Potentiating Effect of Mizoribine on the Anti-Herpes Virus Activity of Acyclovir

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Pharmacological induction of low deoxyribonucleoside triphosphate (dNTP) levels in virus-infected cells could result in an increased antiviral effectiveness of some selective antiviral nucleoside analogues. That could be exploited as a new combined strategy in the treatment of herpes virus infections. From this point of view the alteration of antiherpes activity of acyclovir (ACV) in combination with mizoribine (N′-[β-D-ribofuranosyl]-5-hydroxyimidazole-4-carboxamide) (MZR), an inhibitor which lowers the intracellular pool of dGTP, was studied. MZR applied alone at non-toxic concentrations had no effect on herpes simplex virus type 1 (HSV-1) replication in human embryonic skin-muscle fibroblasts (HESMF). The combination of MZR and ACV acts synergistically, as measured by the virus yield assay in the above mentioned system. The potentiating effect of MZR on the anti-HSV-1 activity of ACV was reversed by guanosine (Guo). In this case dGTP could be considered as the "key metabolite" responsible for the higher effectivity of the combination of drugs.

Introduction

In previous studies we have demonstrated that drugs which decrease the deoxyribonucleoside triphosphate (dNTP) pool in herpes virus-infected cells could enhance the antiviral effectiveness of some antiviral nucleoside analogues (Pancheva, 1991, 1995; Pancheva *et al.*, 1997; Pancheva *et al.*, 1999; Pancheva and Venkova, 1999). It has been shown that inhibitors of inosine monophosphate dehydrogenase (IMPDH), as ribavirin and mycophenolic acid, markedly potentiate the inhibitory effect of acyclovir (ACV). It was therefore of interest to further explore the combined effect with other IMPDH inhibitors as partner drugs. In this study we present results which confirm the above mentioned concept by combining acyclovir, a well known antiherpes drug, with mizoribine (bredinin), an imidazole nucleoside with a similar chemical structure to ribavirin. ACV exhibits very strong and selective antiviral activity against HSV-1. ACV triphosphate act as a competitive inhibitor with respect to the natural substrate dGTP for initial binding of viral DNA polymerase (Furman *et al.*, 1984). Like ribavirin (Streeter *et al.*, 1973), mizoribine (MZR) (Kusumi *et al.*, 1988; Mitchell *et al.*, 1993) inhibits competitively IMPDH, and consequently lowers the intracellular pool of dGTP. The decrease of the cellular pool of dGTP would give a better opportunity to its competitor to interact with the target enzyme HSV-1 DNA polymerase.

Based on this concept, one might expect an enhancement of the anti-herpes virus activity of ACV in the cell, if the level of the natural competing substrate (dGTP) is reduced by MZR. In the present study, we have investigated the effect of a combination of ACV with MZR against HSV-1 in cell cultures of human embryo skin-muscle fibroblasts (HESMF).

Materials and Methods

Compounds, virus and cell culture

Acyclovir was provided by Dr. G. Elion (Burroughs Wellcome Co., Research Triangle Park, NC), mizoribine (N′-[β-D-ribofuranosyl]-5-hydroxyimidazole-4-carboxamide) and guanosine originated from Sigma. Herpes simplex virus type 1 (HSV-1) strain DA was received from Dr. S. Dundarov, Institute of Infectious and Parasitic Diseases, MA, Sofia. The virus was cultivated in diploid cultures of human embryonic skin-muscle fibroblasts (HESMF), prepared by Dr. D. Dundarova, Nat. Cent. Infect. Paras. Dis, MA, Sofia. The

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cells were grown and maintained in Basal Medium Eagle containing 10% calf serum.

Virus yield reduction assay

The activity of the combination of ACV and MZR was evaluated against HSV-1 in HESMF cells by the virus yield reduction method. Investigations were carried out on confluent cell monolayers in 24-well plates infected with 100 $CCID_{50}$ (50% cell culture inhibitory dose) in 0.1 ml. Alone and in combination, the drugs were added after 1 h of virus adsorption and the cells were then incubated for 48 h at 37 °C. Virus titers in samples (pools of 4 wells) were determined by titration in microplate cultures of HESMF cells and expressed in $CCID_{50}$/0.1 ml. Cytotoxicity was monitored by the trypan blue exclusion method.

Reversal assay

The studies were performed by the virus yield reduction assay in HESMF cells infected with the HSV-1 strain DA. Treatment with ACV and/or MZR was done in parallel without and with Guo at different concentrations. Controls containing virus and medium only and virus, medium and Guo were run simultaneously. The results were means of three independent experiments.

Results and Discussion

We had previously established that MZR was not toxic to HESMF cells at concentrations up to 965 μM. No cytotoxicity of the combination of MZR and ACV in the concentrations used in the antiviral assays was observed.

The results obtained for different concentrations of MZR and ACV used in combination are shown on Table I. MZR showed no antiviral activity against HSV-1 in HESMF cells at the used doses. Lack of antiherpes effect of MZR was in accordance with the observation of Mizuno *et al.*, 1974, with HeLa cell cultures. However, MZR enhanced enormously the anti-HSV-1 activity of ACV, at that by using ACV in concentrations with limited activity (1.1 μM ACV reduces 45-times the virus yield). When the drugs were combined in concentrations of 154 μM (40 μg/ml) MZR and 1.1 μM (0.25 μg/ml) ACV complete virus reduction ($> 10^6$-fold) was achieved, indicating a high degree of synergism. By using the non-effective dose of ACV, 0.55 μM (0.125 μg/ml), the virus titer was reduced more than 6000 times.

The inhibition of HSV-1 replication by the two substances in combination was reversed when infection was carried out in excess of guanosine. Fig. 1 presents data of the antiviral effect when

Table I. Potentiating effect of mizoribine on the inhibitory activity of ACV against HSV-1 replication in HESMF cells.

Drugs	Conc. [μM]	Virus yield $\log_{10}$ $CCID_{50}$/0.1 ml mean values ± SD[a]	*n*-fold virus titer reduction[b]
Control		6.33 ± 0.29	1
ACV	1.1	4.67 ± 0.16	45
	0.55	5.50 ± 0.17	6.6
MZR	772	5.47 ± 0.17	7.2
	463	6.00 ± 0.23	2.1
	154	6.33 ± 0.29	1
ACV + MZR	1.1 + 772	0	2.1×10^6
	1.1 + 463	0	2.1×10^6
	1.1 + 154	0	2.1×10^6
	1.1 + 62	3.33 ± 0.29	1000
	1.1 + 31	3.67 ± 0.16	447
	0.55 + 772	2.00 ± 0.23	21000
	0.55 + 463	2.28 ± 0.25	11000
	0.55 + 154	2.50 ± 0.17	6600
	0.55 + 62	4.65 ± 0.14	47
	0.55 + 31	5.50 ± 0.17	6.6

[a] Data are mean values of three independent determinations; SD = standard deviation.

[b] Virus titer of drug-free control divided by virus titer in the presence of drug (s).

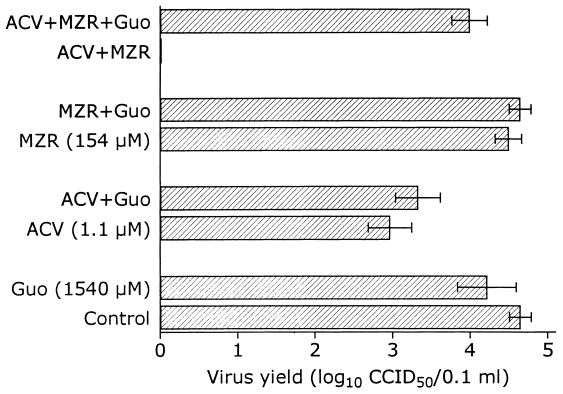

Fig. 1. Reversal of the combined inhibitory effect of ACV and MZR on HSV-1 replication in HESMF cells by Guo.

Guo was administrated together with the drugs. The complete reduction of the virus yield 48 h p.i. attained from 1.1 μM ACV plus 154 μM MZR was recovered 89% in the presence of 1.54 mM Guo (concentration 10-times higher than that of MZR). As Guo is converted within the cells to dGTP, it could be assumed that the potentiating effect of MZR on the antiviral effect of ACV is due to the reduced level of dGTP content because of the inhibitory effect of MZR on IMPDH activity in the cells.

The data shown in this paper confirm the validity of the above mentioned concept as an approach to the combined chemotherapy of viral infections.

Taking also in consideration the fact, that MZR is in wide-spread clinical use in Japan as an immunosuppressive agent, especially registered for prevention of rejection in renal transplantation its combined application with ACV could be of great clinical utility in organ-transplant recipients with herpes virus infections.

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