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Evaluation of the efficacy of 2′,3′-dideoxycytidine against adenovirus infection in a mouse pneumonia model

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Abstract

The antiviral activity of 2′,3′-dideoxycytidine (ddC) has been investigated in a mouse pneumonia model. Consolidation of lung, histopathological changes, DNA synthesis as well as levels of TNFα were assayed. In this in vivo model, the oral administration of ddC twice daily over 4 days, displayed an inhibitory effect. The drug significantly reduced histopathologic responses. Analysis indicated that under treatment pulmonary lesions were less severe than those of untreated controls. These data confirm the in vitro activity of ddC against adenovirus. Thus, ddC represents a potential therapeutic approach for inhibiting adenovirus infection and may offer promise as an anti-adenovirus agent for immunocompromised patients in whom serious adenovirus infection may prove fatal.

Keywords: Adenovirus; Antiviral; ddC; Pneumonia model in mouse

1. Introduction

Adenoviruses (Ad) are responsible for a wide range of clinical diseases worldwide. Infection by adenovirus results in significant morbidity and mortality in both the immunocompetent and immunosuppressed host. Pneumonia and hemorrhagic cystitis are among the most severe complications of adenovirus infection (Hierholzer, 1992; Zarraga et al., 1992; Childs et al., 1998).

Only some compounds were found to be effective against adenovirus. Among them is (*S*)-1-(3-hydroxy-2-phosphonomethoxypropyl)cytosine (HPMPC, cidofovir) with a broad-spectrum antiviral therapeutic potential (De Clercq, 1993; Hui et al., 1994; De Clercq, 1996; De Oliviera et al., 1996; Gordon et al., 1996). No effective antiviral drug for adenovirus infections has been licensed.

Recently we reported that 3′-fluoro-2′-deoxythymidine (FTdR) and 2′,3′-dideoxycytidine (ddC) are the most effective inhibitors of viral replication in FL cells against adenovirus of serotypes 2 and 3 (Mentel et al., 1996a, 1997). Additionally we have shown that these compounds inhibit the adenovirus polymerase and, consequently, possess anti-adenovirus effects and are worthy of further evaluations (Mentel et al.,

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1999). It is difficult to evaluate the anti-adenovirus effects of chemicals in vivo. For topical application ocular animal models in rabbits and cotton rats were established (Tsai et al., 1992; Gordon et al., 1992).

Semipermissive infections of Ad2 in mouse cells were demonstrated. Mouse cells were able to express 72K DNA binding protein, and variable levels of other early proteins, and to replicate viral DNA (Eggerding and Pierce, 1986). Cotton rats and mice have been used to investigate the molecular pathogenesis of adenovirus pneumonia (Ginsberg et al., 1991; Berencsi et al., 1994). Because only early genes are required to produce the disease we used the murine pneumonia model as an alternative to cotton rats which are more difficult to obtain.

This report describes the effects of ddC on the course of adenovirus-induced pneumonia in a mouse model. We found that oral treatment with ddC led to a reduction of events associated with lung inflammation when compared to the control group.

2. Material and methods

2.1. Cells and virus

Monolayer cultures of Fogh and Lund cells (FL cells), American Type Culture Collection (ATCC) CCL 62, were grown in Eagle's minimal essential medium (MEM) containing 7% heat-inactivated fetal calf serum (FCS). L 929 cells were grown in RPMI 1640 medium with 10% FCS. The prototype strain ADV2 (Ad 6: ATCC) was propagated in FL cells using Eagle's MEM supplemented with 2% FCS. Fluorescent focus assay (FFA) was used to quantitate the virus in viral stocks and in lung homogenates as described previously (Liebermann and Mentel, 1994).

2.2. Drug

2′,3′-Dideoxycytidine (ddC) was provided by Sigma-Aldrich Chemie, Taufkirchen. It was prepared in sterile physiological buffered saline solution (PBS) for animal experiments.

2.3. Mice

Female BALB/c mice weighing 18–21 g were obtained from the Institute of Pathophysiology, Department for Experimental Animals in Karlsburg (Germany).

2.4. Animal studies

Mice were anesthetized by intraperitoneal injection of a mixture of xylazinhydrochloride (Rompun 2%) and ketaminhydrochloride (Velonarcon 0.1) and then exposed to 50 μl of virus suspension containing 10^9 focus forming units (FFU) by intranasal instillation. Treatment with ddC was performed by a twice daily peroral administration of the drug. In each drug-treated and/or infected group 10 mice were examined. The placebo group got PBS instead of the drug. Three mice were used in the toxicity control group. Mice were sacrificed at various times postinfection by cervical dislocation to analyze lesions in the lungs and assess the cytokine response.

2.5. Lung pathology

Lungs were prepared at 24, 48, 72 and 96 h postinfection and examined for tissue consolidation by scoring on a scale ranging from 0 (normal) to 4 (maximal consolidation). For histopathological examination lungs were placed immediately into Bouin's solution. Following fixation, lungs were embedded in paraffin, thin sectioned, and stained with hematoxylin and eosin. The extent of the inflammatory perivascular and peribronchiolar responses was also scored. Three histological sections of each lung were analyzed by two observers without knowledge of the treatment group or the time of sacrifice (i.e. scored blind). Sections devoid of inflammatory response received a score of zero, and sections with abundant infiltration of lymphocytes a score of four.

2.6. Viral assay

Lungs were removed aseptically, rinsed with PBS, and frozen at -20°C. For titrations, lungs

were thawed, minced and homogenized to a 10% suspension by sonication (200 W, 10 s). Varying 10-fold dilutions of this suspension were assayed for infectious virus by overlaying FL cells for 60 min at 37°C and checking the FL cells in a FFA after incubation for 48 h as described (Mentel et al., 1996a).

2.7. PCR assay

Adenovirus DNA was detected by nested PCR assay using hexon-oligonucleotide primers. Nucleotide sequences of the primers for the first run of amplification were: H1: 5′GCC GCA GTG GTC TTA CAT GCA CAT C 3′ and H2: 5′CAG CAC GCC GCG GAT GTC AAA GT 3′. The nucleotide sequence of primers for the second PCR were: H3: 5′CCA CCG AGA CGT ACT TCA GCC 3′ and H4: 5′CAC ACG GTT GTC ACC CAC AGC 3′ (Allard et al., 1990).

DNA was extracted from lung tissue using phenol-chloroform extraction. Samples were mixed with PCR buffer, nucleotides including two amplification primers and Taq polymerase, and incubated in a thermocycler. Thirty amplification cycles were carried out in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) starting with of 94°C for 45 s, followed by 55°C for 90 s and 72°C for 90 s. The second PCR was performed under the same conditions. The final amplification products were analyzed on a 3% agarose gel containing ethidium bromide under UV light, and their molecular weight determined, which was calculated for the first PCR being 300 bp and for the second PCR 178 bp, respectively. The limit of detection of adenovirus DNA in this assay was 10^3 mol/ml.

2.8. Cytokine bioactivity assay

Lungs were rapidly removed, frozen immediately in liquid nitrogen and stored until homogenized in 5 vol of 155 mM NaCl at 4°C by sonication. After two centrifugation cycles at 4°C for 30 min, each at $3200 \times g$ and $18\,000 \times g$, respectively, the cell-free supernatant was stored at −70°C. Blood was obtained by puncture of the retroorbital complex and the serum was stored at −70°C.

The TNF α bioactivities in the lungs and serum were measured in a 24 h bioassay that measures cytolysis of actinomycin D-sensitized L-929 murine fibroblasts. L-929 cells were seeded in 96-well microplates at a concentration of 5×10^5 cells/ml in 100 μl RPMI with 10% FCS and incubated for 4 h at 37°C in 5% CO_2. Subsequently 50 μl actinomycin D and 50 μl of sample per well were added in duplicate wells. A recombinant murine TNFα (Biosource, Camarillo, CA) was used as standard. After 20 h incubation at 37°C cytolysis of cells was determined by staining with 50 μl crystal violet for 20 min at room temperature, adding a mixture of 50 μl of 0.1 M KH_2PO_4 and ethanol (1:1) and reading the plates at a wavelength of 550 nm and a reference wavelength of 690 nm.

2.9. Statistical evaluations

Comparisons among groups for histopathology and DNA detection were evaluated by Fisher Exact Test. Lung score reductions were analyzed using the Mann–Whitney-Wilcoxon-test. Differences in lung virus titers were compared with control values using Student's *t*-test.

3. Results

3.1. Effect of orally administered ddC treatment on lung inflammation parameters

The inflammatory response after infection with adenovirus (10^9 FFU) consisted of lymphocytic perivascular and peribronchiolar infiltrations and was observed at 48–96 h after infection. At this time all animals developed histopathological changes of various severity degrees (Fig. 1A, 1B). The pneumonia was not lethal to the mice. The doses of ddC (50 and 75 mg/kg) were not associated with toxicity (Fig. 1C). Animals treated with ddC demonstrated a significant reduction of signs and symptoms of pneumonia after adenovirus infection. Fig. 1D demonstrates an example of a lung of a ddC-treated mouse: it did not reveal histological evidence of inflammation.

Data of overall score of histopathological changes and consolidation of lung in mice after treatment with ddC are summarized in Table 1. After 48 h of infection, ddC treatment (75 mg/kg) reduced the number of animals with measurable pathologic signs by about 50%. Treatment of intranasal adenovirus inoculation of mice with ddC at two doses led to reduction of the number of animals developing signs of pneumonia compared to the placebo control. Therapy with 75 mg/kg resulted in a statistically significant reduction of 60% of animals developing pneumonia in this group, while a dose of 50 mg/kg of ddC concentration resulted in 14.3% prevention of pneumonia (Fig. 2). Lungs of animals were scored according to the extent of consolidation, depending on the percentage of the lung lesions exhibiting typical coloration. Animals treated with ddC showed a reduced lung tissue consolidation. A score of 0.4 was seen after 72 h, while a score of 2.6 was observed at the same time without treatment (Table 1). Furthermore, the lesions after histopathological examination were scored on a scale of 0–4. Animals treated with ddC (75 mg/kg) showed a reduced score of 0.33. In the untreated group, a mean score of 1.66 was

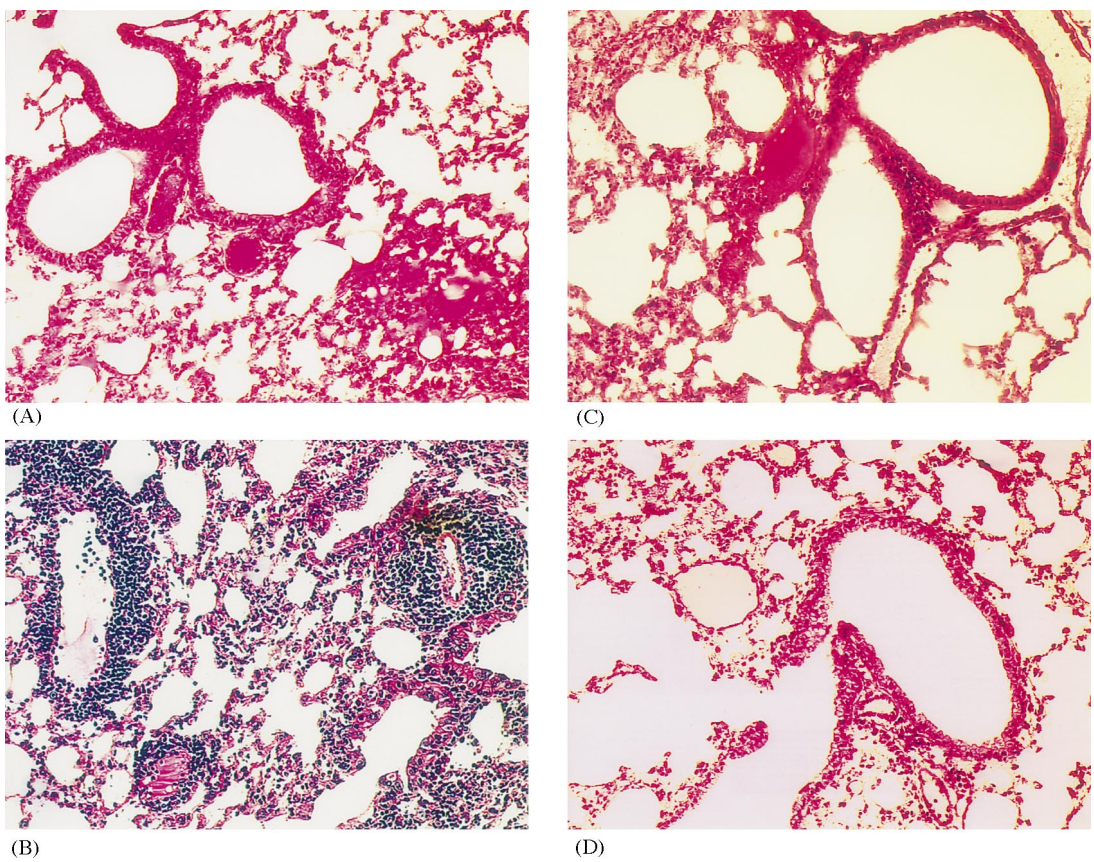

Fig. 1. Effect of orally administered ddC (75 mg/kg) on the histopathologic lesions in lung tissue from mice infected with adenovirus, evaluated for pathological changes 4 days p.i. in hematoxylin-and eosin-stained sections from lungs of mice. (Magnification × 100) (A) without infection and treatment; (B) following intranasal infection with Ad2 (10^9 FFU); (C) following treatment with ddC; and (D) following infection and ddC treatment. In untreated mice abundant invasion of lymphocytes in alveolar membranes and the perivascular and peribronchiolar area was seen (panel B).

Table 1
Effect of oral treatment[a] with ddC on adenovirus type 2 infection in mice

Time after intranasal infection (h)	Consolidation of lung mean score ± SD		Histopathology number of animals with lymphocytic infiltration/total		Adenovirus titer in lung tissue Log_{10}/g		DNA[b] detection number of animals with adenovirus DNA/total		TNFα (pg/ml)	
	treated	untreated	treated	untreated	treated	untreated	treated	untreated	treated	untreated
24	0 ± 0	0 ± 0	1/3	3/3	4.0 ± 0.4	4.0 ± 0.2	2/2	2/2	31.6	104.7
48	0 ± 0	1.4 ± 0.9	4/10[d]	9/10	3.6 ± 0.3	4.0 ± 0.4	1/4	3/3	<5	<5
72	0.4 ± 0.5[c]	2.6 ± 0.5	7/10	10/10	2.5 ± 0.2[f]	4.1 ± 0.5	1/3	4/4	<5	<5
96	0.6 ± 0.9	2.4 ± 0.8	4/10[e]	10/10	1.7 ± 0.2[g]	2.5 ± 0.3	1/3	3/3	<5	<5

[a] Twice daily treatment schedule with 75 mg/kg ddC.
[b] PCR result.
[c] Differences from control $P<0.01$ by Mann–Whitney-Wilcoxon-Test.
[d] $P=0.0286$.
[e] $P=0.0054$ by Fisher Exact Test.
[f] $P<0.01$.
[g] $P<0.05$ by t-Test.

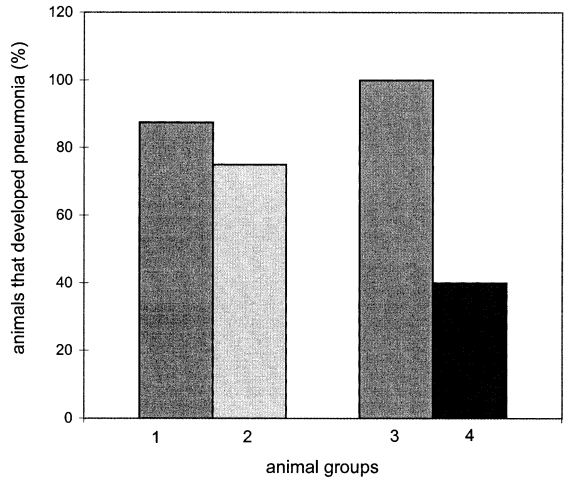

Fig. 2. Effect of oral treatment with different concentrations of ddC after adenovirus type 2 infection. Lung histopathology was scored at 96 h after intranasal infection. Development of pneumonia was scored in comparison with the group receiving placebo (score of eight mice in groups 1 and 2 and score of 10 mice in groups 3 and 4). (1, 3) placebo group; (2) treatment with ddC at 50 mg/kg; and (4) treatment with ddC at 75 mg/kg.

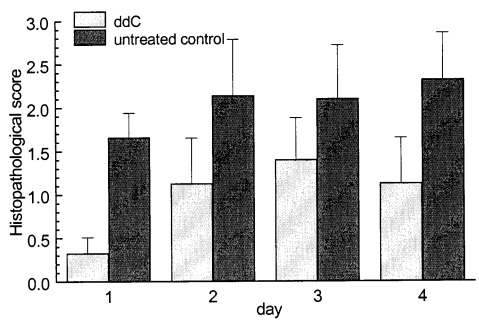

Fig. 3. Influence of treatment with ddC (75 mg/kg) on development of adenovirus induced pneumonia. Mice were intranasally infected with Ad2 and evaluated for pulmonary lesions. Mean histopathological scores from three (24 h p.i.) and 10 animals (48, 72, 96 h p.i.), respectively, are shown. Error bars represent standard deviation.

determined 24 h post infection. The ddC treated group showed scores up to 50% below the value of the placebo group (Fig. 3). The differences in histopathological scores were not significant.

3.2. Virus concentration in the lung

The initial viral titer declined from a level of approximately 4.0 $\log_{10}$ on day 1 after infection to 2.5 $\log_{10}$ on day 4 in the placebo treated controls (Table 1). Treatment with ddC influenced this decline. We observed a significant difference in virus clearance on day 3 and 4. Animals treated with ddC had an Ad titer of 1.7 $\log_{10}$ on day 4.

In lung tissue of infected mice the presence of adenovirus DNA was tested by PCR. In the untreated group DNA was detected in all animals at 24, 48, 72 and 96 h after infection. The treatment was associated with reduction in the number of animals with a positive PCR.

At 48 h after infection in only one of four animals DNA could be detected. In animals which were not infected (and not treated with ddC) adenovirus DNA was not detected under the same conditions (data not shown).

3.3. Effect of ddC treatment on TNF production

TNFα was assayed in lung homogenates and plasma by a cytokine bioactivity method. Specimens from uninfected animals were also assayed. TNFα was not detectable in the peripheral blood of the infected mice (data not shown). In the infected lungs, TNFα was determined at 24 h after infection. Upon treatment with 75 mg/kg ddC, the TNF production was clearly reduced with concentrations in lung tissue of 104 pg/ml in untreated as compared to 31.6 pg/ml for treated mice (Table 1). This suggests an influence of ddC on the development of the early phase of inflammation.

4. Discussion

The observation that several sugar-modified nucleoside analogues have potent antiviral activity against adenovirus in vitro led us to investigate ddC in a murine pneumonia model. The substance ddC was selected for this study because of its prominent in vitro capacity against replication of adenoviruses type 2 and 3 (Mentel et al., 1997). Furthermore, ddC is a licensed drug for the treat-

ment of HIV infection and has a potent inhibitory effect on replication of duck hepatitis B virus (De Clercq, 1998; Kassianides et al., 1989).

The results presented here show that ddC treatment of adenovirus infection reduced the number of animals showing lung lesions after infection. The reductions in the inflammatory response in treated animals correlated with a suppression of TNFα synthesis.

TNFα is induced during Ad infections and plays an important role in the early phase of the pathogenic process. It interacts in a cytokine network that produces adherence of lymphocytes to endothelial cells to permit their transport into the lungs. Macrophages liberate the inflammatory mediators from Ad-infected bronchiolar epithelial cells. These mediators include leukotrienes that act as chemoattractants and prostaglandins that increase vascular permeability (Ginsberg et al., 1991; Ginsberg and Prince, 1994; Sparer et al., 1996). The amount of TNFα in the ddC treated groups was reduced threefold. The histopathological lung score was also reduced two- to threefold as compared with placebo-treated control animals. Oral application of ddC (75 mg/kg twice daily) did not only significantly reduce histopathologic visible lesions in the lungs, but also the extent of lung consolidation.

Ad clearance from the lung in the presence of ddC was higher than that of untreated controls, with 1.6 and 0.8 log lower virus yields at 72–96 h after infection (Table 1). These observations were also supported by DNA data using polymerase chain reaction (Table 1). This suggests that the virus inoculum was sufficient in these experiments for early gene expression. Only early gene expression is required to produce pneumonia. In in situ hybridization studies Berencsi et al. (1994) estimated strong signals in the mouse epithelial cells: these signals demonstrated the susceptibility of these cells to early and/or late Ad virus gene expression. Our results with Ad DNA in the mouse lung may indicate abortive infection in mice. Abortive infection may arise from an early block, any early step including DNA replication, or a late block, any step after the onset of DNA replication (Lucher, 1995).

The histopathological alterations of the lung alveolar structure in the examined smears appear to be a sensitive indicator for monitoring the effect of ddC in the adenovirus murine pneumonia model. The development of pneumonia depends on the amount of the infectious dose. In former studies for development of animal models for testing antiviral drugs using $10^{4.5}$ FFU of Ad5 the feasibility using hamsters as a model for adenovirus-induced respiratory infection was demonstrated. We did not observe histopathologic changes in mice under the same experimental conditions (Mentel et al., 1993). In the present experiments in BALB/c mice all animals showed signs of inflammation when 10^9 FFU were inoculated. The histopathologically visible lesions following Ad2 infection resembled those previously described in hamsters after intranasal inoculation of Ad5. Both Ad5 and Ad2 belong to group C. The fiber of Ad, located at the 12 vertices of the viral icosahedron, is responsible for the specific binding of virions to the cell receptor and determines the tissue tropism. The fiber knob causes the first step in the interaction of Ad with cell membrane receptor. Antisera against the fiber knob possess virus neutralization capacity (Liebermann et al., 1996, 1998).

Sparer et al. (1996) demonstrated that the induction of pulmonary pathology requires the expression of viral genes subsequent to infection. When mice were inoculated with UV-inactivated virus the histopathological score resembled that of noninfected mice. Therefore, the authors concluded that the observed pneumonia was not due to the toxic effect of input virion proteins.

Despite profound knowledge of the molecular biology of adenovirus, no specific antiviral substance has been licensed for treatment in adenovirus infections. This is in contrast with clinical use of nucleoside analogues in herpes simplex virus and human immunodeficiency virus infections.

The search for antiviral compounds has led to some promising compounds against adenovirus infection. Among them are (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA] and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine [(*S*)-HPMPC], two acyclic nucleoside

phosphonates (De Clercq et al., 1987; Baba et al., 1987; Gordon et al., 1991). In a New Zealand rabbit ocular model, high antiviral activity of Cidofovir (HPMPC, GS-504) was demonstrated. Topical application led to suppression of viral replication and decreased duration of virus shedding (Gordon et al., 1996). The efficacy of this compound in systemic adenovirus infections, i.e. adenovirus bronchopneumonia in humans, has not been reported.

The mechanism of action of ddC is the inhibition of Ad viral polymerase and, in particularly, reverse transcriptase (Mentel et al., 1999; De Clercq, 1998). The compound is well absorbed orally and is generally well tolerated. In humans the dose limiting toxicity has been a peripheral neuropathy that can be significantly reduced by lowering the dose (Kassianides et al., 1989). The toxicity profile of ddC differs substantially from that of zidovudine, which induces bone marrow suppression (Hirsch and D'Aquila, 1993). Embryonic cytotoxicity of ddC was also evaluated; no effect was seen with ddC or ddI at concentrations up to 100 μM. Cytotoxic effects were significantly less compared to zidovudine at equivalent concentration (Toltzis et al., 1994). This finding is in agreement with our previous observations (Mentel et al., 1997). Pharmacokinetic studies have demonstrated that oral absorption of ddC is rapid, with relatively high tissue levels in kidney, liver, pancreas and lungs of mice (Kelley et al., 1987).

The ability of Ad to establish persistent infections has been also recognized. Lymphocytes can interact with target cells for Ad2 (Mentel et al., 1996b). Like cytomegalovirus latent adenovirus infection is thought to be reactivated under some conditions. Disseminated adenovirus disease with multiorgan involvement ocurred in immunocompromised and immunocompetent children (Munoz et al., 1998). Therefore a specific antiviral agent would be an invaluable addition to the current non-specific therapeutic options. The ability of ddC to inhibit in vivo infection induced by adenovirus provides strong evidence for the potential therapeutic efficacy of ddC in the treatment of human adenovirus infection.

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