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RAPID COMMUNICATIONS

EFFICIENCY OF PHOSPHORYLATION OF THE CYCLOBUT-G (A-69992) ENANTIOMERS BY HSV-1 THYMIDINE KINASE DOES NOT CORRELATE WITH THEIR ANTI-HERPESVIRUS ACTIVITY

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A novel class of antiviral compounds has been developed which is based on the structure of oxetanocin, an unusual nucleoside isolated from *Bacillus megaterium* which contains an oxetane ring in an N-glycoside linkage with adenine (1). Analogues of oxetanocin have been synthesized by several independent laboratories and shown to have potent antiviral activity (2-4). Cyclobut-G (A-69992)† (see inset of Fig. 2) is a carbocyclic oxetanocin analogue which has a guanine base and shows considerable promise as a useful therapeutic agent against HIV-1 (2, 5) and herpesviruses (including cytomegalovirus) (2-4, 6). Because of its potency, novel structure, and activity against viruses that are not known to encode a thymidine kinase, we have begun to investigate the mode of action of cyclobut-G.

The only reported mechanistic study of cyclobut-G was done with a racemic mixture of enantiomers (see legend to Fig. 2 for description of enantiomeric configurations). It was shown that Herpes Simplex Virus 1 (HSV-1) thymidine kinase readily phosphorylates (±)-cyclobut-G, suggesting that this virally-encoded enzyme catalyzes the initial phosphorylation reaction essential for antiviral activity against HSV-1 (4). We have extended this work by assessing the relative potency of each cyclobut-G enantiomer against HSV-1 and correlating these data with the ability of viral thymidine kinase to phosphorylate each form. We report here that the antiviral activity of cyclobut-G against HSV-1 may be attributed to just a single enantiomer. Surprisingly, it is the opposite enantiomer that is preferentially phosphorylated by HSV-1 thymidine kinase. The implications that these data have on the mode of action of cyclobut-G are discussed.

MATERIALS AND METHODS

Cyclobut-G (A-69992). All compounds were synthesized at Abbott Laboratories. (±)-Cyclobut-G was prepared as described (2). The methods used for the synthesis of the individual enantiomers and the confirmation of configuration by X-ray diffraction will be described elsewhere.¤ [^{3}H]Cyclobut-G forms were prepared from the 8-bromo analogues of (+)-, (-)-, and (±)-cyclobut-G by dehydrohalogenation.§

Virus growth and assay. HSV-1 strains (E377 and R3820) were grown and titered in monolayers of Vero cells. Plaque reduction assays were also done with Vero cells in 6-well culture plates. Confluent monolayers were infected with virus for 1 hr, washed, and overlayed with maintenance medium containing carboxymethylcellulose and the concentrations of drug indicated in Fig. 1. After 3-4 days the monolayers were fixed, stained with crystal violet, and counted. Assays were done in duplicate. Control wells routinely had approximately 150 viral plaques.

HSV-1 thymidine kinase. Thymidine kinase was partially purified from Vero cells infected with HSV-1 (R3820), a strain that induces a several-fold excess of viral thymidine kinase (7). Cells were infected prior to confluency and grown for 18 hr at 34°. After removal of growth medium the cells were extracted with buffer containing NP-40 as described (8), centrifuged, processed by DE-52 chromatography, and used without further

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† Also known as BHCG and SQ 33,054.
¤ Norbeck D, Herrin T, Hsiao C-N and Spanton S, manuscript in preparation.
§ Rotert G and Thomas S, manuscript in preparation, cited with permission.

purification. A standard DE-81 paper assay was used to quantitate thymidine and cyclobut-G kinase activity (9) in 100-μL reaction mixtures containing 20 μM nucleoside substrate and the other components listed below for the large-scale preparation of cyclobut-G monophosphate.

Preparation and purification of cyclobut-G monophosphate. Cylcobut-G was phosphorylated in 7.5-mL reaction mixtures containing: 200 mM Tris-Cl, pH 7.5; 2 mM ATP; 4 mM $MgCl_2$; 10 mM dithiothreitol; 10 mM NaF; 4 mM phosphocreatine; 100 μM (±)-cyclobut-G containing [^{3}H]-(±)-cyclobut-G to follow the reaction; and 40 units/mL phosphocreatine kinase (Sigma). The reaction was started by the addition of 1.4 mL of partially purified viral thymidine kinase, run at 37° for 3 hr, and passed through a 3-mL column of AG1-X4 resin (Bio-Rad). The column was washed with 50 mL H_2O and enough 10 mM HCl to bring the effluent to pH 2. The cyclobut-G monophosphate was eluted with 30 mM HCl. A 1 M HCl wash of the column did not elute any additional counts, indicating that no additional phosphorylation took place. Fractions containing cyclobut-G monophosphate were combined, neutralized, lyophilized, and desalted using a Sep-Pak cartridge (Baker) (10). The sample was then purified by reversed-phase HPLC on a Waters μBondapack C_{18} column using a volatile triethylammonium bicarbonate buffer (10). Control studies confirmed that the (+)-, (-)-, and (±)-cyclobut-G forms had identical retention times. The triethylammonium salt of the cyclobut-G monophosphate fraction was prepared for NMR spectroscopy by drying, followed by successive washing and drying cycles using methanol, and then D_2O. The final sample was dissolved in 0.5 mL of 99.9% D_2O. The yields of the monophosphate were typically 25-30% of the starting material under these conditions.

NMR. All NMR spectra were recorded on a Bruker AM500 NMR spectrometer. The double quantum filtered COSY spectrum (11) was acquired as 263 complex t_1 values of 1024 complex points using a spectral width of 5000 Hz. The NMR data were processed with a CSPI minimap array processor interfaced to a Vax 8350 computer using in-house software written in the format of the FTNMR program of Dr. D. R. Hare. A sinebell window function shifted by 45 degrees was applied in both dimensions before Fourier transformation.

The ^{31}P-edited ^{1}H NMR spectrum (12) was acquired using a reverse probe with the outer coil tuned to 202.5 MHz (^{31}P) using a pulse sequence that has been described previously (13,14) except that no ^{31}P was applied during the acquisition time. A delay of 30 msec was used to create proton magnetization antiphase with respect to ^{31}P. On alternate scans a 180 degree ^{31}P pulse was applied, and the receiver phase was inverted, yielding a proton NMR spectrum containing only those protons coupled to ^{31}P.

The spectra shown in Fig. 2 were acquired with a sample containing approximately 250 nmol cyclobut-G monophosphate prepared as described above.

RESULTS

The anti-herpesvirus activity of the different cyclobut-G enantiomeric forms and their rate of phosphorylation by HSV-1 thymidine kinase are shown in Fig. 1. The plaque-reduction assay (panel A) was done with HSV-1 (E377), and it shows that the anti-herpesvirus activity of cyclobut-G can be attributed to the (+) enantiomer. Both the (+) enantiomer and the racemic mixture had IC_{50} values significantly less than 1 μM against this strain of HSV-1, which is in agreement with previous results (2,4,6). In contrast, the (-) enantiomer of cyclobut G had no detectable effect on virus production at concentrations at which the (+) enantiomer completely prevented plaque formation ($IC_{50} > 10$ μM). The slight increase in potency observed with the (+) enantiomer, when compared to the racemate, likely reflects a higher concentration of the biologically active form of the drug.

The mode of action and possible routes of intracellular activation of cyclobut-G are of considerable interest since this drug is active against viruses that do not appear to encode a thymidine kinase (2,3,5). In an attempt to reconcile these observations with the possible role for HSV-1 thymidine kinase suggested by studies with the cyclobut-G racemate (4), we examined the rate of phosphorylation of each cyclobut-G enantiomer by this enzyme. Surprisingly, HSV-1 thymidine kinase efficiently phosphorylated the biologically-inactive (-) enantiomer, but demonstrated very low activity when the (+) enantiomer was used as substrate (Fig. 1B). As indicated, an intermediate rate of phosphorylation was observed with the racemate. The rate of phosphorylation shown with the (+) enantiomer at 20 μM was 1/20 of the rate shown for the (-) enantiomer at the same concentration. The K_m values for this reaction were 150 and 350 μM for the (-) and (+) enantiomers respectively. The apparent V_{max}/K_m

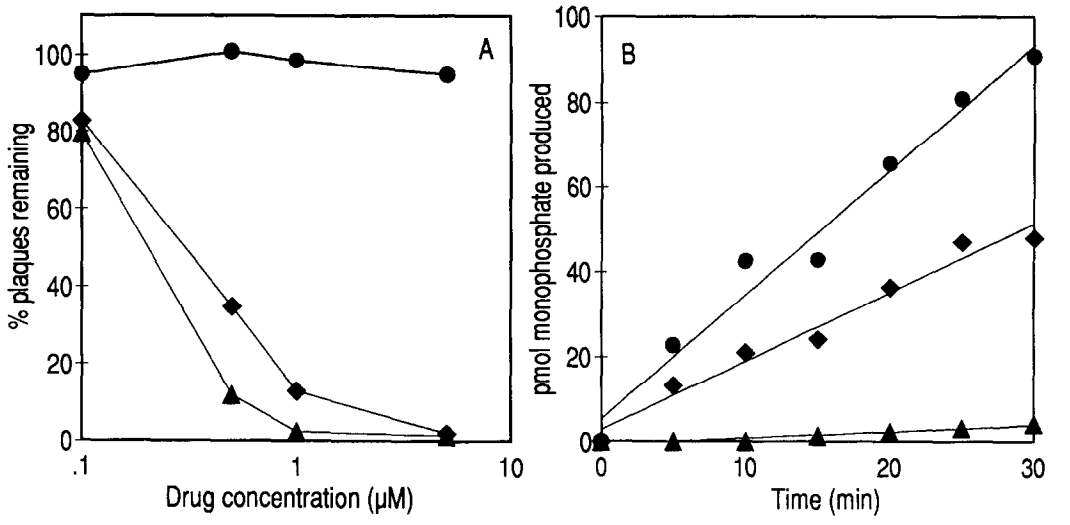

Fig. 1. (A) Plaque-reduction assay of the different cyclobut-G forms using HSV-1(E377). Compounds were added at the indicated concentrations to the maintenance medium of Vero cell monolayers after viral infection. The numbers of viral plaques were determined as described in Materials and Methods. (B) Time course of phosphorylation of the different cyclobut-G forms (20 μM) by partially purified HSV-1 thymidine kinase. The scale indicates the amounts of monophosphate produced in 100-μl assay mixtures. The (+)-, (-)-, and (±)-cyclobut G forms are indicated by (▲), (●) and (◆), respectively, in both panels.

obtained with the (+) enantiomer as substrate was 1/30 of the value obtained with the (-) enantiomer (data not shown), confirming that the (+) enantiomer is indeed a poor substrate for HSV-1 thymidine kinase.

In view of these results and the unusual structure of cyclobut-G, we wished to determine which hydroxyl was phosphorylated on the different enantiomeric forms by HSV-1 thymidine kinase. An NMR study was first done with the purified monophosphate species generated by HSV-1 thymidine kinase-catalyzed phosphorylation of (±)-cyclobut-G. Figure 2 depicts a portion of the 2D double-quantum filtered COSY spectrum in which the resonances of the cyclobutane group are found (spectrum A). The off-diagonal peaks are due to through-bond coupling and they allow the indicated proton assignments to be made. The overlay (spectrum B) is a phosphorus-edited proton NMR spectrum of the product formed by thymidine kinase in which only those protons that are coupled (three bond coupling) to the phosphorus are detected. The only significant resonance that was observed in the phosphorus-edited experiment (spectrum B) can be unambiguously assigned by comparison of chemical shifts to the methylene protons of the side chain at C-3, establishing this position as the site of phosphorylation of the (-) enantiomer. Since it was difficult to establish the level of (+)-cyclobut-G monophosphate in this sample, a proton NMR spectrum was taken of a small sample (25 nmol) of purified (+)-cyclobut-G monophosphate generated during an extended incubation with HSV-1 thymidine kinase. The proton chemical shifts of the cyclobutane ring in (+)-cyclobut-G monophosphate were identical to those shown in Fig. 2 (data not shown). These data establish the site of phosphorylation at the pseudo 5' position* for both the (-) and (+) enantiomers, and they rule out the interesting possibility that differences in the site of phosphorylation account for differences in the antiviral activity seen with these two cyclobut-G forms. There was no evidence in the NMR spectra for any modification of the guanine base during these incubations.

DISCUSSION

Our studies demonstrate that the (+) enantiomer of cyclobut-G is the form that has potent antiviral effects against HSV-1, whereas the (-) enantiomer has little detectable activity in a therapeutically useful range. Cyclobut-G is unusual in that HSV-1 thymidine kinase is much more efficient at phosphorylating the less active (-) enantiomer. The difference in antiviral activity of these two cyclobut-G forms does not result from different phosphorylation sites since our NMR data indicated that both enantiomers are phosphorylated at the C-3 CH_2OH

* A similar result was noted without experimental detail in Ref. 4, which appeared during the latter stages of these studies.

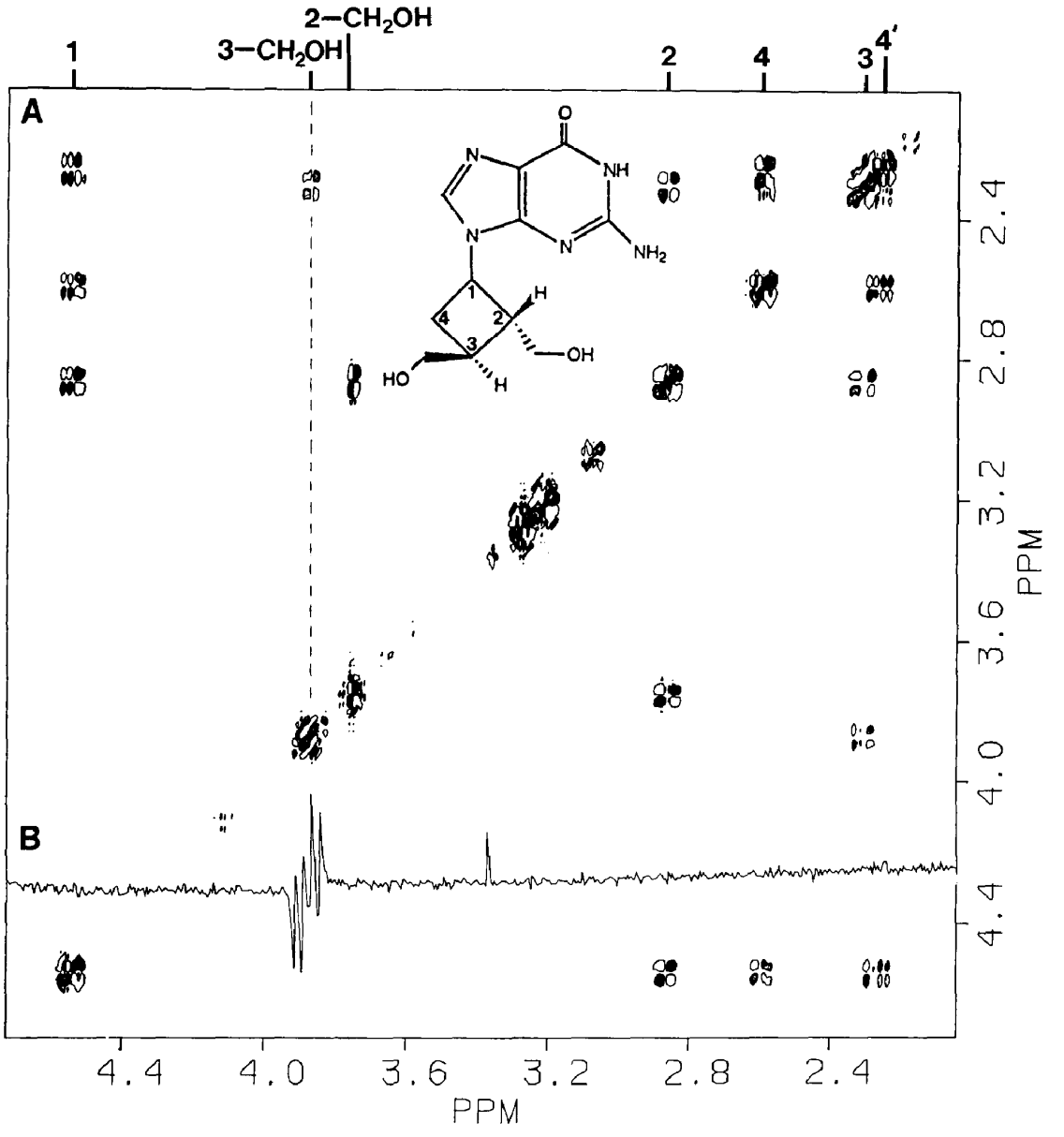

Fig. 2. NMR spectra of cyclobut-G monophosphate. The inset shows the structure of the biologically active (+)-cyclobut-G enantiomer. Proton resonances are labeled according to which carbon atom the proton is bound to. The resonances designated as CH_2OH refer to the methylene protons in the alkyl side chains. (-)-Cyclobut-G (not shown) has a CH_2OH group at the indicated C-4 position (which would be redesignated as C-2) in the "down" configuration rather than at the indicated C-2 position. The enantiomers cannot be distinguished by NMR spectroscopy; thus, the designated resonances are identical for both enantiomers. (Spectrum A) 2D Contour plot of a phase-sensitive double quantum filtered COSY NMR spectrum of the cyclobut-G monophosphate formed by herpes thymidine kinase using (±)-cyclobut-G as substrate. (Spectrum B) ^{31}P-edited ^{1}H-NMR spectrum of the same sample. The vertical dashed line indicates the coupling between the phosphate and the methylene protons at C-3 (pseudo 5' position).

group by HSV-1 thymidine kinase. These results have important implications for the therapeutic applications of cyclobut-G and they demonstrate a distinct stereoselectivity by HSV-1 thymidine kinase, which is commonly viewed as a relatively nonspecific enzyme. It is interesting to note that thymidine kinase stereoselectively phosphorylates a prochiral center in ganciclovir yielding a single enantiomer of the chiral monophosphate product (15).

Although the (+) enantiomer is not phosphorylated efficiently by HSV-1 thymidine kinase and cyclobut-G is active against viruses that do not encode a thymidine kinase (2,3,5), our data do not rule out the possibility that viral thymidine kinase can enhance the antiviral effects of cyclobut-G. Indeed, some loss of potency is seen against tk^- HSV mutants. In studies to be reported elsewhere*, an approximately 5-fold difference was seen between IC_{50} values obtained with (±)-cyclobut-G against tk^+ and tk^- strains derived from HSV-1 and HSV-2. The antiviral effects against the tk^- mutants were seen at drug concentrations far below the minimum toxic dose of drug against the host (Vero) cells, which is consistent with the drug effects being virus-specific rather than being a result of host-cell toxicity. In a separate study, (±)-cyclobut G was reported to be 25-fold less potent against a tk^- HSV-2 mutant (4). Although the reason for these different ratios is unclear, these data do suggest that sufficient levels of (+)-cyclobut-G derivatives can be generated by HSV thymidine kinase which have a detectable effect on the observed antiviral activity. Such involvement of HSV thymidine kinase could be best accommodated if (+)-cyclobut-G metabolites have very potent antiviral effects and if the activation of cyclobut-G by cellular enzymes is relatively inefficient as well. Consistent with this, no phosphorylation of (+)-cyclobut-G could be detected, at the present level of sensitivity, using extracts from mock-infected cells. Cyclobut-G could thus be effective in the absence of viral thymidine kinase and have enhanced activity in its presence. However, establishing the relative contributions from viral and host-cell kinases in activating cyclobut-G requires a more in-depth study of the residual kinase activity in the tk-deficient HSV mutants.

Addressing the questions raised by these data will require careful comparison of intracellular pool levels of the phosphorylated derivatives of each enantiomer, identification of cellular enzymes capable of generating cyclobut-G derivatives, and assessment of the potency of these derivatives against various targets that may interfere with virus production. It is also necessary to further define what, if any, role (-)-cyclobut-G and its derivatives play in the antiviral action of cyclobut-G, and to determine if any synergistic effects on antiviral activity occur when both enantiomers are present. Such studies should provide further insight into the mode of action of this important new class of nucleoside analogues and yield additional information that may prove useful in the design of new therapeutic agents directed against HIV and other AIDS-related viruses.

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