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RIBONUCLEOTIDE REDUCTASE INDUCED BY VARICELLA ZOSTER VIRUS

CHARACTERIZATION, AND POTENTIATION OF ACYCLOVIR BY ITS INHIBITION

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Abstract—An enzyme that catalyzes the conversion of CDP to 2′-dCDP in the presence of dithiothreitol (DTT) was detected in ammonium sulfate fractionated-extracts of varicella zoster virus (VZV)-infected cells. This ribonucleotide reductase was antigenically distinguishable from the isofunctional eucaryotic enzyme as well as the ribonucleotide reductases induced by herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). The VZV-induced enzyme was purified to the extent that most of the contaminating enzymes, which would significantly deplete the substrate, were removed. The VZV-induced ribonucleotide reductase exhibited maximum activity in the absence of ATP and/or magnesium and was only weakly inhibited by 2′-deoxynucleoside triphosphates. Furthermore, ADP, UDP and GDP competitively inhibited CDP reduction with K_i (K_m) values of 15, 20, 1.8 and 0.88 μM, respectively. These kinetic properties were very similar to those of the correspondingly purified ribonucleotide reductases induced by HSV-1 [Averett *et al.*, *J. biol. Chem.* **258**, 9831 (1983)] and HSV-2 [Averett *et al.*, *J. Virol.* **52**, 981 (1984)] and were dissimilar to the allosterically regulated mammalian enzyme. A723U, an inactivator of HSV-1 ribonucleotide reductase that potentiates the anti-HSV-1 activity of acyclovir [Spector *et al.*, *Proc. natn. Acad. Sci. U.S.A.* **82**, 4254 (1985)], also appeared to inactivate this VZV-induced ribonucleotide reductase and to potentiate the anti-VZV activity of acyclovir.

Herpes simplex viruses (HSV)† induce unusual ribonucleotide reductases that differ from their mammalian and bacterial counterparts in that they are not sensitive to allosteric regulators. Studies with ribonucleotide reductase from crude extracts [1], ammonium sulfate fractionated-extracts [2–4], and preparations purified to the extent that the substrates were not extraneously depleted [5, 6] demonstrated that the HSV enzymes are neither inhibited nor activated by the deoxynucleoside and ribonucleoside triphosphates that exquisitely regulate the mammalian and bacterial enzymes (reviewed in Ref. 7). In lieu of allosteric regulation, the HSV ribonucleotide reductases appear to be regulated by competition between substrates. All four diphosphate nucleosides compete for reduction at a common catalytic site, which is fully active in the absence of magnesium and/or any nucleotide effector [5, 6].

The findings that HSV ribonucleotide reductases are virally encoded ([8], reviewed in Ref. 9) and appear to be very important for virus replication [10, 11] emphasize the importance of these enzymes as potential chemotherapeutic targets. Recent studies have demonstrated that inhibition of the HSV ribonucleotide reductases correlates with inhibition of HSV replication [12, 13] and that certain inactivators of HSV-1 ribonucleotide reductase are capable of potentiating the antiviral activity of acyclovir (ACV) [12].

Varicella zoster virus (VZV), another member of the herpes virus group, induces a thymidine kinase [14] and DNA polymerase [15, 16] that are similar to those induced by HSV [17]. Furthermore, it was reported recently that an antibody directed against the carboxy terminal amino acids of the small subunit of HSV-1 ribonucleotide reductase precipitates a protein of similar molecular weight from extracts of VZV-infected cells [18]. Another report described the sequence of the VZV genome, which contains regions of predicted amino acid sequences that correspond to and share some homology with the small and large subunits of HSV-2 ribonucleotide reductase [19]. In view of these observations and the importance of VZV as a human pathogen, the following study was initiated. A ribonucleotide reductase, which is antigenically different from HSV and mammalian ribonucleotide reductases, was detected and partially purified from VZV-infected cells. Its kinetic properties are very similar to those of the HSV enzymes. Furthermore, A723U appeared to inactivate this enzyme and to potentiate the anti-VZV activity of ACV.

† Abbreviations: HSV, herpes simplex virus: HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; ACV, acyclovir (9-[(2-hydroxyethoxy)methyl]-guanine); ACV-TP, acyclovir triphosphate; VZV, varicella zoster virus; DTT, dithiothreitol; A723U, 2-acetylpyridine 4-(2-morpholinoethyl)thiosemicarbazone; and HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Reagents. Polyclonal anti-HSV-2 ribonucleotide reductase rabbit antiserum R1 was generously pro-

vided by Dr. Sylvia Bacchetti. Rat monoclonal anti-tubulin antibody YL1/2 (as ascites fluid) was purchased from Seralab. IgG from these sources was prepared by ammonium sulfate precipitation followed by column chromatography on protein A-Sepharose [20]. Rat monoclonal YL1/2 did not bind strongly to protein A-Sepharose but was adequately retarded to allow purification. Following elution from the protein A-Sepharose, fractions containing IgG were detected by direct ELISA, pooled, concentrated by ammonium sulfate precipitation, and dialyzed against phosphate-buffered saline. The IgG fractions were diluted to a final protein concentration of 1 mg/ml and stored at 5°. HSV-1 [6], HSV-2 [5] and eucaryotic (isolated from human D98 cells [21]) ribonucleotide reductases were purified as described in those references. All other reagents were obtained as previously described [5, 6].

Enzyme assays. Ribonucleotide reductase activity was assayed according to the method of Steeper and Steuart [22] for CDP reduction as modified in this laboratory [6]. Standard reaction mixtures contained 200 mM potassium HEPES, pH 7.7*, 10 mM dithiothreitol (DTT), and the concentration of [^{14}C]CDP as noted. The [^{14}C]CDP was purified and reactions were terminated with EDTA and hydroxyurea in order to achieve blanks (enzyme omitted) with less than 0.02% of the total radioactivity [6]. One unit of ribonucleotide reductase reduces one nmol CDP per hr. Phosphatase activity was determined from the amount of CMP and Cyd formed from CDP by the TLC procedure previously described [6]. Nucleoside diphosphate kinase was determined by TLC analysis [6] of the amount of CTP produced from CDP in the presence of 5 mM $MgCl_2$ and 5 mM ATP.

Cell culture and viral infection. Procedures for the growth of human lung fibroblasts (MRC-5) and propagation of VZV (Oka strain) have been described previously [23]. VZV ribonucleotide reductase was isolated from infected cultures that had been established by mixing VZV-infected cells and uninfected cells at a ratio of 1:3. After the infection was allowed to proceed for about 24 hr, the cells were washed with phosphate-buffered saline and harvested by scraping. The cell pellet was stored at −70°.

ELISA assay for VZV. The inhibitory effects of ACV and A723U on the replication of VZV (Oka strain) in MRC-5 cells were assessed by an ELISA procedure [24] that was modified as follows. Infections were initiated in the presence of drug, rather than before drug addition. At the end of the 3-day incubation, the 96 well plates were centrifuged for 5 min at 200 *g* to sediment detached cells prior to glutaraldehyde fixation. The ELISA used an alkaline phosphatase-conjugated anti-human IgG as the second antibody. The rate of cleavage of *p*-nitrophenyl phosphate by bound alkaline phosphatase was determined as previously described [25]. Uninfected cells were used to obtain the blank reaction rates, which were subtracted from the rates obtained with virus present. This assay was suitable to detect progeny virus antigen in cultures that were initially infected with 15 to 3600 infectious particles/well.

The concentration of drugs resulting in 50% of control values (IC_{50}) was calculated with a computer program which analyzes the data according to the log[drug] versus percent inhibition algorithm. Data in the range of 20 to 80% of control were used in these calculations.

Enzyme purification. The purification procedure was the same as that used for HSV-1 and HSV-2 ribonucleotide reductases [5, 6] except for the preparation of the extracts. VZV-infected cells were lysed in 50 mM Tris–Cl, pH 7.5, containing 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose, and 5 mM 2-mercaptoethanol. A protease inhibitor mixture (individual inhibitors purchased from the Sigma Chemical Co.) consisting of 50 μg/ml each of aprotinin, leupeptin, benzamidine, ovo-mucoid, and soybean trypsin inhibitor was added to the lysis buffer. The supernatant fraction from a 105,000 *g* centrifugation was brought to 50% saturation by the addition of 0.29 g solid ammonium sulfate/ml extract. The precipitate from this fractionation was suspended and dialyzed. The clarified retentate was then applied to an ATP-agarose affinity column that retains nucleoside diphosphate kinases but not ribonucleotide reductase [5, 6]. Fractions were assayed for ribonucleotide reductase, nucleoside diphosphate kinase, and phosphatase. Fractions containing ribonucleotide reductase were dialyzed against 50 mM potassium Hepes, pH 7.6, containing 2 mM dithiothreitol (DTT) and stored at −70°.

Miscellaneous. Kinetic constants were calculated by computer analysis [26] of initial velocities where the amount of product formed was linear with respect to time. Inhibition data were tested for conformity to the common models of enzyme inhibition [27]. Protein concentrations were determined by the refined Coomassie blue method [28].

RESULTS AND DISCUSSION

Detection. The resuspended and dialyzed precipitate of ammonium sulfate-fractionated VZV-infected cell extracts was assayed for CDP reducing activity using the standard assay with 10 μM CDP and no Mg^{2+} or ATP. A time-dependent positive signal was clearly detected. In contrast, no activity was detected (<3% of the activity observed in infected extracts) in uninfected cell extracts assayed under the same conditions. This detection of a VZV-induced ribonucleotide reductase supports the recent reports predicting the presence of this enzyme [18, 19].

The VZV-induced ribonucleotide reductase could be distinguished from the isofunctional enzymes from other sources by antibody studies. The polyclonal anti-HSV-2 ribonucleotide reductase antibody (R1) inhibited the ribonucleotide reductases from the following sources with the following order of potency: HSV-2 > HSV-1 > VZV. The eucaryotic enzyme (isolated from human D98 cells) was not inhibited (Fig. 1A). The monoclonal anti-tubulin antibody, which was found to bind to the carboxy

* This buffer is identical to that used in the previous studies of HSV-1 and HSV-2 ribonucleotide reductases. Although the pH of the 1 M stock is 8.0, the actual pH of the 0.20 M buffer in the reaction at 37° was 7.7.

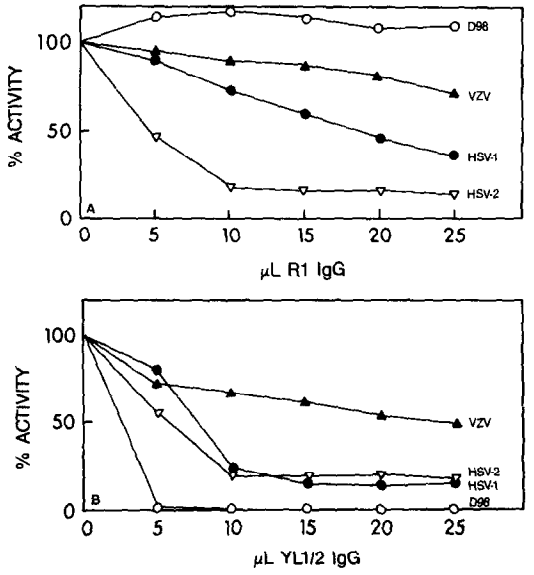

Fig. 1. Inhibition of ribonucleotide reductases from various sources by antibodies. The ribonucleotide reductases (0.01 units) were incubated at 37° for 1 hr in 50-μl mixtures containing 10 mM potassium phosphate, pH 7.3, 46 mM NaCl, 1 mM DTT, and the indicated volumes of stock solutions (1 mg/ml) of IgG. Aliquots were then assayed for CDP reduction. (A) R1 IgG, a polyclonal anti-HSV2 ribonucleotide reductase antibody (rabbit). (B) YL1/2, a monoclonal anti-tubulin antibody (rat), which also neutralizes eucaryotic (D98) ribonucleotide reductase.

terminus of the M2 subunit of eucaryotic ribonucleotide reductases [29, 30], was also studied. The potency of inhibition was human ≫ HSV-2 ≅ HSV-1 > VZV for ribonucleotide reductases from these sources (Fig. 1B). These antigenic dissimilarities between HSV and VZV ribonucleotide reductases are consistent with the data of the recent study mentioned above [18]. Their antibody [18] to the small HSV-1 subunit was incubated with VZV-infected cellular extracts. Although this antibody coprecipitates the small and large subunits of HSV-1 ribonucleotide reductase, it only precipitates one protein from the VZV-infected extracts [18]. This protein had a molecular weight similar to the small subunit of HSV-1 ribonucleotide reductase.

Purification. The enzyme from the ammonium sulfate-fractioned preparation was further purified on an ATP-agarose affinity column. Ribonucleotide reductase eluted in a sharp symmetrical peak just after the void volume. Its specific activity varied with the concentration of protein in the assay (Fig. 2). For example, it was 0.20 units/mg protein at 0.1 mg/ml and 0.80 units/mg protein at 1 mg/ml when assayed at 10 μM CDP. Although it is difficult to compare quantitatively the various preparations, the amount of enzyme in these preparations was much less than that obtained by the same method from cells infected with HSV-1 [6] or HSV-2 [5]. Because VZV infections are not propagated by the addition of cell-free virus (see Materials and Methods), as are HSV infections, the multiplicity of infection may be considerably lower, which may account for this difference.

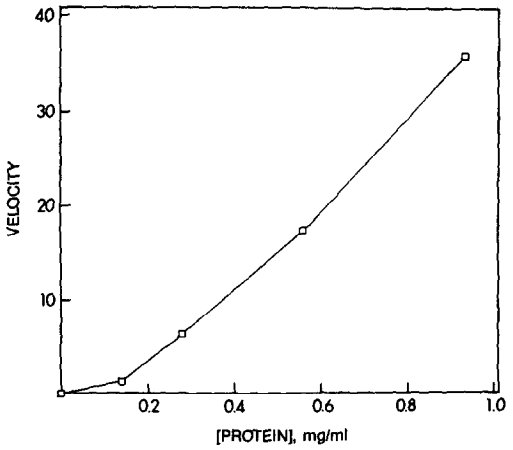

Fig. 2. Dependence of amount of product formation on the concentration of VZV-induced ribonucleotide reductase. CDP reduction was measured in reactions containing 200 mM HEPES (pH 7.7), 10 mM dithiothreitol, 10 μM [^{14}C]CDP, and the indicated concentrations of partially purified ribonucleotide reductase.

The residual nucleoside diphosphate kinase, which was not retained by the ATP column, was present at 0.58 units/mg when assayed in the presence of 5 mM ATP and $MgCl_2$. It was not detectable in the absence of ATP and $MgCl_2$.

Some fractions collected from the ATP-agarose column had ratios of ribonucleotide reductase to CDP phosphatase of >4:1, and >98% of the substrate and/or product remained as the diphosphate at the end of the standard 20-min reaction. Other fractions contained significant phosphate cleaving activity. The addition of 0.4 mM dTTP inhibited this phosphatase by 90% while inhibiting the ribonucleotide reductase by less than 10%. The dTTP improved the stability of CDP in assays of these fractions to the same level (97–99%) as in reactions with fractions that were relatively free of phosphatase. Furthermore, similar K_m values for CDP were obtained in the presence of 0.4 mM dTTP with enzyme samples that contained high levels of phosphatase and in the absence of dTTP with enzyme samples having negligible phosphatase. Thus, it was concluded that dTTP could be used to inhibit the phosphatase without interfering with the kinetic studies of ribonucleotide reductase.

The identity of the ribonucleotide reductase reaction product was verified (following snake venom treatment) for each batch of enzyme by HPLC analysis [6]. Greater than 90% of the radiolabel eluting from the Dowex-1-borate column co-eluted with dCyd.

The enzyme was relatively stable during reactions at 37°. Deviation from linearity was <10% over 20-min reactions for CDP concentrations ranging from 0.5 to 12 μM.

Properties. Although the paucity of obtainable enzyme limited the depth of an investigation of its properties, it was feasible to characterize the key features that distinguish the VZV ribonucleotide reductase. To begin, the reaction conditions were optimized with respect to buffer concentration, pH, and concentration of DTT. The reaction rate

Table 1. Inhibition of VZV-induced ribonucleotide reductase*

Inhibitor	% Inhibition	
	1 mM	5 mM
dATP	12	55
dTTP	19†	60†
dCTP	38	84
dGTP	63	95
ACV-TP	42	81
$MgCl_2$	22	28
ATP	24	71
$MgCl_2$-ATP‡	45	60

* Assayed in duplicate at 1.8 μM CDP (two times K_m).

† The reported values of 1 and 5 mM dTTP are in addition to the 0.4 mM dTTP present in all reactions as a phosphatase inhibitor.

‡ The [ATP–Mg^{2+}] complex was calculated [35] to be 0.88 mM and 4.73 mM in solutions containing 1 mM $MgCl_2$ and 1 mM ATP or 5 mM $MgCl_2$ and 5 mM ATP respectively.

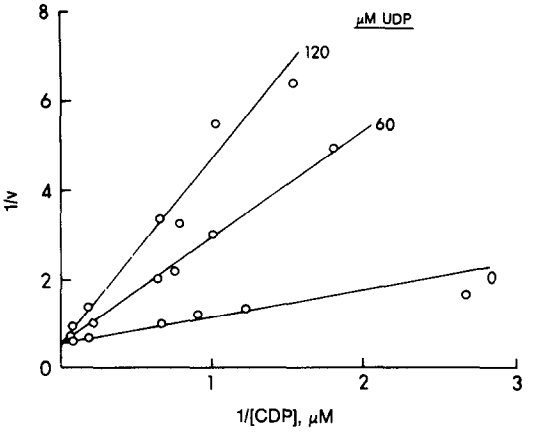

Fig. 3. Competitive inhibition of CDP reduction by UDP. Reactions contained 200 mM HEPES (pH 7.7), 10 mM dithiothreitol, 0.4 mM dTTP, partially purified VZV-ribonucleotide reductase, and the indicated concentrations of [^{14}C]CDP and UDP.

increased sharply (3.4-fold) with increasing concentration of HEPES between 50 and 200 mM at pH 7.7. The rate at 400 mM HEPES was only 12% greater than the rate at 200 mM HEPES. The activity varied slightly between pH 7.1 and 7.9 with maximum activity at pH 7.7.*

CDP reduction was dependent upon DTT. The optimum concentration of DTT is in the range of 5 to 10 mM. Inhibition occurred above this range. The standard assays therefore contained 200 mM HEPES, pH 7.7, and 10 mM DTT.

Hydroxyurea, an inhibitor of HSV ribonucleotide reductases [3, 5, 6, 31] and most other ribonucleotide reductases (reviewed in Ref. 32), also inhibited the VZV-induced enzyme. An IC_{50} value of 250 ± 30 μM was obtained when the initial concentration of CDP was 1.7 μM.

As had been observed with the HSV-induced enzymes [2, 5, 6], the amount of product formed was not a linear function of enzyme concentration (Fig. 2). A plot of velocity versus the square of the enzyme concentration did not linearize the graph as was observed previously with data from some other ribonucleotide reductases [33]. It is believed that nonlinear response with those enzymes was due to dissociation of the subunits [33].

The VZV ribonucleotide reductase is similar to the HSV (reviewed in Ref. 7) enzymes and different from mammalian ribonucleotide reductases [29, 34] in that it is refractory to inhibition by dTTP. An IC_{50} value of 2 mM dTTP was obtained for the inhibition of the VZV-induced enzyme with an initial CDP concentration of 2 μM. As seen in Table 1, it appears to be somewhat more sensitive to inhibition by some of the other deoxynucleoside triphosphates and acyclovir triphosphate (ACV-TP) than by dTTP. However, it is possible that the apparent inhibition by these triphosphates was actually caused by trace decomposition of these compounds to their corresponding diphosphates, which are inhibitors of HSV-1 ribonucleotide reductase [6].

The effects of $MgCl_2$, ATP, and the ATP–Mg^{2+} complex on the reaction rate were also examined as a way of distinguishing the VZV-induced ribonucleotide reductase from the cellular enzyme. Eucaryotic ribonucleotide reductases have an absolute requirement for ATP–Mg^{2+} as an allosteric activator (reviewed in Refs. 36 and 37), whereas the HSV enzymes are inhibited by ATP or $MgCl_2$, with the inhibition being partially overcome by formation of an ATP–Mg^{2+} complex [2, 4–6]. The VZV-induced ribonucleotide reductase was similar to the HSV enzymes in that it exhibited maximum activity in the absence of ATP and/or Mg^{2+}. However, it differed from HSV-ribonucleotide reductase in that the ATP–Mg^{2+} complex, as well as uncomplexed ATP and uncomplexed Mg^{2+}, was inhibitory (Table 1). In fact, 1 mM ATP–Mg^{2+} complex produced more inhibition than either 1 mM Mg^{2+} or ATP.

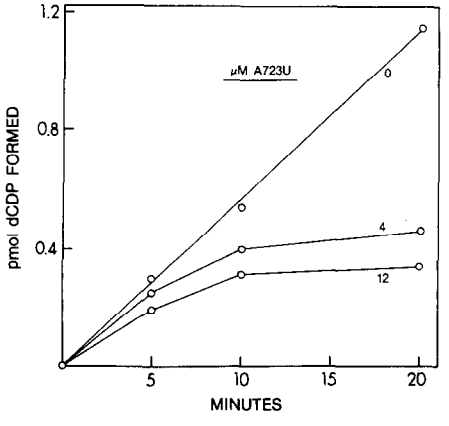

Fig. 4. Apparent inactivation of VZV-induced ribonucleotide reductase by A723U. Reactions contained 200 mM HEPES (pH 7.7), 10 mM dithiothreitol, 2 μM [^{14}C]CDP, partially purified ribonucleotide reductase, and the indicated concentrations of A723U.

* This buffer is identical to that used in the previous studies of HSV-1 and HSV-2 ribonucleotide reductases. Although the pH of the 1 M stock is 8.0, the actual pH of the 0.20 M buffer in the reaction at 37° was 7.7.

Table 2. Potentiation of the anti-VZV activity of ACV by A723U

A723U (μM)	IC_{50} of ACV (μM) Calculated* (additive inhibition)	Observed (±SE)	Potentiation (fold)†
0	30	30 ± 5	
4.5 (0%)‡	30	21 ± 2	1.4
6.8 (5%)	28	3.5 ± 3	8.0
10 (36%)	11	0.6 ± 0.4	18

* The theoretical IC_{50} for independent (additive) interactions between ACV and A723U was calculated by analysis of curves generated by multiplying the fraction of the control observed for A723U alone by the fraction of the control observed for ACV alone, at multiple concentrations of ACV.

† The ratio of the calculated IC_{50} divided by the observed IC_{50}.

‡ Numbers in parentheses are the percent inhibition obtained for A723U in the absence of ACV. The IC_{50} for A723U was 12 μM.

The average K_m (three determinations) for CDP was 0.88 μM (range = 0.72 to 1.08 μM ± 5–16% SE), which is similar to that for the HSV-1 and HSV-2-induced enzymes [5, 6] and lower than for mammalian ribonucleotide reductase [34]. As with HSV-1 ribonucleotide reductase [6], UDP, ADP, and GDP were found to be competitive inhibitors of CDP reduction with K_{is} values of 20 ± 2, 15 ± 3 and 1.8 μM ± 0.3 μM respectively. The pattern of Fig. 3, with UDP as the inhibitor, is a typical representation of inhibition by these putative alternative substrates. The K_{is} values for these nucleoside diphosphates are similar to the values obtained with the HSV-1 enzyme, where it was found that the K_{is} value of each substrate was approximately equal to its K_m value [6]. A similar relationship between CDP and ADP was observed with HSV-2 ribonucleotide reductase [5]. The conclusions reached for the HSV ribonucleotide reductases [5, 6], that the reduction of all four substrates occurs at a common site and that substrate-competition affords a means of regulation, are probably applicable to the VZV enzyme.

Inactivation. The 2-acetylpyridine thiosemicarbazone, A723U, which inactivates HSV-1 ribonucleotide reductase [12] also appeared to inactivate the VZV-induced enzyme (Fig. 4). This type of progressive inhibition, which increases as the reaction proceeds, is characteristically the result of enzyme inactivation.

Inhibition of VZV replication and potentiation of ACV. The replication of VZV *in vitro* was assessed by the ELISA described in Materials and Methods. A723U inhibited replication with an IC_{50} of 12 ± 2 μM. The finding that inhibition of replication occurs at concentrations of A723U that readily inactivate the induced ribonucleotide reductase suggests that this enzyme may be important to the replication cycle of VZV. Furthermore, the combination of A723U with ACV resulted in a marked synergistic inhibition of VZV replication. The ability of different concentrations of A723U to decrease the IC_{50} of ACV beyond the decrease expected for independent (additive) antiviral activities is shown in Table 2.

Since unhealthy cells could have impaired capacity to support virus replication, it was necessary to test A723U for cytotoxic effects. Mock-infected MRC-5 monolayers (40,000 cells/well) were incubated with A723U and ACV in the same manner as were the infected cells. After the 3-day period, the cells were stained with the vital stain neutral red to assess cell number.

A723U, at concentrations of 3–23 μM, appeared to be modestly cytotoxic, causing an approximate 25% decrease in staining compared to untreated control monolayers. However, when tested against actively growing cultures of uninfected MRC-5 cells (initiated at 1/10 the standard cell number), A723U was quite inhibitory with an IC_{50} of 4 μM. It is possible that the apparent cytotoxicity observed with "confluent" monolayers was due to the growth inhibition of residual cell replication within the monolayer. It is important to note that ACV, at concentrations from 0.3 to 260 μM, did not alter the effects of A723U on uninfected MRC-5 cells. Therefore, although quantitation of the antiviral potency of A723U may be affected by its apparent mild cytotoxicity to monolayers, the above determinations of ability of A723U to potentiate ACV were clearly valid assessments.

A723U and ACV were found previously to synergistically inhibit HSV-1 replication [12]. Inactivation of HSV-1 ribonucleotide reductase by A723U not only decreases the pool size of dGTP, but also, by an unknown mechanism, markedly increases the pool of ACV-triphosphate [12]. The net result would be facilitation of the binding of ACV-triphosphate to its target enzyme, the viral DNA polymerase. It is likely that similar events account for the synergistic inhibition of VZV replication. It is hoped that this combined therapy will prove useful in the treatment of human VZV disease.

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