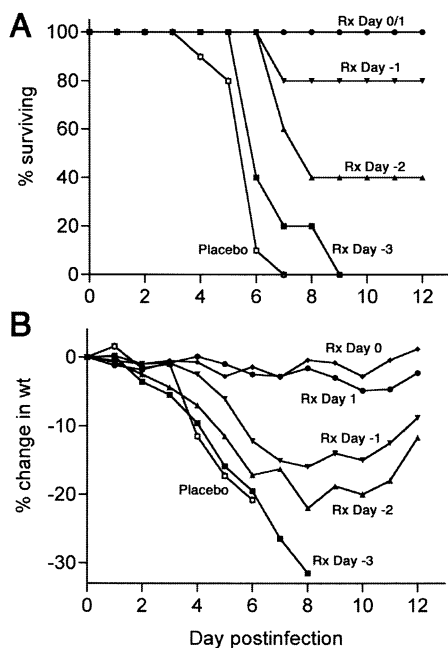


Fig. 1. Percent survival (A) and percent change in mean body weight (B) of groups of mice infected with mouse-adapted EBO-Z and treated on day -3, -2, -1, 0, or 1 with c^3 -Npc A, or with a placebo on day 0.

3.2. Viremia and cytokine responses in mice

In another experiment, treatment with c^3 -Npc A on day 1 was again highly protective; the weight gain of infected, treated mice did not differ significantly from that of uninfected, untreated ('naïve') mice (Fig. 2A). In this experiment, mice

treated on day 0 went through a period of weight loss, but all survived infection. As previously observed, serum viral titers of mice treated on day 0 or 1 were 10–100 000 times lower than placebo controls on days 3 and 4 (Fig. 2B; Bray et al., 2000). Serum IFN- α levels of placebo-treated mice did not become elevated until day 3, when all of the animals were already losing weight and were visibly ill (Fig. 2C). Mean values rose from baseline on day 2 to 147 pg/ml on day 3 and 182 pg/ml on day 4. Treatment with c^3 -Npc A caused IFN- α to appear earlier in the serum and to reach higher peak levels. Mice treated on day 0 showed a steady increase in the serum IFN- α level from baseline on day 0 to 18 pg/ml on day 1, 50 pg/ml on day 2 and 185 pg/ml on day 3 (Fig. 2C). In marked contrast, treatment on day 1 caused the IFN- α concentration to rise suddenly to a mean of 1300 pg/ml on day 2. The level remained elevated on day 3.

In another experiment, groups of infected or uninfected mice were treated with c^3 -Npc A or PBS on day 1 or 2 and exsanguinated on day 2 or 3. Treatment on day 1 reduced mean serum viral titers on day 2 by 100-fold and on day 3 by 100 000-fold, compared with placebo controls (Fig. 3A), and resulted in a mean serum IFN- α level of 1420 pg/ml on day 2 and 1830 pg/ml on day 3 (Fig. 3B). By contrast, infected placebo-treated mice had mean serum IFN- α levels of only 50 and 280 pg/ml on days 2 and 3, respectively. Treatment on day 2 reduced the serum viral titer on day 3 four-fold, with respect to placebo con-

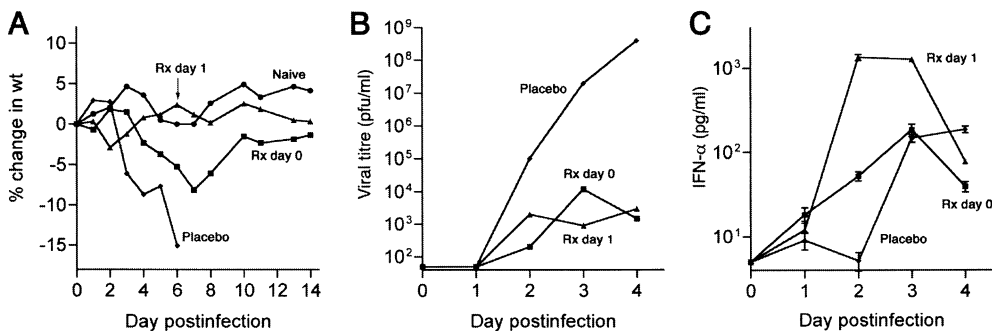


Fig. 2. Percent change in mean body weight (A), serum viral titers (B) and mean serum IFN- α concentrations (C) of groups of mice infected with mouse-adapted EBO-Z and treated on day 0 or 1 with c^3 -Npc A or with placebo on day 0. Bars, standard error (S.E.) of the mean (S.E.M.).

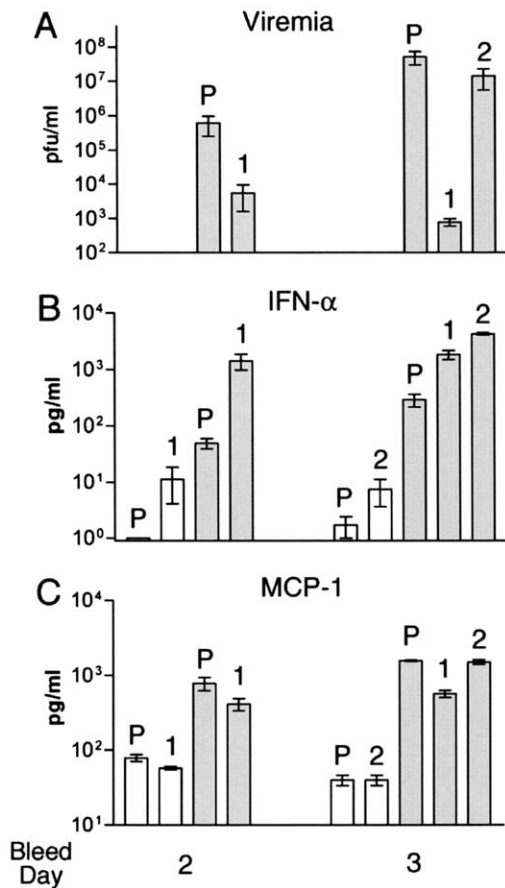


Fig. 3. Mean serum viral titers (A), serum IFN- α concentrations (B) and serum MCP-1 concentrations (C) on day 2 or 3 of groups of five mice infected with mouse-adapted EBO-Z (grey bars), or left uninfected (open bars), and treated either with a placebo on day 0 ('P') or with c^3 -Npc A on day 1 or 2, as indicated. Bars, S.E.M.

trols, but resulted in a striking elevation of the mean serum IFN- α level to 4300 pg/ml. No IFN- α was detected in the sera of 9 of 10 uninfected, PBS-treated mice on day 2 or 3; the one positive animal had a value of 5 pg/ml (Fig. 3B). Similarly, 5 of 10 uninfected, c^3 -Npc A-treated mice were negative on days 2 and 3; the other five had values ranging from 6 to 37 pg/ml.

In contrast to the marked increase in IFN- α induced by c^3 -Npc A in infected mice, the drug did not cause a rise in serum levels of MCP-1, compared with the placebo group (Fig. 3C). Treatment on day 1, in fact, resulted in a slight

decrease in the MCP-1 concentration from its baseline level on days 2 and 3. Mice treated on day 2 had MCP-1 levels similar to placebo controls on day 3. In all experiments, it was evident that the MCP-1 level increased or decreased in parallel with serum viral titers, and was thus a marker for the progression of infection. Treatment with c^3 -Npc A also did not increase the production of interferon-gamma (IFN- γ) or tumor necrosis factor-alpha (TNF- α) in infected mice (data not shown).

The effect of therapy on day 2 was further examined in an experiment in which groups of 3 mice were exsanguinated daily through day 10. Infected, drug-treated mice initially lost weight, but began to recover on day 8; 80% of them survived infection (Fig. 4A). Placebo- and drug-treated mice had similar viral titers on day 3, but the titers of the placebo controls continued to rise on day 4, exceeding 10^{10} pfu/ml on day 5 (Fig. 4B). Viral titers in treated mice began to decline on day 4 and were negative by day 8. Infected, treated mice had a mean serum IFN- α concentration of 4360 pg/ml on day 3, compared with only 58 pg/ml for placebo-treated infected controls (Fig. 4C). The serum IFN- α level of the treated mice fell rapidly and was undetectable by day 6. No IFN- α was detected in the sera of any of the uninfected, drug-treated mice.

3.3. Pathology, immunohistochemistry and electron microscopy

We examined the effect of c^3 -Npc A therapy on the progression of infection in major target organs by killing drug- or placebo-treated mice on day 4 and examining the liver and spleen. Routine light microscopic studies of placebo-treated mice revealed lysis of MPS cells throughout marginal zones of the spleen, infection of Kupffer cells throughout the liver and multifocal hepatocellular necrosis, as previously described (Gibb et al., 2001). By contrast, mice treated on day 0 or 1 showed minimal evidence of injury to the liver or spleen, while those treated on day 2 showed lysis of MPS cells within the marginal zones of the spleen and occasional foci of hepatocellular necrosis.

Immunohistochemistry studies revealed striking differences in the quantity and distribution of EBO-Z antigen between placebo- and drug-treated mice. The liver of placebo-treated mice contained large amounts of viral antigen in great numbers of hepatocytes and in nearly all Kupffer cells in every field examined (Fig. 5A). By contrast, mice treated on day 0 showed only small amounts of antigen in scattered Kupffer cells (Fig. 5B). The same pattern was seen in mice treated on day 1. Larger amounts of antigen were observed in Kupffer cells of mice treated on day 2, but only occasional positive hepatocytes were found (Fig. 5C). Treatment on day 0 or 1 also markedly reduced the amount of viral antigen in splenic MPS cells (not shown). Little difference in the quantity of antigen was noted between placebo- and day-2-treated mice.

EM studies revealed typical EBO-Z inclusions in hepatocytes and Kupffer cells of placebo-treated mice (not shown) (Geisbert and Jahrling, 1995). Such inclusions were very rare in mice treated on day 0 or 1. In mice treated on day 2, evidence of viral infection of the liver was confined to Kupffer cells, and no inclusions were observed in hepatocytes, reflecting the rarity of antigen-positive cells in IHC studies. The morphologic features of viral inclusion bodies, budding virus and free virions all were normal in c^3 -Npc A-treated mice.

4. Discussion

The ability of c^3 -Npc A to induce massively increased production of IFN- α in virus-infected animals has not previously been observed. The finding has confirmed our suspicion that additional mechanisms of action, besides impaired methylation of viral mRNA, contribute to the drug's potent antiviral effect. In support of the important role of IFN- α in preventing disseminated EBO-Z infection in mice, we have also found that the administration of murine leukocyte IFN or the IFN-inducer polyICLC is partially protective, and that treatment with the chimeric B/D form of recombinant human IFN- α , which has cross-species activity in mice, can prevent the development of viremia, illness and death (M Bray et al., manuscript in preparation).

Other studies of EBO-Z pathogenesis have demonstrated the decisive role of the type I IFN response in determining the outcome of EBO-Z infection in mice (Bray, 2001; Bray and Paragas, 2002). Normal adult mice do not become ill after inoculation of filoviruses that have not been 'adapted' to mice through sequential passage. However, both knockout mice lacking a functional type I response and normal mice treated with antibodies to murine IFN- α/β develop rapidly lethal infection after inoculation of non-mouse-adapted EBO-Z, EBO Sudan and Marburg

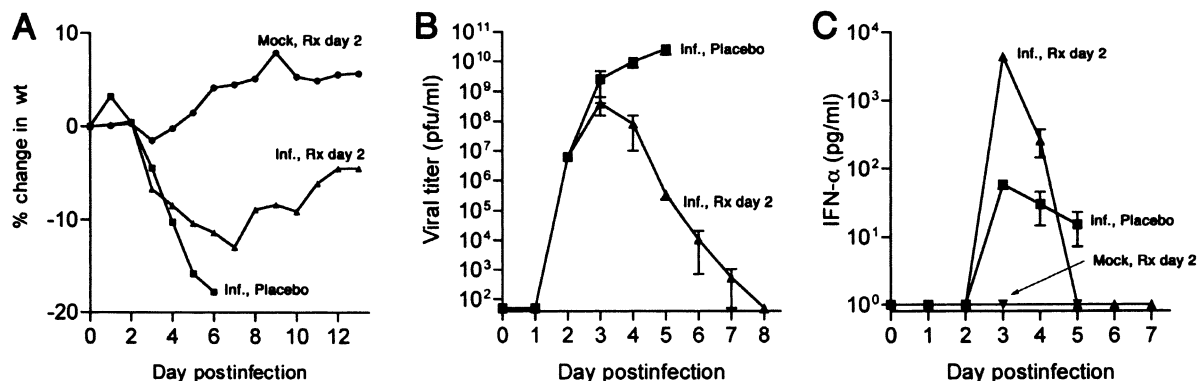


Fig. 4. Percent change in mean body weight (A), circulating serum viral titer (B) and serum IFN- α level (C) of groups of mice infected with mouse-adapted EBO-Z and treated with c^3 -Npc A on day 2. Bars, S.E.M.

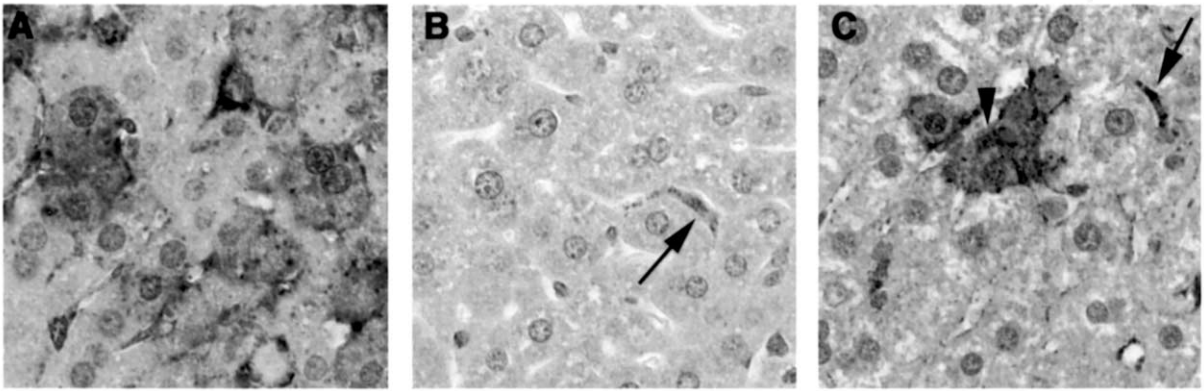


Fig. 5. Immunohistochemical staining of EBO-Z viral antigen in the liver of mice killed on day 4 $\times$ 60. A. Placebo-treated. Large numbers of hepatocytes and Kupffer cells contain viral antigen. B. Treated with c^3 -Npc A on day 0. Antigen is present within an occasional Kupffer cell (arrow), but not in hepatocytes. C. Treated on day 2. Antigen is present in Kupffer cells (arrow) and occasional hepatocytes (arrowhead).

viruses. The nature of the interactions between filoviruses and innate antiviral responses in primates are less well understood, but there is evidence that the EBO-Z VP35 protein is able to suppress production of Type I IFN in infected cells (Basler et al., 2000). Reversal of such virus-induced suppression is a potentially rewarding goal of antiviral therapy.

We earlier noted that the effect of c^3 -Npc A treatment on day 0 or 1, but not on day 2, could be blocked by injecting the mice with anti-IFN- α/β antibodies (Bray, 2001). It is clear that the outcome of treatment with a combination of c^3 -Npc A and anti-IFN antibodies is determined by a dynamic relationship among viral replication, innate antiviral responses, the effect of the drug and the timing and quantity of the antibodies. In the experiments previously reported, it appears that the IFN- α induced by c^3 -Npc A treatment on day 0 or 1 was completely neutralized by the quantity of antibodies administered, but the very much larger amount of IFN induced by treatment on day 2 was not entirely blocked.

Pathology observations of the quantity and distribution of EBO-Z antigen and viral inclusion bodies in tissues are consistent with a scenario in which IFN- α induced by c^3 -Npc A therapy results in the induction of an 'antiviral state' in uninfected cells, preventing further viral dissemination. Earlier studies in BALB/c mice infected with

mouse-adapted virus showed that viral replication cannot be detected in tissues on day 1 postinfection, but can be found in a moderate number of MPS cells of the lymph nodes and the spleen on day 2 (Gibb et al., 2001). By day 3, innumerable MPS cells in lymphoid tissues and the liver are infected, as are large number of hepatocytes. In the present study, treatment with c^3 -Npc A on day 0 or 1 drastically limited the progression of infection, so that only a few scattered MPS cells were positive on day 4. Treatment on day 2 permitted the virus to disseminate to a large number of MPS cells, but prevented its further spread to hepatocytes.

Two types of results indicate that c^3 -Npc A induces massively increased IFN- α production by virus-infected cells, but little or no IFN production by uninfected cells. First, no IFN- α was detected in the serum of the majority of uninfected, drug-treated mice over several days post-treatment. In the experiment shown in Fig. 3, 5 of 10 mice had very low levels of IFN on day 2 or 3, while in the experiment shown in Fig. 4, no IFN was detected in the serum of any uninfected, drug-treated mouse on any day post-treatment. These results indicate that c^3 -Npc A induces, at most, a very small amount of IFN- α production in uninfected cells. Second, the magnitude of the peak IFN- α level increased markedly when treatment was deferred from day 0 to day 1 and to day

2, by which time the virus had disseminated to a very large number of cells. It thus appears that, unlike such IFN-inducers as polyICLC, which stimulates IFN- α production in both uninfected and infected cells, c³-Npc A induces massive IFN- α production only in virus-infected cells. The mechanism by which the compound produces this effect is not known. The fact that treatment 24 h before infection was protective is a significant finding in this regard. Since c³-Npc A has been shown to have a serum half-life in mice of only 17 min, and a tissue half-life of a few hours (Coulombe et al., 1995), it appears that the drug itself does not need to be present at the time of infection. Rather, exposure to c³-Npc A causes a long-lasting intracellular change. If this modification results from the inhibition of SAH hydrolase, it may mean that EBO-Z is only able to suppress innate antiviral responses if a cellular target molecule is methylated.

No previous report has described an effect of adenosine analogs on innate antiviral responses. However, a number of investigators have described alterations of other aspects of immune system function, suggesting that the intracellular change(s) induced by c³-Npc A have multiple effects. Most changes are immunosuppressive in nature; some may be mediated through changes in NF- κ B activity (Jeong et al., 1999). Thus, several compounds reduced the production of TNF- α by endotoxin-treated macrophages and protected mice against endotoxin challenge (Parmely et al., 1993; Jeong et al., 1996). Adenosine analogs also inhibited the activation of T cells and decreased the phagocytic activity of macrophages (Sung and Silverstein, 1985; Wolos et al., 1993a,b; Lambert et al., 1995). Some studies have shown a positive correlation between the SAH/SAM ratio and changes in a given cellular function, but none has yet identified the mechanism by which the drugs produce their effect.

In an initial study, we have found that treatment with c³-Npc A does not appear to cause increased production of IFN- α in nonhuman primates infected with wild-type EBO-Z (data not shown). This divergence in drug effect between mice and primates may be related to a difference in the ability of mouse-adapted and wild-type

Ebola viruses to suppress innate antiviral responses (Bray, 2001; Bray and Paragas, 2002). Additional experiments will include an examination of the effect of c³-Npc on the replication of mouse-adapted EBO-Z in nonhuman primates (Bray et al., 2001). It is essential to understand these basic virus–host relationships in order to design effective therapies (Garcia-Sastre, 2001; Levy and Garcia-Sastre, 2001). Even a partial reversal of suppression of innate antiviral responses in infected cells could tip the balance in favor of the host, by providing additional time for the mobilization of antigen-specific immune responses. Such a form of therapy would be particularly useful for treating persons accidentally exposed to a filovirus in the laboratory or during an outbreak. Further research aims to examine and compare interactions between filoviruses, innate antiviral responses and SAH hydrolase inhibitors at the molecular level in mice and primates, in order to find effective approaches to the therapy of human filovirus infections. We hope that the discovery of this additional mechanism of action of SAH hydrolase inhibitors will also lead to progress in the therapy of other human viral infections.

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