ANTI-HERPESVIRUS ACTIVITY OF CARBOCYCLIC OXETANOCIN G *IN VITRO*

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A series of new compounds, carbocyclic oxetanocins, have been synthesized and their anti-herpesvirus activity determined. Carbocyclic oxetanocin G (OXT-G) was most active against herpes simplex virus (HSV) and human cytomegalovirus (HCMV) among carbocyclic oxetanocins tested; the median effective concentrations (EC_{50}) for HSV-1, -2, and HCMV were 0.23, 0.04 and 0.40 μg/ml, respectively. The EC_{50} value of carbocyclic OXT-G against HSV-2 was significantly lower than those of acyclovir, ganciclovir (DHPG) and OXT-G, while the value for HCMV was comparable to those of DHPG and OXT-G. Carbocyclic OXT-G showed much higher activity against TK^+ HSV-2 than against a TK^- mutant, suggesting that this compound is a good substrate for HSV-2-induced TK. The antiviral activity of the compound was only partially reversed even by the addition of 100-fold excess deoxyguanosine. The results suggest that the mode of action of carbocyclic OXT-G is different from that of OXT-G.

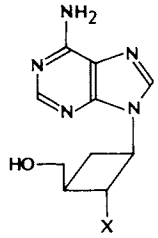
Acyclovir (ACV) is the most successful antiviral agent that has been licenced for the treatment of herpes simplex virus (HSV) infections, and has proved beyond all doubt that antiviral therapy with minimal toxicity is an achievable goal. But this agent is not so active against HSV type 2 (HSV-2) as against HSV type 1 (HSV-1)[1,2], and has shown little efficacy in the treatment of human cytomegalovirus (HCMV) infections[1~4]. Ganciclovir (DHPG), a potent inhibitor against HCMV *in vitro*, is currently undergoing clinical trials for the treatment of HCMV infections in bone marrow transplant recipients and in patients with aquired immunodeficiency syndrome (AIDS)[5~8]. Although ganciclovir has demonstrated significant antiviral activity against HCMV in these patients, the therapeutic efficacy is limited by its toxicity, principally ganciclovir-induced myelosuppression[9~11]. Therefore it is necessary to keep on searching more selective and potent agents against these pathogens. Recently we have reported that oxetanocin G (OXT-G), a novel nucleoside having an oxetanocyl-*N*-glycoside linkage, has a potent and selective antiviral activity against HCMV *in vitro*[12]. OXT-G was also highly effective against systemic HSV-2 and murine CMV infections in mice[13]. As part of our antiviral chemotherapy program, we had an interest in carbocyclic analogs of oxetanocins. In the present study, we investigated the effect of these compounds against HSV and HCMV *in vitro*.

Materials and Methods

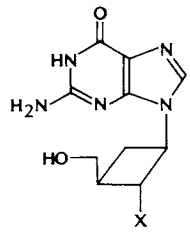
Chemicals

OXT-G were synthesized as described previously[14]. NNU-018 and -019 were synthesized from (+)-(1α,3β)-3-(*tert*-butyldiphenylsilyloxymethyl)cyclobutane-1-yl methansulfonate with adenine or

Fig. 1. Structures of carbocyclic oxetanocins.



NNU-018 X=H
NNU-011 X=OH
NNU-022 X=CH_2OH



NNU-019 X=H
NNU-013 X=OH
NNU-023 X=CH_2OH

2-amino-6-(2-methoxyethoxy)purine, followed by deprotection. NNU-011 and -013 were synthesized from (+)-(1α,2β,4α)-2-(*tert*-butyldiphenylsilyloxymethyl)-5-oxa-bicyclo[2.1.0]pentane with adenine or 2-amino-6-(2-methoxyethoxy)purine, followed by deprotection. Procedures for chemical synthesis of these four compounds will be described in detail elsewhere. NNU-022 and -023 were synthesized from (+)-(1*S*,2*S*,3*S*)-2,3-bis(*tert*-butyldiphenylsilyloxymethyl)cyclobutane-1-yl methansulfonate with adenine or 2-amino-6-(2-methoxyethoxy)purine, followed by deprotection as reported previously[15]. Optical purities of NNU-022 and -023 were determined from NMR spectrum of bis-(*R*)-α-methoxy-α-trifluoromethylphenylacetate of an intermediate (−)-(2*S*,3*S*)-2,3-bis(hydroxymethyl)-1,1-bis(methylthio)cyclobutane, and were >98% ee. All compounds were characterized by 400 MHz NMR, UV, and high-resolution fast atom bombardment (HRFAB)-MS. Purities of these compounds were determined by HPLC, and were >98%. The chemical structures of carbocyclic oxetanocins used in this study are shown in Fig. 1. ACV and DHPG were provided by Burroughs Wellcome Co., Research Triangle Park, N.C., and Syntex Laboratories, Inc., Palo Alto, Calif., respectively.

Cells and Viruses

Vero cells, a line of African green monkey kidney cells, and human embryonic fibroblasts (HEF) were used in this study and were grown in EAGLE's minimum essential medium (MEM) supplemented with 7% calf serum (CS) and 10% fetal calf serum (FCS), respectively. HSV-1 strain HF, HSV-2 strain 186 (wild type [TK^+] and thymidine kinase deficient [TK^-]), and HCMV strain AD169 were propagated in HEF cell monolayers as described previously[16,17].

Plaque Reduction Assays

Confluent monolayers of Vero or HEF cells in plastic dishes (diameter, 35 mm) were infected with 100 to 150 plaque forming units (PFU) of HSV or HCMV. After a 1-hour adsorption period at 37°C, the cultures were overlaid with 2 ml of 0.5% agarose in MEM containing 3% FCS and various concentrations of drugs. The cultures infected with HSV or HCMV were fixed and stained at 1 or 2 and 9 or 10 days after infection, respectively. In the case of HCMV-infected cultures, the second agarose overlay containing appropriate concentrations of drugs was added 5 days after infection. Plaque numbers were counted by using a dissecting microscope at ×20 magnification.

Yield Reduction Assays

Monolayers of Vero or HEF cells were infected with HSV-2 or HCMV at a multiplicity of about 10 PFU per cell and treated with various concentrations of drugs after a 1-hour virus adsorption period at 37°C. HSV-2 and HCMV were harvested 24 hours and 5 days postinfection, respectively. After freeze-thawing followed by low speed centrifugation, the supernatant was assayed for virus infectivity.

Results

Antiviral Activities of Carbocyclic Oxetanocins

The antiviral activities of carbocyclic oxetanocins were measured by the plaque reduction assays and

Table 1. Antiviral activities of carbocyclic oxetanocins.

Compound	EC_{50} (μg/ml)[a]		
	HSV-1	HSV-2	HCMV
NNU-018	>20	>20	>20
NNU-011	3.4	4.2	0.45
NNU-022	8.0	7.5	12
NNU-019	8.0	2.0	2.6
NNU-013	0.74	0.12	0.95
NNU-023	0.23	0.04	0.40

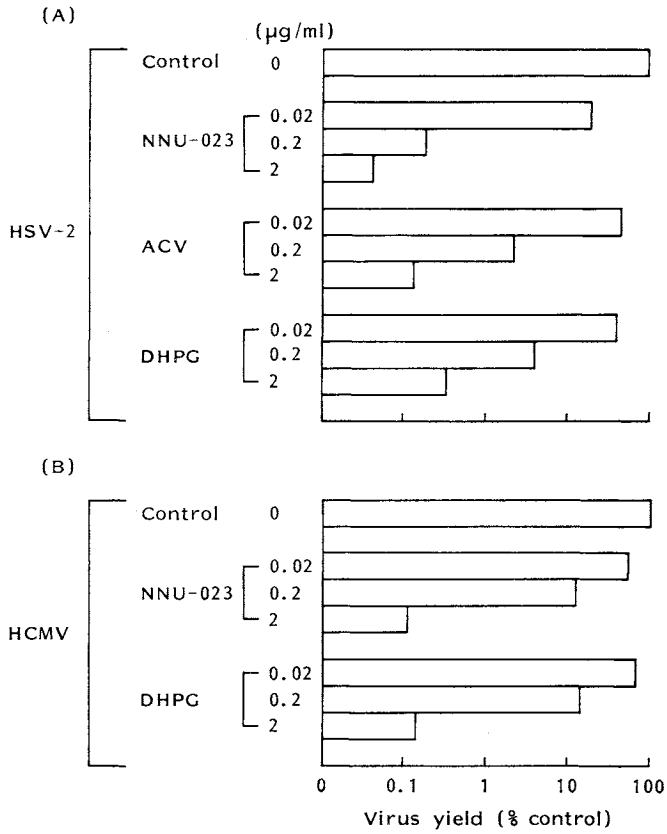
[a] Results are the average of two or three different experiments. Each experiment was carried out in duplicate or triplicate.

Table 2. Inhibitory effects of NNU-023, OXT-G, ACV, and DHPG against wild-type and TK^- HSV-2.

Compound	EC_{50} (μg/ml)[a]	
	HSV-2 (TK^+)	HSV-2 (TK^-)
NNU-023	0.04	1.4
OXT-G	2.0	2.4
ACV	0.18	12
DHPG	0.30	64

[a] Results are the averages of two experiments. Each experiment was carried out in duplicate or triplicate.

Fig. 2. Inhibitory effect of NNU-023, ACV, and DHPG on the production of HSV-2 (A) and HCMV (B).



Vero or HEF cells were infected with either HSV-2 or HCMV at a multiplicity of infection of approximately 10 PFU/cell, as described in Materials and Methods. The titers were the average of triplicate samples.

were expressed in terms of median effective concentrations (EC_{50}) which were defined as the drug concentrations that reduced viral replication to 50%. The results are shown in Table 1. Among six compounds tested, NNU-023, carbocyclic OXT-G, was found to have the most potent anti-herpetic activity; the EC_{50} of this compound against HSV-2 and HCMV were approximately 0.04 and 0.40 μg/ml, respectively.

The EC_{50} value of NNU-023 against HSV-2 was significantly lower than those of ACV and DHPG, while the EC_{50} value of this compound against HCMV was comparable to that of DHPG[18~20]. Similar results were obtained in yield reduction assays where cells were infected with HSV-2 and HCMV at a relatively high multiplicity (10 PFU/cell) (Fig. 2), namely NNU-023 was much more active against HSV-2 than ACV and DHPG.

Table 3. Reversal effects of anti-herpetic activity of carbocyclic oxetanocin by exogenous nucleosides.

Overlay	No. of plaques (% control)
None	100
NNU-023[a]	4.6
NNU-023 plus 10×dA[b]	4.0
plus 100×dA[b]	3.5
plus 10×dC	4.0
plus 100×dC	4.4
plus 10×dG	6.5
plus 100×dG	15
plus 10×dT	5.0
plus 100×dT	7.1
plus 10×4dN	7.7
plus 100×4dN	41

[a] Concentration of NNU-023 is 0.1 μg/ml.
[b] 10× or 100× indicates a 10- or 100-fold greater concentration of deoxyribonucleosides relative to concentration of NNU-023.
dA: Deoxyadenosine, dC: deoxycytidine, dG: deoxyguanosine, dT: deoxythymidine, 4dN: four deoxyribonucleosides.

Effect of Carbocyclic OXT-G against Thymidine Kinase Deficient (TK^-) Mutant of HSV-2

We compared the activity of NNU-023 with those of OXT-G, ACV and DHPG against wild-type and TK^- HSV-2. As reported previously[12], OXT-G exhibited equal potencies against TK^+ and TK^- HSV-2, while the antiviral activity of ACV and DHPG was strikingly weakened by the lack of HSV-induced TK. Although carbocyclic OXT-G was still most active among four compounds against TK^- HSV-2, the EC_{50} value increased about 35-fold compared with that against TK^+ HSV-2 (Table 2).

Reversal of Anti-herpetic Activity of NNU-023 by Exogenous Nucleosides

The effect of the addition of exogenous nucleosides on the antiviral activity of NNU-023 was investigated to characterize the mode of action of this compound. Confluent monolayers of Vero cells were infected with about 100 PFU of HSV-2, and after a 1-hour virus adsorption period, cells were overlaid with 0.5% agarose containing NNU-023 and deoxyribonucleosides. As shown in Table 3, the antiviral activity of NNU-023 was partially reversed by the addition of 100-fold excess deoxyguanosine (dG). The addition of four deoxynucleosides (4dN) all together was much more effective in reversing the antiviral effect of NNU-023 than that of dG alone. However, even 100-fold excess of 4dN did not induce complete reversion of antiviral effect of carbocyclic OXT-G.

Discussion

NNU-023 (carbocyclic OXT-G) was most potent against HSV-2 and HCMV among carbocyclic oxetanocins tested; the EC_{50} of HSV-2 and HCMV was 0.04 and 0.40 μg/ml, respectively. The activity of this compound against HSV-2 was significantly more potent than those of ACV and DHPG. However, the inhibitory concentration of the agent for the growth of Vero cells was lower than those of ACV and DHPG; NNU-023 inhibited the growth of Vero cells by 50% at the concentration of about 5 μg/ml (data not shown) and then the selectivity index was 125. While OXT-G exhibited equal potencies against TK^+ and TK^- HSV-2, carbocyclic OXT-G was about 35-fold more active against TK^+ HSV-2 than a TK^- mutant. This observation suggests that carbocyclic OXT-G, unlike OXT-G, is a good substrate for HSV-2-induced TK. Since carbocyclic OXT-G still showed the high activity against TK^- HSV-2, this nucleoside analog must be well phosphorylated by a nucleoside kinase of Vero cells too. Antiviral activity of NNU-023 was only partially reversed even with 100-fold excess of dG, and the addition of 4dN was shown to be much more effective in reversing antiviral activity than that of dG alone.

These results suggest that the antiviral effect of carbocyclic OXT-G can not be solely due to its ability to act as a dG analog. On the other hand, the antiviral activity of OXT-G can be reversed more effectively by the addition of dG alone (unpublished observation). It appears that the mode of action of carbocyclic OXT-G is different from that of OXT-G. In addition to these observations, our preliminary *in vivo* studies have shown that the administration of NNU-023 at a dose of 5 mg/kg/day is highly effective in reducing the mortality rate of HSV-2-infected mice. When evaluated under the same conditions, on the other hand, ACV had little or no effect on the mortality rate even at the dose of 50 mg/kg/day[13]. We also found that NNU-023 did not induce any overt signs of toxicity in mice, which received intraperitoneally the drug at a dose of 20 mg/kg/day for 10 days. We thus conclude that NNU-023 is a compound worth further investigation.

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