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Anti-herpesvirus activity of (1′*S*,2′*R*)-9-[[1′,2′-bis(hydroxymethyl)-cycloprop-1′-yl]methyl] × guanine (A-5021) in vitro and in vivo

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Abstract

The novel nucleoside analog (1*S*′,2*R*′)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1-yl]methyl]guanine (A-5021) was previously shown to be a potent inhibitor of the replication of herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV), both in vitro and in vivo (J. Med. Chem. 41, 1284–1298; Antimicrob. Agents Chemother. 42, 1666–1670). Here we demonstrate that A-5021 is also a potent inhibitor of Epstein-Barr virus (EBV) and human herpes virus 6 (HHV-6A and HHV-6B), but that the compound lacks activity against HHV-8. A-5021, in comparison to acyclovir, was also assessed for protective activity against HSV-1-induced mortality in SCID mice. The compounds were administered at 50 mg/kg per day by subcutaneous injection for four consecutive days and treatment was initiated at either 2 h, 1 or 2 days post infection (p.i.). When administered from day 0 to 4 p.i., A-5021 conferred complete protection against the infection (as assessed at 22 days p.i.), whereas acyclovir delayed virus induced mortality by only 5 days. When treatment was begun on day 1 or 2, A-5021 still afforded marked protection against the infection, whereas acyclovir was virtually devoid of any activity under these conditions. Our data underline that A-5021 may offer great promise for the treatment of herpesvirus infections.

1. Introduction

The novel nucleoside analog (1′*S*,2′*R*)-9-[[1′,2′-bis(hydroxymethyl)cycloprop-1-yl]methyl]guanine (A-5021) is a potent inhibitor of the replication of several herpesviruses including herpes simplex virus type 1 (HSV-1), HSV-2 and varicella-zoster virus (VZV), with an antiviral activity superior to that of acyclovir (Sekiyama et al., 1998; Iwayama et al., 1998). The compound depends for its activation (phosphorylation) on the HSV- or VZV-encoded thymidine kinase and hence demonstrates lesser activity against TK-deficient strains of HSV-1, HSV-2 and VZV. Following phosphorylation to the 5′-monophosphate, A-5021 is further converted to its 5′-triphosphate which is a competitive inhibitor of HSV-1 and

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HSV-2 DNA polymerases with respect to dGTP (Ono et al., 1998). 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). The compound proved, following intravenous treatment, also much more effective than acyclovir in the treatment of (intracerebral) HSV-1 infections (Iwayama et al., 1999). We have now further elaborated on the antiviral activity spectrum and the in vivo efficacy of A-5021, relative to that of acyclovir.

2. Materials and methods

2.1. Viruses

The GS strain of HHV-6A (a kind gift from Dr R. Gallo, when at NCI, Bethesda, MD) was propagated in human T-lymphoblast HSB-2 cells (ATCC, CCL-120-1), whereas the Z-29 strain of HHV-6B was grown in human T-lymphoblast MOLT-3 cells (both from ABI Technologies, Columbia, MD). Mouse cytomegalovirus (MCMV) (Smith strain) was cultured in C127I cells (ATCC; CRL-1616) and the murine γ-herpesvirus MHV-68 [clone G2.4 (kindly provided by Dr A.A. Nash, Edinburgh, UK)] was cultured in Vero-B cells (ATCC; CCL-81). The EBV-carrying P3HR-1 cells and HHV-8-carrying BCBL-1 cells from the NIH AIDS Research and Reference Reagent Program. HBV-carrying Hep AD38 cells (Ladner et al., 1997, in which viral replication is under the control of a tetracycline responsive element) were a kind gift from Dr Ph. Furman (Triangle Pharmaceuticals, Durham, NC).

2.2. Compounds

A-5021 was synthesized as described previously (Sekiyama et al., 1998) and kindly provided by Dr T. Tsuji from Ajinomoto Co (Kawasaki, Japan). Acyclovir was from Glaxo Wellcome (Aalst, Belgium) and cidofovir from Gilead Sciences (Foster City, CA).

2.3. Antiviral activity assays for HCMV, MCMV and MHV-68

Confluent cultures of either C127I or Vero cells were inoculated with 50 PFU of MCMV (C127I cells) or MHV-68 and HSV-1 (Vero cells), as described previously (Neyts et al., 1993; Neyts and De Clercq, 1998a). Compounds, either alone or in combination, were added after a 2-h virus adsorption period. Virus-induced cytopathic effect (CPE) was recorded microscopically at 2–3 days post infection (p.i.) for HSV at 5 days for MCMV and at 7 days for HCMV and MHV-68.

2.4. Antiviral assays for EBV, HHV-6, HHV-8 and HBV

HSB-2 cells and MOLT-3 cells were inoculated with, respectively, the GS and Z-29 strain of HHV-6 at a multiplicity of infection of 0.001 $CCID_{50}$ per cell. Virus was allowed to adsorb for 2 h incubation at 37°C. After centrifugation, the infected cells were resuspended and transferred to 48-well microtrays, containing different dilutions of the antiviral compounds. The final cell density was 0.8×10^6 cells per ml. Cultures were incubated at 37°C and subcultivated on days 4 and 7, by 2-fold dilution in medium containing fresh compound. On day 10–14, (i) cells were examined microscopically to score viral CPE (visible by the appearance of large ballooning cells) and drug toxicity, and (ii) total DNA was extracted from the infected cells with the QIAamp Blood kit (Qiagen, Germany).

Anti-EBV activity was assessed as described previously (Meerbach et al., 1998). Briefly, exponentially growing P3HR-1 cells were seeded at a density of 10^6 cells per ml in 25 cm^2 cell culture flasks and the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) was added at a concentration of 30 ng/ml to induce virus replication. TPA was not added to the cultures that served as negative control. Cultures were incubated with different dilutions of the compounds for 7 days, after which total cellular DNA was extracted as described above for HHV-6.

Anti-HHV-8 activity was assessed as described previously (Neyts and De Clercq, 1997). Briefly,

exponentially growing BCBL-1 cells were seeded at a density of 3×10^5 cells per ml and 30 ng/ml of TPA was added to induce viral replication. No TPA was added to the cultures that served as negative control. Cell cultures were incubated with different dilutions of the compounds, medium was replenished at day 3 and total cellular DNA was extracted at 5 days p.i. as described above for HHV-6.

Anti-HBV activity was assessed as described previously (Ying et al., 1999, 2000). Briefly, Hep AD38 cells were grown to confluency in the presence of 0.3 μg/ml tetracycline. When confluent, tetracycline-containing medium was removed (by extensive washing) and cultures were further incubated in tetracycline-free medium containing different dilutions of the test compounds. Total cellular DNA was extracted 6 days later as described above for HHV-6.

Digoxigenin-labelled probes specific for HHV-6 (strain GS or Z-29), EBV, HHV-8 and HBV were generated in a polymerase chain reaction (PCR) in the presence of digoxigenin-11-dUTP using as template DNA extracted from HHV-6-infected HSB-2 cells, or P3HR-1, or BCBL-1 or Hep AD38 cells, respectively. For HHV-6 an amplicon of 259 bp delimiting a sequence within the U67 gene was amplified. For HHV-8 a 213-bp sequence within the viral capsid antigen was generated (Neyts and De Clercq, 1997), for EBV a 124-bp sequence within the EBV internal repeat 1 (BamHI W region, Meerbach et al., 1998) and for HBV a 523 bp fragment of the capsid gene (Ying et al., 1999). The digoxigenin containing amplified products were separated on agarose gel and purified.

For slot-blot quantitation of viral DNA, 5-μg aliquots of total cellular DNA were boiled and vacuum-blotted onto nylon membranes. All reagents for hybridization and chemiluminescence detection were from Boehringer Mannheim (Germany). Membranes were UV-cross linked and prehybridized with DIG easy Hyb solution, after which hybridization with 30 ng/ml of the digoxigenin-11-dUTP labeled virus-specific probe was carried out overnight at 42°C. The membranes were then washed at high stringency [$2 \times$ SSC − 0.1% SDS] for 10 min at room temperature followed by two washes of 15 min each at $0.1 \times$ SSC − 0.1% SDS at 65°C. Following treatment with blocking buffer the membrane was incubated for 1 h with alkaline phosphatase-conjugated anti-digoxigenin antibody. Chemiluminescence detection was performed by standard methods using CSPD as substrate. The intensity of the viral DNA bands was determined by densitometric scanning.

2.5. HSV-1 infections in mice

SCID mice (15–20 g) were inoculated intraperitoneally with 10^3 PFU of HSV-1. The compounds were administered by subcutaneous injection in a 0.2 ml volume. Animals were monitored daily for mortality. Statistical significance of the differences in the mean day of death and the number of survivors was assessed by means of the Student's t-test and χ^2-test with Yate's correction, respectively.

3. Results

3.1. Antiviral activity of A-5021

Previous in vitro studies showed that A-5021 is more potent in vitro than acyclovir and penciclovir against HSV-1 and VZV and to be about equipotent as these reference compounds against HSV-2 (Iwayama et al., 1998). We now studied the effect of A-5021 on the replication of several other human herpesviruses i.e. EBV, HHV-6 and HHV-8 and the murine viruses MCMV and MHV-68 (Table 1). A-5021 was equally active as acyclovir against EBV but was, like acyclovir, devoid of activity against HHV-8 (Table 1). A-5021 proved 10-fold more potent than acyclovir and equipotent to cidofovir against HHV-6A and HHV-6B (as assessed by means of CPE reduction assay and detection of viral DNA). A-5021 was more active than acyclovir against the murine γ-herpes virus MHV-68, but less active than acyclovir against MCMV. The compound was devoid of anti-HBV activity (Table 1).

Table 1
Activity of A-5021, acyclovir and cidofovir against HBV and various herpesviruses

Compound	EC_{50} (μg/ml)[a]								
	MCMV	EBV	MHV-68	HHV-6A		HHV-6B		HHV-8	HBV[b]
	CPE	DNA	CPE	CPE	DNA	CPE	DNA	DNA	DNA
A-5021	15 ± 8	1.0 ± 0.8	1.0 ± 0.5	2.6 ± 1.3	3.4 ± 1.3	3.4 ± 1.1	3.5 ± 1.0	>20	>20
Ayclovir	1.7 ± 0.0	1.4 ± 0.2	15 ± 0.2	27 ± 8	41 ± 8	36 ± 21	41 ± 16	≥25	>20
Cidofovir	0.1 ± 0.02	0.4 ± 0.3	1.0 ± 0.7	2.0 ± 0.9	2.9 ± 0.5	1.9 ± 1.5	3.1 ± 1.2	2.1 ± 0.7	–

[a] Concentration required to reduce virus-induced CPE or viral DNA synthesis by 50%.
[b] Under the experimental conditions used, adefovir inhibited HBV replication by 50% at a concentration of 0.04 μg/ml.

3.2. Comparative activity of A-5021 and acyclovir on HSV-1 induced mortality in SCID mice

We next studied the effect of treatment with A-5021 or acyclovir on lethal HSV-1 infections in SCID mice. This model may provide more relevant information with regard to the treatment of opportunistic herpesvirus infections in immunodeficient patients than infection models in immunocompetent mice. When treatment with acyclovir or A-5021 (at 50 mg/kg) was initiated at 2 h p.i., A-5021 offered complete protection against virus-induced morbidity and mortality (Table 2). Under the same conditions, acyclovir proved much less protective, i.e. only two out of ten mice survived; the mean day of death was delayed by about 5 days. When the start of A-5021 treatment was delayed till 1 day after infection, 80% of the mice were protected against virus-induced mortality (as assessed at 22 days p.i.). When A-5021 treatment was started at 2 days after infection, an important delay (12 days) in the mean day of death was observed although 70% of the animals finally succumbed. Under these conditions (treatment started at 1 or 2 days after infection), little or no protective effect was noted for acyclovir. Mice that had been treated with A-5021; and for which treatment was initiated at 2 h p.i. were further monitored for 4 months. At that time they were sacrificed and liver, lungs and kidneys were removed. No infectious virus was recovered (as assessed by titration of 10% w/v organ homogenates), nor were viral DNA sequences detectable (by means of an HSV-1 specific PCR on DNA isolated from these organs).

4. Discussion

A-5021 was recently shown to possess potent and selective activity against herpesviruses. In vitro the compound proved superior to acyclovir in its efficacy against HSV-1, HSV-2 and VZV, and the in vitro antiviral activity lasted longer than is the case for acyclovir (Iwayama et al.,

Table 2
Effect of A-5021 or acyclovir on HSV-1-induced mortality in SCID mice when treatment was started at different times p.i.[a]

Condition	MDD[b]	Mortality[c]
Control	8.6 ± 1.0	12/12
A-5021 (50 mg/kg per day)		
Day 0–4	–	0/10***
Day 1–5	14.5 ± 0.7*	2/10^{NS}
Day 2–6	21.0 ± 4.7***	7/10^{NS}
Acyclovir (50 mg/kg per day)		
Day 0–4	12.5 ± 5.0NS	8/10^{NS}
Day 1–5	12.1 ± 2.6**	10/10^{NS}
Day 2–6	10.6 ± 1.3**	8/10^{NS}

[a] The compounds were administered subcutaneously once daily at the indicated dose for five consecutive days as indicated. NS, not significant ($P>0.05$); *, $P<0.05$; **, $P<0.005$; ***, $P<0.001$.
[b] Mean day of death.
[c] As assessed at 22 days p.i.

1998). We now extended the panel of herpesviruses against which the activity of A-5021 was evaluated. A-5021 proved equipotent to acyclovir against EBV. Although it has been reported that the EBV thymidine kinase (TK) does not phosphorylate acyclovir (Gustafson et al., 1998), acyclovir elicits marked activity against EBV, as noted here, and in other studies (Lin and Machida, 1988; Meerbach et al., 1998). Both A-5021 and acyclovir were devoid of activity against HHV-8. The lack of activity of acyclovir against HHV-8 (Neyts and De Clercq, 1997; Kedes and Ganem, 1997) may be in agreement with the fact that also HHV-8 TK is unable to phosphorylate acyclovir (Gustafson et al., 2000). It remains to be studied whether A-5021 is a substrate for the EBV- and HHV-8 encoded TK. Although A-5021 and acyclovir have a comparable activity profile against EBV and HHV-8, A-5021 proved much more potent than acyclovir against HHV-6A and HHV-6B. The HHV-6 U69 gene is homologous to the HCMV UL97 gene, the product of which has been shown to play a major role in the phosphorylation of ganciclovir in HCMV-infected cells. Likewise the HHV-6 U69 gene was shown to confer ganciclovir sensitivity to baculoviruses (Ansari and Emery, 1999). Whether A-5021 is a substrate for the HHV-6 U69-encoded protein remains to be studied. Besides the role of the virus-encoded kinases in the metabolic activation of A-5021, a key determinant in the susceptibility of a particular virus to A-5021 will be the sensitivity of the viral DNA polymerase to A-5021 triphosphate. It will be of interest to study which factor (kinase and/or polymerase) is (are) responsible for the lack of activity of A-5021 against HHV-8.

Recently, the potent activity of A-5021 against HSV-1 and HSV-2 infections (following either intracutaneous, intraperitoneal and intracerebral inoculation) in immunocompetent mice was reported (Iwayama et al., 1999). When A-5021 was given orally to HSV-1-infected mice, the compound proved to be more effective than acyclovir, despite its limited oral availability. Oral A-5021 was also more effective against intracutaneous HSV-1 infection and intravenously administered A-5021 proved more effective than acyclovir against intracerebral HSV-1 infections (Iwayama et al., 1999).

In the present study, we evaluated the effect of A-5021 in SCID mice that had been infected with HSV-1. Since opportunistic herpesvirus infections are often life-threatening in immunodeficient patients (AIDS patients and organ transplant recipients), an animal model in immunodeficient mice may provide more relevant information regarding the potential use of a new agent (such as A-5021) for the treatment of herpesvirus infections in immunodeficient patients. When compared with acyclovir in the HSV-1/SCID model, A-5021 proved to be much more potent. When start of treatment was initiated 2 h after infection, acyclovir resulted in a delay of virus induced mortality but had little or no effect on overall mortality rate. Under the same condition, A-5021 completely protected the animals against virus-induced mortality (up to at least 4 months p.i.). At that time no infectious virus, nor viral DNA could be detected in the organs of these animals. Even when start of treatment was delayed for 1 or 2 days after virus infection, A-5021 still conferred significant protection. In fact, when start of treatment with A-5021 was delayed for 2 days after virus inoculation, the compound proved even more effective than acyclovir treatment initiated at 2 h p.i. No side-effects were observed in mice that had been treated with A-5021 at the indicated regimens. A-5021 was also shown to have little, if any, inhibitory effect on bone marrow progenitor cells and colony formation (both human and rodent, Iwayama et al., 1998; Hasegawa et al., 2000).

The data reported here and previously (Ono et al., 1998; Iwayama et al., 1998, 1999) point to the exquisitely potent anti-herpesvirus activity of A-5021, in particular in HSV-infected mice. Phase I clinical studies with A-5021 have been successfully completed. Further development of this very potent compound as an anti-herpesvirus drug is clearly warranted.

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