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Potentiating effect of ribavirin on the anti-retrovirus activity of 3′-azido-2,6-diaminopurine-2′,3′-dideoxyriboside in vitro and in vivo

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Summary

3′-Azido-2,6-diaminopurine-2′,3′-dideoxyriboside (AzddDAPR) is a potent and selective inhibitor of human immunodeficiency virus (HIV) replication in vitro. It also inhibits Moloney murine sarcoma virus (MSV)-induced transformation of murine C3H/3T3 embryo fibroblasts. AzddDAPR causes a marked dose-dependent suppression of MSV-induced tumor formation and mortality therewith associated in newborn mice infected with MSV. Combination of AzddDAPR with ribavirin resulted in a marked potentiation of its anti-retrovirus activity in vitro and a significant enhancement of its inhibitory effect on MSV-induced tumor formation in vivo. A slight increase in the in vivo toxicity of AzddDAPR was noted when combined with ribavirin.

3′-Azido-2,6-diaminopurine-2′,3′-dideoxyriboside (AzddDAPR); Ribavirin; 2′,3′-Dideoxynucleoside; Human immunodeficiency virus (HIV); Moloney murine sarcoma virus (MSV)

Introduction

Since the identification of human immunodeficiency virus (HIV) as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983; Gallo et al., 1984), various 2′,3′-dideoxynucleoside analogues have been synthesized for evaluation of their inhibitory effect on HIV replication. Introduction of a 3′-azido group in the sugar moiety of 2′,3′-dideoxynucleosides has yielded

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a number of novel molecules which are endowed with marked anti-retrovirus activity in vitro (Mitsuya et al., 1985; Baba et al., 1987a; Herdewijn et al., 1987a; Balzarini et al., 1988a,b). For example, when evaluated for their inhibitory effect on HIV-induced cytopathogenicity in MT-4 cells, the 3′-azido-substituted 3′-azido-2′,3′-dideoxythymidine (AzddThd, AZT), 3′-azido-2′,3′-dideoxyuridine (AzddUrd), 3′-azido-2′,3′-dideoxyguanosine (AzddGuo) and 3′-azido-2,6-diaminopurine-2′,3′-dideoxyriboside (AzddDAPR) proved 1500-, 600-, 5.4- and 12-fold more effective as anti-HIV agents than the corresponding unsubstituted 2′,3′-dideoxynucleoside counterparts. In contrast, 3′-azido-2′,3′-dideoxycytidine (AzddCyd) and 3′-azido-2′,3′-dideoxyadenosine (AzddAdo) were markedly less active against HIV than ddCyd and ddAdo, respectively (Herdewijn et al., 1987b; Balzarini et al., 1988a). The 50% effective doses (ED_{50}) of AzddThd, AzddUrd, AzddGuo and AzddDAPR against HIV replication in MT-4 cells are 0.004, 0.36, 2.8 and 0.3 μM, respectively. When evaluated for their inhibitory effect on Moloney murine sarcoma virus (MSV)-induced transformation of murine C3H/3T3 embryo fibroblasts, ED_{50} values of 0.02, 15, 6.2 and 1.0 μM were recorded. Thus, AzddDAPR proved to be a promising anti-HIV compound (Balzarini et al., 1988b) and it seemed imperative to further examine AzddDAPR for its efficacy against retrovirus replication. Since ribavirin enhances the anti-HIV activity of purine 2′,3′-dideoxynucleosides (i.e. ddAdo and ddGuo) in vitro, while antagonizing the anti-HIV effects of the pyrimidine 2′,3′-dideoxynucleosides AzddThd and ddCyd (Baba et al., 1987b), it also seemed worth investigating whether ribavirin might potentiate the anti-retrovirus activity of AzddDAPR. We have now examined the effect of ribavirin on the anti-MSV activity of AzddDAPR in vitro. In addition, the combination of ribavirin with AzddDAPR was also evaluated in newborn NMRI mice infected with MSV. We found that AzddDAPR inhibited MSV-induced cell transformation in vitro and MSV-induced tumor formation in vivo. Ribavirin enhanced the anti-retrovirus activity of AzddDAPR both in vitro and in vivo. Our data provide an experimental basis for the combined use of purine 2′,3′-dideoxynucleosides (i.e. AzddDAPR) and ribavirin in the treatment of retrovirus infections.

Materials and Methods

Compounds

Ribavirin (Virazole®) was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). AzddDAPR was synthesized as described previously (Balzarini et al., 1988b).

Cells

Murine embryo fibroblast C3H/3T3 cells were grown in Eagle's minimum essential medium supplemented with 10% (v/v) inactivated fetal calf serum (Gibco, Glasgow, Scotland, U.K.), 2 mM L-glutamine (Flow Laboratories, Irvine, Scot-

land, U.K.) and 0.075% (v/v) $NaHCO_3$ (Flow Laboratories). MT-4 (HTLV-I-transformed human T4-lymphocyte) cells were cultured in RPMI-1640 medium supplemented with the same ingredients as described for C3H/3T3 cells.

Viruses

Moloney murine sarcoma virus (MSV) was prepared from tumors induced by in vivo infection of 10-day-old NMRI ('Naval Medical Research Institute') mice according to the procedure described by De Clercq and Merigan (1971). Human immunodeficiency virus (HIV, strain $HTLV\text{-}III_B$) was derived from a pool of American AIDS patients as described previously (Popovic et al., 1984).

Inhibitory effects of test compounds on HIV-induced cytopathogenicity in MT-4 cells

The procedure for the anti-HIV assay has been described previously (Balzarini et al., 1987, 1988a,b). Briefly, MT-4 cells were adjusted to 5×10^5 cells/ml and infected with HIV at 400 $CCID_{50}$/ml. Then, 100 μl of the infected cell suspension were brought into the wells of a microtiter tray containing 100 μl of varying dilutions of the test compounds. After 5 days incubation at 37°C, the number of viable cells was recorded microscopically in a hematocytometer by trypan blue exclusion. The percentage of protective effect of the test compounds on the survival of the MT-4 cells exposed to the virus was determined by the following formula: $100 \times$ [(number of total viable cells exposed to HIV and cultured in the presence of the test compound) − (number of total viable cells exposed to HIV and cultured in the absence of the test compound)]/[(number of total viable cells not exposed to HIV and cultured in the absence of the test compound) − (number of total viable cells exposed to HIV and cultured in the absence of the test compound)].

Inhibitory effects of test compounds on MSV-induced transformation of murine C3H/3T3 embryo fibroblasts

The anti-MSV assay was carried out as described previously (Balzarini et al., 1987). Briefly, murine C3H/3T3 embryo fibroblast cells were seeded at 5×10^5 cells/ml into 1 cm^2 wells of a 48-well microplate. Twenty-four hours later, the cell cultures were infected with 80 focus-forming units of MSV for 60 to 90 min at 37°C. Then, the medium was replaced by 1 ml of fresh culture medium containing varying concentrations of the test compounds. After 6 days, transformation of the cell cultures was examined microscopically.

Inhibitory effects of test compounds on the initiation of MSV-induced tumor formation in NMRI mice and on the survival of MSV-inoculated NMRI mice

Two- to three-day-old NMRI mice (weighing ± 2 gram) were inoculated subcutaneously (s.c.) in the left hind leg with 50 μl MSV (100 focus-forming units, as

based upon virus-induced transformation of murine C3H/3T3 embryo fibroblast cells). At 4 to 6 days post-infection, tumors developed and rapidly increased in volume with further aging of the mice. Within 9 to 11 days post-infection, mice died from the viral infection. Drug treatment [intraperitoneally (i.p.)] was started 2 to 3 hours prior to virus inoculation and continued daily for an additional 4 days.

Results

Anti-retrovirus activity of AzddDAPR and ribavirin in vitro

AzddDAPR and ribavirin were evaluated for their inhibitory effects on the HIV-induced cytopathogenicity in MT-4 cells and MSV-induced transformation of C3H/3T3 cells. In these experiments ddAdo was included as reference product (Mitsuya and Broder, 1986; Balzarini et al., 1987). AzddDAPR was a 20- to 35-fold more potent inhibitor of HIV replication in MT-4 cells and MSV-induced C3H/3T3 cell transformation than ddAdo. However, since ddAdo was less toxic to MT-4 cells than AzddDAPR, both compounds showed a comparable selectivity index (ratio of CD_{50} to ED_{50}) (Table 1). Ribavirin was active against MSV at an ED_{50} of 29 μM. However, it was inactive against HIV at concentrations that were not toxic to the host cells.

Effects of ribavirin on the anti-MSV activity of AzddDAPR

Different concentrations of ribavirin (i.e. 20, 10, 5, 2.5 and 1.25 μM) were combined with varying concentrations of AzddDAPR to examine whether ribavirin might influence the anti-retrovirus potency of AzddDAPR (Table 2). Ribavirin, when applied as such, inhibited MSV-induced C3H/3T3 cell transformation at an ED_{50} of 29 μM. When added at a 10- to 20-fold lower concentration than its ED_{50},

TABLE 1

Inhibitory effect of AzddDAPR and ribavirin on HIV-induced cytopathogenicity in MT-4 cells and MSV-induced transformation of murine C3H/3T3 embryo fibroblasts

Compound	HIV-infected MT-4 cells			MSV-infected C3H/3T3 cells	
	ED_{50}[a] (μM)	CD_{50}[b] (μM)	SI[c]	ED_{50}[a]	(μM)
AzddDAPR[d]	0.3 (± 0.04)	44 (± 25)	147	1.0	(± 0.6)
Ribavirin	> 20	26 (± 13)	< 1.3	29	(± 7.2)
ddAdo[e]	6.3 (± 2.4)	890	141	35	(± 27)

[a]Fifty percent effective dose or dose required to protect HIV-infected cells by 50% or inhibit MSV-induced C3H/3T3 cell transformation by 50%.
[b]Fifty percent cytotoxic dose or dose required to reduce the number of mock-infected cells by 50%.
[c]Selectivity index or ratio CD_{50}/ED_{50}.
[d]Data taken from Balzarini et al. (1988b).
[e]Data taken from Balzarini et al. (1987).

TABLE 2

Combined inhibitory effects of AzddDAPR and ribavirin on the transformation of murine C3H/3T3 embryo fibroblasts by MSV

Ribavirin concentration (μM)	AzddDAPR ED_{50} (μM)
0	1.23 (± 0.75)
1.25	0.76 (± 0.38)
2.5	0.63 (± 0.31)
5.0	0.47 (± 0.17)
10	0.32 (± 0.07)
20	0.25 (± 0.11)

The ED_{50} of ribavirin for MSV-induced C3H/3T3 cell transformation was 29 μM.

ribavirin caused a 2-fold increase in the anti-retrovirus potency of AzddDAPR. With increasing concentrations of ribavirin (up to 20 μM), the ED_{50} of AzddDAPR further decreased to 0.25 μM, which is about a 5-fold lower dose than the initial ED_{50} of AzddDAPR (Table 2). When plotted by the isobologram method, the combination of ribavirin and AzddDAPR resulted in what according to this method should be defined as a subsynergistic antiviral activity (Fig. 1).

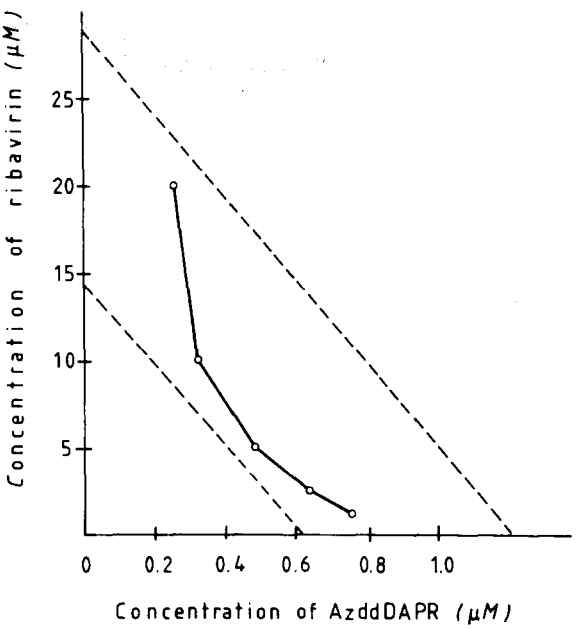

Fig. 1 Isobologram representation of the combined inhibitory effects of AzddDAPR and ribavirin on MSV-induced transformation of murine C3H/3T3 cells. Broken lines represent the unity lines for the fractional inhibitory concentration (FIC) equal to 1 and 0.5, respectively. The data correspond to the average values obtained in 4 independent experiments.

TABLE 3

Inhibitory effects of AzddDAPR and ribavirin on the initiation of tumor formation, and mortality associated therewith, in NMRI mice inoculated with MSV

Compound	Dose[a] (mg/kg/day)	No. of mice at the start of the experiment	Mean day of tumor initiation (± SD)	Mean day of animal death (± SD)
None (control)	-	33	5.1 (± 0.89)	9.6 (± 1.67)
AzddDAPR	125	14	8.4 (± 1.74)	14.8 (± 6.0)
AzddDAPR	25	20	6.7 (± 1.60)	11.9 (± 2.17)
AzddDAPR + ribavirin	125 50	19	12.7 (± 2.94)	10.7 (± 7.14)
AzddDAPR + ribavirin	25 50	20	9.6 (± 1.74)	10.0 (± 5.23)
Ribavirin	50	18	7.7 (± 1.89)	11.5 (± 3.42)

[a]Intraperitoneal drug treatment was started 2–3 h prior to virus inoculation and continued daily for an additional 4 days.

Single and combined effects of ribavirin and AzddDAPR on tumor formation in newborn (NMRI) mice infected with MSV

Treatment of MSV-infected mice with AzddDAPR during 5 subsequent days caused a marked delay in the tumor initiation time (Table 3; Fig. 2, panel A). With an AzddDAPR dose of 125 and 25 mg/kg/day, the mean tumor initiation time was increased to 172 and 132%, respectively ($P < 0.0005$). Also ribavirin at a dose of 50 mg/kg/day afforded a 49% increase in tumor initiation time. At lower doses (i.e. 40 or 20 mg/kg/day) ribavirin had no effect on MSV-induced tumor formation, while at higher doses (i.e. 75 or 100 mg/kg/day) it was toxic to the mice (Balzarini et al., 1989). When combined with AzddDAPR, ribavirin (50 mg/kg/day) markedly increased the anti-retrovirus activity of AzddDAPR. The mean day of tumor initiation increased from 8.4 days to 12.7 days, if AzddDAPR was used at a dose of 125 mg/kg/day, and from 6.7 to 9.6 days, if AzddDAPR was used at a dose of 25 mg/kg/day. Thus, the combined use of ribavirin (50 mg/kg/day) and AzddDAPR at 125 or 25 mg/kg/day resulted in a 2.5- and 1.9-fold increase in mean tumor initiation time, respectively, as compared to the untreated controls (Table 3).

Single and combined effects of ribavirin and AzddDAPR on mortality of newborn NMRI mice infected with MSV

AzddDAPR was clearly effective in prolonging the survival of MSV-infected mice when administered at a dose of 125 or 25 mg/kg/day: at these doses (given from day 0 till day 4 post-infection), the life span of the MSV-infected mice was increased to 154 and 124%, respectively ($P < 0.0005$) (Table 3). Ribavirin at 50 mg/kg/day also increased the life span of the MSV-infected mice to 121%. However, when AzddDAPR was combined with ribavirin, the mean day of animal death decreased from 14.8 to 10.7 days (when AzddDAPR was used at 125 mg/kg/day)

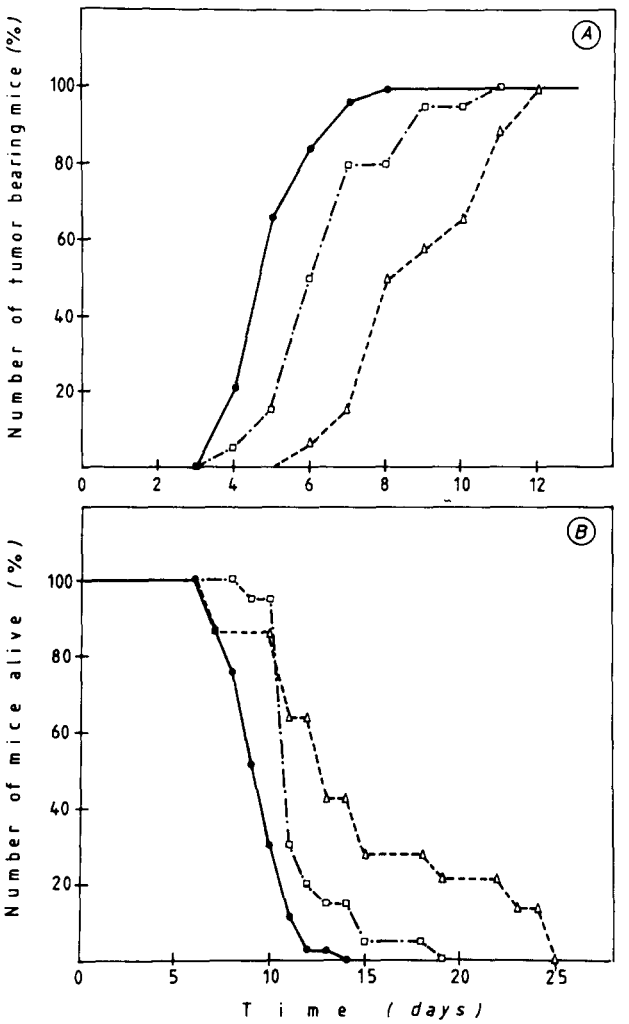

Fig. 2. Effect of AzddDAPR on tumor formation in NMRI mice inoculated with Moloney murine sarcoma virus (MSV) at the 3rd day after birth (panel A) and on the survival of the MSV-inoculated NMRI mice (panel B). The compound was given i.p. daily, starting 2 to 3 h prior to MSV infection and continued till the 4th day after MSV infection. AzddDAPR concentration: 125 mg/kg/day, △---△; 25 mg/kg/day, □---□; control: ●—●.

and from 11.9 to 10.0 days (when AzddDAPR was used at 25 mg/kg/day) (Table 3). Statistical significance (a) between the survival time of mice receiving AzddDAPR (125 mg/kg/day) and AzddDAPR (125 mg/kg/day) + ribavirin (50 mg/kg/day), (b) between ribavirin (50 mg/kg/day) and AzddDAPR (125 mg/kg/day) + ribavirin (50 mg/kg/day), (c) between AzddDAPR (125 mg/kg/day) and control (no treatment), and (d) between AzddDAPR (125 mg/kg/day) + ribavirin (50 mg/kg/day) and control, was $P < 0.05$, > 0.25, < 0.0005 and < 0.25, respectively.

The decreased survival times noted following the combined use of ribavirin and AzddDAPR may be ascribed to an increased toxicity of the drug combination.

Discussion

Combination of antiviral drugs may lead to an increased efficacy of these drugs, whether or not accompanied by a reduction in their acute or chronic toxicity. Recently, several drug combinations have been evaluated for their anti-HIV activity in vitro. Hartshorn et al. (1986) demonstrated a synergistic anti-HIV action of AzddThd and interferon-αA in human peripheral blood lymphocytes. Mitsuya and Broder (1987) reported that acyclovir potentiated the anti-HIV activity of AzddThd in ATH8 cells. However, Vogt et al. (1987) and Baba et al. (1987b) found that ribavirin antagonized the inhibitory effects of pyrimidine 2′,3′-dideoxynucleosides (i.e. AzddThd) on HIV replication in vitro. The latter observations could actually be extended to the in vivo situation. When ribavirin (at 50 mg/kg/day) was combined with AzddThd (at 5 and 25 mg/kg/day) in the treatment of MSV-infected mice, a decreased inhibitory effect on tumor formation was achieved (data not shown), thus indicating an antagonistic interaction between these two drugs. These observations caution against the combined use of ribavirin and AzddThd in the treatment of retrovirus infections.

Baba et al. (1987b) also reported that combination of ribavirin with either ddAdo or ddGuo resulted in a synergistic inhibitory effect on HIV replication in MT-4 cells. We have now demonstrated that this synergistic effect also occurs with combination of ribavirin and other purine 2′,3′-dideoxynucleosides such as AzddDAPR and the potentiating effect of ribavirin on the anti-retrovirus activity of AzddDAPR in vitro also extends to the in vivo situation.

AzddDAPR, like ddAdo, is a potent and selective inhibitor of HIV replication in vitro and it may be considered as a potential chemotherapeutic agent for the treatment of AIDS (Balzarini et al., 1988b). AzddDAPR is about 20- to 35-fold more effective than ddAdo, an inhibitor of both HIV-induced cytopathogenicity in MT-4 cells and MSV-induced transformation of C3H/3T3 cells. The mechanism by which AzddDAPR inhibits HIV replication remains subject for further study. For some 2′,3′-dideoxynucleosides (i.e. AzddThd, ddCyd, ddAdo), it has been ascertained that their corresponding 5′-triphosphate derivatives selectively interfere with HIV reverse transcriptase and may inhibit further DNA chain elongation by acting as DNA chain terminators (Furman et al., 1986; Cheng et al., 1987; Hao et al., 1988). In analogy, it is likely that the anti-retrovirus activity of AzddDAPR is based on the same mechanism of action. However, it is not clear why AzddDAPR has a substantially greater anti-retroviral and cytostatic effect than ddAdo. One possible explanation may be the faster entry of AzddDAPR into the cells by virtue of the greater lipid solubility conferred on AzddDAPR by the 3′-azido group (Balzarini et al., 1989b). Also, it has been reported by Cooney et al. (1987) and Ahluwalia et al. (1987) that the anti-retroviral activity of ddAdo, which is rapidly deaminated to ddIno, depends on the fraction of the compound that undergoes

direct or indirect anabolism to ddATP. In contrast, AzddDAPR may be partially deaminated to AzddGuo and act as an anti-retrovirus agent both through the AzddDAPR 5′-triphosphate and AzddGuo 5′-triphosphate (Balzarini et al., 1988b). As demonstrated by the present results, AzddDAPR is also an efficient inhibitor of murine retrovirus replication in vivo. In this respect, AzddDAPR proved clearly more effective in delaying the mean day of MSV-induced tumor initiation as well as the mean day of death resulting from MSV-induced tumor progression than did ddAdo, a drug that has already been the subject of clinical trials in AIDS patients (Balzarini et al., 1989a).

Of major importance is the fact that combined use of ribavirin with AzddDAPR resulted in a marked potentiation of the in vitro and in vivo anti-MSV activity of these compounds. Indeed, combination of ribavirin with AzddDAPR significantly increased the mean day of MSV-induced tumor initiation. However, as reflected by a shortening in the life span of the mice, combination of ribavirin with AzddDAPR might also have resulted in an increased toxicity for the host. The fact that the incidence of tumor initiation in the individual mice varied much less than the incidence of animal deaths suggests that the tolerance for the compound combinations may vary considerably in the individual animals. Treatment of mice with AzddDAPR at 125 mg/kg/day as a single agent resulted in 15% killing of the mice before 50% of the tumors appeared. In contrast, combination of ribavirin with AzddDAPR at 125 mg/kg/day or 25 mg/kg/day resulted in 70% and 55% death of the mice (respectively), before appearance of 50% of the tumors. Ribavirin alone caused animal death in ± 10% of the mice before appearance of the tumors (data not shown). These observations explain why the means were shorter for survival than for tumor initiation in the mice treated with AzddDAPR (125 mg/kg/day) + ribavirin (50 mg/kg/day) and suggest that the shorter survival times found for the combined agents compared to the agents used individually may be the result of increased toxicity. Nevertheless, our findings provide an experimental basis for the further pursuit of AzddDAPR in combination with ribavirin as a therapeutic modality for the treatment of retrovirus infections including AIDS.

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