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The anti-herpesvirus activity of (1′*S*,2′*R*)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1′-yl]methyl] guanine is markedly potentiated by the immunosuppressive agent mycophenolate mofetil

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Abstract

Mycophenolic acid (MPA), the active form of the immunosuppressive agent mycophenolate mofetil (MMF), was found to markedly potentiate the anti-herpesvirus activity of the novel anti-herpesvirus agent A-5021, (1′*S*,2′*R*)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1′-yl]methyl]guanine. For example, at a concentration of 1 μg/ml MPA, the activity of A-5021 against HSV-1, HSV-2 and TK^- HSV-1 increased by a factor of 130, 14 and ≥ 189, respectively. Exogenously added guanosine reversed this potentiating effect, suggesting that a depletion of the endogenous dGTP pools enhanced the inhibitory effect of the 5′-triphosphate metabolite of A-5021 on the viral DNA polymerase. The combined effect of A-5021 and MPA on the growth of uninfected Vero cells was additive rather than synergistic. The combination of topically applied MMF (5%) with 0.05% A-5021 (a subactive concentration) completely protected against HSV-1-induced cutaneous lesions in hairless mice, whereas therapy with either compound used alone had no protective effect. These findings may have implications for those transplant recipients that receive MMF as (part of) their immunosuppressive therapy and that develop intercurrent herpesvirus infections for which they need treatment.

1. Introduction

A-5021 (1′*S*,2′*R*)-9-[[1′,2′-bis(hydroxymethyl)-cycloprop-1-yl]methyl]guanine is a novel nucleoside analogue that potently inhibits the replication of several herpesviruses including herpes simplex virus type 1 (HSV-1), HSV-2, varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and herpesvirus type 6 (HHV-6), but lacks activity against HHV-8. The antiviral activity of A-5021 is superior over ACV against HSV-1, HSV-2, VZV and HHV-6 (Sekiyama et al., 1998; Iwayama et al., 1998; Neyts et al., 2000). HSV- or VZV-encoded thymidine kinase, specifically phosphorylate A-5021; hence, the compound demon-

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strates lesser activity against TK-deficient strains of HSV-1, HSV-2 and VZV. The 5′-triphosphorylated form A-5021-TP is a selective inhibitor of HSV-1 and HSV-2 DNA polymerases and does so competitively with respect to dGTP. A-5021-TP is incorporated into viral DNA at dGMP sites and appears to act as a 'pseudo'-chain terminator. The compound strongly terminates DNA elongation at tandem dGMP analogues (Ono et al., 1998). Of interest is that A-5021 has, unlike ganciclovir, no inhibitory effect on bone marrow progenitor cell and colony formation (Hasegawa et al., 2000). A-5021 was shown to be more antivirally effective than either acyclovir or famciclovir, when given orally, in protecting mice against HSV-1 infections (either following intraperitoneal or intracutanous inoculation). When administered to HSV-1 infected SCID mice, A-5021 proved much more effective than ACV (Neyts et al., 2000). Also, following intravenous treatment, A-5021 proved much more potent than acyclovir in the treatment of intracerebral HSV-1 infections in mice (Iwayama et al., 1999).

Mycophenolate mofetil (MMF), the morpholinoethyl ester of mycophenolic acid (MPA) is an immunosuppressant that is used in solid organ transplantation (Bullingham et al., 1998). After oral administration, MMF is hydrolysed to MPA, the active immunosuppressive agent, which is a potent inhibitor of inosine monophosphate (IMP) dehydrogenase. Inhibition of this enzyme results in a depletion of the intracellular dGTP pools. The immunosuppressive effect of MPA has been ascribed to depletion of the dGTP pools (Fulton and Markham, 1996). We recently demonstrated that the antiherpesvirus activity of acyclovir (ACV), penciclovir (PCV) and ganciclovir (GCV) is markedly increased by MPA and its oral prodrug MMF. Here we report that the antiviral efficacy of A-5021 is also markedly enhanced, both in vitro and in vivo following the addition of mycophenolic acid.

2. Materials and methods

2.1. Viruses

Human cytomegalovirus (HCMV strain Davis ATCC; VR807) was obtained from ATCC and was propagated in human embryonic lung cells (CCL-137). The origin of HSV-1 (strain KOS) and HSV-2 (strain G) and TK- HSV-1 (strain B2006) has been described before (De Clercq et al., 1980). Stocks of these viruses were prepared in Vero cells (ATCC; CCL-81).

2.2. Compounds

A-5021 was synthesised as described previously (Sekiyama et al., 1998) and was kindly provided by Dr T. Tsuji from Ajinomoto Co (Kawasaki, Japan). Mycophenolic acid (MPA) was purchased from Sigma (St. Louis, MO) and mycophenolate mofetil (MMF) was kindly provided by Roche (Palo Alto, CA).

2.3. Antiviral activity assays for HSV-1, HSV-2, TK^- HSV-1 and HCMV

All the experiments were carried out on confluent cells in 96-well microtiter trays. HEL or Vero cells were inoculated with 100 times the $CCID_{50}$ (50% cell culture infective dose) of the different HSV-strains. Confluent cultures of HEL cells were inoculated with 100 plaque forming units (PFU) of HCMV. Following a 2-h virus adsorption period, the inoculum was removed and cultures were incubated with the appropriate concentration of (combinations of) the different drugs. Virus-induced cytopathic effect (CPE) was recorded microscopically at 2–3 days for HSV and at 7 days for HCMV.

2.4. Antiviral evaluation of drug combinations

The inhibitory effects of the drugs combined on HCMV-induced cytopathicity were examined by checker-board combinations of various concentrations of the test compounds. The drug combination effect was analysed as previously described (Baba et al., 1987). In this analysis, the EC_{50} was

used for calculating the fractional inhibitory concentration (FIC). When the minimum FIC index, which corresponds to the FIC of compounds combined (e.g. $FIC_x + FIC_y$), is equal to 1.0, the combination is assumed to act in an additive fashion; when it is between 1.0 and 0.5, the combination would act subsynergistically and when it is < 0.5 it should act synergistically.

2.5. Determination of cytostatic activity

Vero cells were seeded at a density of 4000 cells per well in 96-well plates in MEM containing 10% FCS and various combinations of A-5021 and MPA. Cells were allowed to proliferate for 4 days at 37°C after which they were trypsinised and counted with a Coulter counter.

2.6. HSV-1 infections in mice

Hairless mice were inoculated intracutaneously (i.c.) at the lumbrosacral area (by scratching the skin with a scarificator) with HSV-1 (KOS) at 10^4 PFU per 0.05 ml per mouse. The mice were then treated for 5 days, starting at 2 h after the infection. Test compounds were applied topically twice a day at the indicated concentrations in dimethyl sulfoxide (DMSO) in a volume of 0.05 ml over an area of 1.5 cm^2. Mice were monitored daily for the development of herpetic skin lesions and mortality. Lesions were scored daily on a scale from 0–4 with increments of 0.5. Statistical significance of the differences in the mean day of death and the number of survivors was assessed by means of the Students' t-test and χ^2-test with Yates' correction, respectively.

3. Results

3.1. In vitro potentiation of the anti-herpesvirus activity of A-5021 by mycophenolic acid

We have shown previously that the anti-herpesvirus activity of guanine-based nucleoside analogues is markedly potentiated by mycophenolic acid (MPA), the active metabolite of the immunosuppressive agent mycophenolate mofetil (MMF) (Neyts et al., 1998a,b; Neyts et al., 1999). Here we studied whether MPA also potentiates the anti-herpesvirus activity of A-5021 (Table 1). At concentrations ranging from 0.25 to 25 μg/ml, MPA potentiated the anti-HSV-1 activity of A-5021, 65–600-fold, whereas at the concentrations used, MPA had little or no effect on viral CPE progression. The anti-HSV-2 activity of A-5021 was potentiated 8–50-fold upon addition of MPA. Of interest is that, when combined with MPA, A-5021 became particularly active against a TK-deficient strain of HSV-1. In fact, when combined with MPA at 2.5 μg/ml (a concentration that is attainable in human plasma upon oral dosing of 1.5–3 g MMF), the EC_{50} value for inhibition of HSV-1 TK^- replication decreased from ≥ 70 to 1.3 μg/ml, which is similar to the activity of the A-5021 when used alone against wild type HSV-1. Interestingly, MPA was found to exhibit by itself some anti-HCMV activity (EC_{50}: ~ 1 μg/ml) (data not shown). We, therefore, analysed the combined effect of MPA and A-5021 on HCMV replication. The combination of both drugs resulted in a subsynergistic effect (minimum FIC index: 0.6) on HCMV replication (data not shown).

To prove evidence that depletion of intracellular dGTP pools is, as postulated, responsible for the potentiating effect of MPA on the anti-herpesvirus activity of A-5021, guanosine (at 100 μg/ml) was added to the culture medium. As shown in Fig. 1, exogenously added guanosine efficiently reversed the potentiating effect of MPA on the anti-HSV-1 activity of A-5021.

We next determined whether the combination of A-5021 with MPA increased the cytostatic effect of A-5021. As can be derived from Fig. 2, A-5021 alone had only a moderate cytostatic effect (EC_{50}: 84 μg/ml). Addition of MPA at concentrations around the CC_{50} (50% cytostatic concentration) for Vero cell growth resulted in an additive, rather than synergistic inhibition of cell growth.

Table 1
Effect of mycophenolic acid (MPA) on the anti-herpesvirus activity of A-5021 in Vero cells[a]

Virus	EC_{50} (μg/ml)					
	A-5021 Alone	A-5021 + MPA at 25 μg/ml	A-5021 + MPA at 10 μg/ml	A-5021 + MPA at 2.5 μg/ml	A-5021 + MPA at 1.0 μg/ml	A-5021 + MPA at 0.25 μg/ml
HSV-1 (KOS)	1.3 ± 1.0	0.0021 ± 0.0066	0.0027 ± 0.0008	0.0056 ± 0.0012	0.0094 ± 0.0002	0.02 ± 0.005
HSV-2 (G)	1.5 ± 0.7	0.03 ± 0.01	0.03 ± 0.01	0.08 ± 0.02	0.11 ± 0.04	0.19 ± 0.7
HSV-1 TK^-	≥ 70	0.6 ± 0.1	0.7 ± 0.2	1.3 ± 0.3	3.9 ± 1.0	7.0 ± 0.0

[a] Data are mean values for at least three separate experiments. At the indicated concentrations (0.25–25 μg/ml) MPA alone had little or no effect on viral replication.

3.2. MMF potentiates the anti-HSV-1 activity of A-5021 in intracutaneously infected mice

Hairless mice were inoculated intracutaneously on the back with HSV-1 (KOS) (Table 2). The animals were treated two times daily for a period of 5 consecutive days, starting 2 h after the infection, with either vehicle [dimethylsulfoxide (DMSO)], a 0.05% A-5021 ointment (a subactive concentration), 5% MMF ointment (that causes no protection), or the combination of 0.05% A-5021 plus 5% MMF ointment. Those animals that received the combined treatment were completely protected against infection and associated mortality (Table 2). Also in this group no signs of toxicity of MMF or local irritation from treatment with MMF were observed and the infected area healed rapidly.

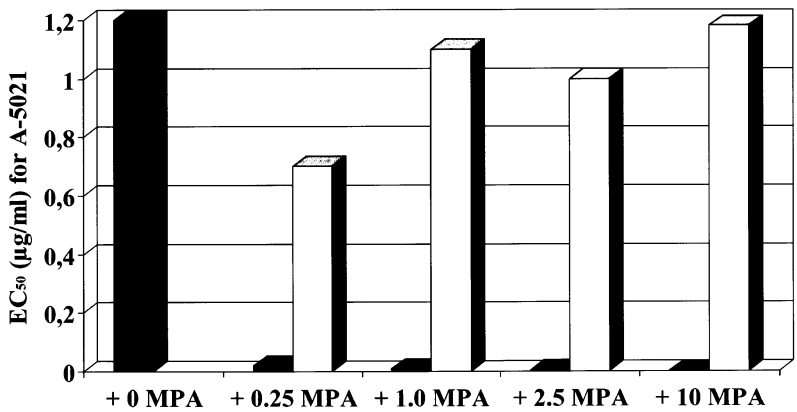

Fig. 1. Effect of exogenously added guanosine on the potentiating effect of MPA on the anti-HSV-1 activity of A-5021 in Vero cells. Black bars: EC_{50} of A-5021 + MPA (at 0, 0.25, 1.0, 2.5 or 10 µg/ml); open bars, similar conditions as for the black bars but + 100 µg/ml guanosine. Data are mean values for the two separate experiments.

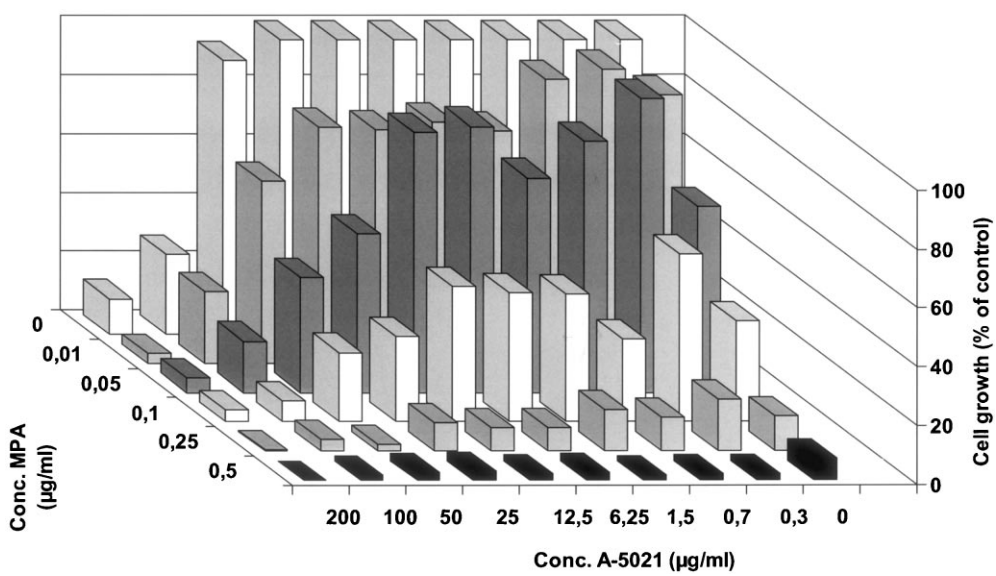

Fig. 2. Effect of the combination of A-5021 + MPA on the growth of uninfected Vero cells.

Table 2
Effect of topically applied mycophenolate mofetil (MMF) on the antiviral efficacy of A-5021 against intracutaneous HSV-1 infections in hairless mice[a]

Condition	MDL	Number with lesion	MDD	Mortality
Control (DMSO)	5.4 ± 1.3	9/10	9.0 ± 1.8	9/10
0.05% A-5021	6.5 ± 0.9^{NS}	$7/10^{NS}$	9.7 ± 2.3^{NS}	$4/10^{NS}$
5% MMF	6.6 ± 2.0^{NS}	$9/9^{NS}$	9.7 ± 2.6^{NS}	$9/9^{NS}$
0.05% A-5021 + 5% MMF	–	0/10***	–	0/10***

[a] Treatment was started at 2 h after infection and was repeated twice daily from day 0 till day 4 post infection; MDL: mean day of lesion development (assessed daily); MDD, mean day of death; NS, not significant ($P > 0.05$); *** $P < 0.001$.

4. Discussion

We have previously shown that mycophenolate mofetil (MMF), the morpholinoethyl ester of mycophenolic acid (MPA) markedly potentiates the anti-herpesvirus activity of guanine based nucleoside analogues such as acyclovir, penciclovir, ganciclovir, and lobucavir (Neyts et al., 1998a,b; Neyts and De Clercq, 1998, 1999). The reason for this potentiation is assumed to reside in the depletion of the intracellular dGTP pools by MPA (a potent inhibitor of IMP-dehydrogenase), thus resulting in an increased inhibitory effect of the acyclic nucleoside triphosphates on the viral DNA polymerase. Akin to acyclovir, penciclovir and ganciclovir, A-5021 is a guanine-based nucleoside analogue that competes with dGTP in the DNA polymerisation reaction. The compound is incorporated into viral DNA instead of dGTP and terminates chain elongation at tandem GMP repeats (Ono et al., 1998). As expected, MPA markedly enhanced the anti-herpesvirus activity of A-5021. The observation that exogenously added guanosine reversed the potentiating effect supports the hypothesis that depletion of dGTP pools is responsible for this potentiation.

Of particular interest is also the observation that the EC_{50} of A-5021 for inhibition of the replication of a TK^- HSV-1 strain decreased, when combined with MPA, from concentrations that may not be attainable in plasma (≥ 70 μg/ml) to concentrations that may be easily reached in plasma (~ 1 μg/ml). The concentration required for MPA to potentiate the antiviral activity of A-5021 was not more than 0.25 μg/ml; concentrations of 1 μg/ml, or even higher are reached in human plasma upon oral dosing with 1.5–3 g of MMF (Bullingham et al., 1998). It should be noted here that the MPA/A-5021 combination experiments were carried out in Vero cells and that the anti-HSV activity of A-5021 is even more pronounced in fibroblast cells (Iwayama et al., 1998). Similarly, acyclovir, ganciclovir and penciclovir were 20–150-fold more active against HSV-1 and HSV-2 in fibroblasts than in Vero cells (Neyts et al., 1998a). The anti-HCMV activity of ganciclovir was also markedly potentiated upon combination with MPA.

The observation that we made in vitro were extended to the in vivo situation, in mice infected intracutanously with HSV-1. Topical treatment of 5% MMF plus a subactive concentration of A-5021 (0.05%) proved to be highly protective against intracutanous HSV-1 infections, whereas treatment with either compound alone caused no protective effect. Phase I clinial studies with A-5021 have been successfully completed. In case the compound would be used for the treatment of herpesvirus infections in transplant recipients it may be used concomitantly with MMF that is being used as (part of) an immunosuppressive therapy. In such setting MMF may potentiate the antiviral efficacy of A-5021. Although we did not observe any potentiation of the cytostatic effect of A-5021 on Vero cell growth by MPA, we suggest that, in case A-5021 and MPA would ever be used concomitantly, patients should be carefully monitored for a potential increase in side effects.

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