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2-Acetylpyridine 5-[(dimethylamino)thiocarbonyl]-thiocarbonohydrazone (A1110U), a potent inactivator of ribonucleotide reductases of herpes simplex and varicella–zoster viruses and a potentiator of acyclovir

(antiviral chemotherapy/drug potentiation)

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ABSTRACT 2-Acetylpyridine 5-[(dimethylamino)thiocarbonyl]thiocarbonohydrazone (A1110U) was found to be a potent inactivator of the ribonucleotide reductases (EC 1.17.4.1) encoded by herpes simplex virus types 1 and 2 and by varicella–zoster virus and to be a weaker inactivator of human ribonucleotide reductase. It also markedly potentiated the antiherpetic activity of acyclovir against these viruses in tissue culture. A1110U both decreased the dGTP pool that builds up when infected cells are treated with acyclovir and induced a large increase in the pool of acyclovir triphosphate. The resultant 100-fold increase in the ratio of the concentrations of acyclovir triphosphate to dGTP should facilitate the binding of the fraudulent nucleotide to its target enzyme, herpes virus-encoded DNA polymerase, and could account for the synergy between A1110U and acyclovir. A similar change in the acyclovir triphosphate-to-dGTP ratio was previously reported to be induced by another ribonucleotide reductase inhibitor, 2-acetylpyridine 4-(2-morpholinoethyl)thiosemicarbazone (A723U). However, A1110U is considerably more potent and may have better clinical potential. Synergistic toxic interactions between A1110U and acyclovir were not detected in uninfected cells.

Herpes simplex viruses (HSVs) encode distinct ribonucleotide reductases (EC 1.17.4.1) (ref. 1 and reviewed in ref. 2) that are very important to viral replication (3–5). These enzymes are unusual because they are not subject to the allosteric regulation (reviewed in ref. 6) that is characteristic of the isofunctional mammalian and bacterial (reviewed in refs. 7 and 8) ribonucleotide reductases. Recently, a ribonucleotide reductase was also partially purified from cells infected with varicella-zoster virus (VZV) (9). Its kinetic properties were very similar to those of the correspondingly purified HSV-1 (10) and HSV-2 (11) ribonucleotide reductases in that physiological concentrations of deoxy- or ribonucleotide triphosphates neither stimulated not inhibited its activity. The absence of inhibitory regulation permits these enzymes to catalyze the formation of high concentrations of deoxynucleotides for viral DNA synthesis. Unfortunately, unrestricted synthesis of dGTP can potentially antagonize the antiherpetic activity of acyclovir (ACV) because the fully activated form of ACV, acyclovir triphosphate (ACVTP), competes with dGTP for binding to the viral DNA polymerases (12–16). To prevent this competition, inhibitors of the viral ribonucleotide reductases were sought. 2-Acetylpyridine 4-(2-morpholinoethyl)thiosemicarbazone (A723U) was found to inactivate HSV-1 ribonucleotide reductase (17) as well as VZV ribonucleotide reductase (9) and to potentiate the antiherpetic activities of ACV. In addition to producing the expected decrease in the dGTP pool, A723U also caused the pool of ACVTP to increase by 10-fold (17).

In the present study, the properties of 2-acetylpyridine 5-[(dimethylamino)thiocarbonyl]thiocarbonohydrazone (A1110U) were investigated. This compound was found to be considerably more potent than A723U. At submicromolar concentrations, A1110U inactivated the ribonucleotide reductases of these three herpes viruses. It also markedly potentiated the activity of ACV against the viruses *in vitro*. Furthermore, as reported elsewhere (§, ¶), A1110U and ACV produced significantly synergistic therapy as topical treatment of HSV-infected animals.

MATERIALS AND METHODS

Reagents. All reagents not described below were obtained as previously described (11).

Synthesis of A1110U. A mixture of 24.2 g (0.203 mol) of 4,4-dimethylthiosemicarbazide, 26.6 g (0.220 mol) of 2-acetylpyridine, 2.5 ml of glacial acetic acid, and 100 ml of 95% (vol/vol) ethanol was refluxed for 1.5 hr and then incubated overnight at room temperature. Thick orange needles contaminated with a small amount of a light-colored solid were collected, washed with 20 ml of ethanol, and, while still damp, added to 450 ml of boiling methanol. After 10 min, the mixture was filtered. The undissolved solid was re-incubated with 50 ml of boiling methanol for 3 min and then filtered. After 15 min at room temperature, the yellow product recrystallized from the combined filtrates and was collected by filtration. Yield 1.47 g (5%, nonoptimized); mp 152.5°C (evolved a gas, resolidified, and remelted at 175–181.5°C); NMR (^{1}H, deuterated dimethyl sulfoxide) δ 2.43 (s, 3H), 3.28 (s, 6H), 7.40 (m, 1H), 7.86 (m, 1H), 8.37 (m, 1H), 8.62 (m, 1H), 9.65 (br s, 1H), 10.40 (br s, 1H), and 10.83 ppm (br s, 1H); UV (pH 7.0) λ_{max} 242 (ε 19,300), 311 (23,000), 324 sh (17,800). Analysis: calculated for $C_{11}H_{16}N_6S_2$: C, 44.57; H, 5.44; N, 28.35. Found: C, 44.75; H, 5.52; N, 28.45.

Enzyme Purification and Assays. HSV-1 (10), HSV-2 (11), VZV (9), and human [isolated from human D98 cells (18, 19)] ribonucleotide reductases were purified and assayed as described in those references. The substrates, [^{14}C]CDP and [^{14}C]ADP, were purified, and the ribonucleotide reductase

Abbreviations: HSV, herpes simplex virus; VZV, varicella–zoster virus; A1110U, 2-acetylpyridine 5-[(dimethylamino)thiocarbonyl]thiocarbonohydrazone; A723U, 2-acetylpyridine 4-(2-morpholinoethyl)-thiosemicarbazone; ACV, acyclovir (9-[(2-hydroxyethoxy)methyl]-guanine); ACVTP, ACV triphosphate.

§Ellis, M. N., Lobe, D. C. & Spector, T., 26th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1986, New Orleans, abstr. 625.

¶Lobe, D. C., Spector, T. & Ellis, M. N., 27th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1987, New York, abstr. 1157.

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reactions were terminated with EDTA and hydroxyurea to produce blanks (enzyme omitted) with less than 0.02% of the total radioactivity (10). First-order rate constants for the inactivation by A1110U were calculated as described (20).

Cell Culture and Viral Infection. Inhibition of HSV replication in confluent cultures of Vero cells was assessed by the plaque reduction method of Collins and Bauer (21) with the following modification. The infected cells were overlaid with Eagle's minimal essential medium (MEM; GIBCO) containing 2% fetal bovine serum (Hyclone) and 0.5% human immune globulin (ganastan, Cutter). Patton HSV-1 and MS HSV-2 strains were used.

Inhibition of VZV replication *in vitro* was assessed by the plaque reduction method in human lung diploid fibroblasts (MRC-5) as described (22). The vaccine strain Oka was used as cell-associated virus to infect MRC-5 cells. These cells and virus were obtained from the American Type Culture Collection and were negative for mycoplasma as assessed by both culture and antibody methods (Hoest stain).

Effect of ACV on the Toxicity of A1110U to Mammalian Cells. Vero cells were grown in MEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), glutamine at 1 mg/ml, minimal essential amino acid supplement (GIBCO), and gentamycin at 50 μg/ml in 96-well plates at an initial density of 2500 cells per well. After the cells had been incubated for 24 hr at 37°C, A1110U and ACV were added and the incubation was continued for an additional 72 hr. The cells were then stained by vital neutral red to assess the number of viable cells (23).

RESULTS

Inactivation of Ribonucleotide Reductases. The ability of A1110U to inhibit HSV-1 and HSV-2 ribonucleotide reductases was investigated. The data of Fig. 1 demonstrate that submicromolar concentrations of A1110U caused the rate of production of dCDP to decelerate rapidly. This premature cessation of catalysis appeared to be due to inactivation of the enzyme, as the addition of a small volume of fresh ribonucleotide reductase induced a second cycle of product formation (data not shown). A maximum first-order rate constant for the apparent inactivation of about 12 per hr was achieved at 1–2 μM A1110U. Further increases in concentration did not increase the rate of inactivation. Although the paucity of enzyme obtainable from VZV-infected cells limited the detail in which it could be studied, a similar trend of apparent inactivation was demonstrated with this enzyme (Fig. 2). Progressive inhibition was also observed with human ribonucleotide reductase (Fig. 3); however, about 10-fold higher concentrations of A1110U were required to induce it. As with the HSV ribonucleotide reductase reactions, a second burst of CDP reduction was produced by the addition of fresh human ribonucleotide reductase to inhibited reaction mixtures.

To test if inactivation occurs during the reduction of a purine substrate as well as a pyrimidine substrate, the ability of A1110U to inhibit HSV-1 ribonucleotide reductase-catalyzed reduction of ADP was also studied. The results (data not shown) were virtually identical to those observed when CDP was the substrate. Inactivation occurred with a maximum first-order rate constant of 11–14 per hr at 1–2 μM A1110U.

Potentiation of the Anti-Herpetic Activity of ACV. A1110U inhibited the replication (plaque formation) of HSV-2 with an IC_{50} of 1.1 ± 0.067 μM. Furthermore, even at concentrations below those required of antiviral activity, it significantly decreased the IC_{50} of ACV. For example, 0.25 μM A1110U, which produced less than 10% inhibition of HSV-2 replication, caused the IC_{50} of ACV to decrease by about one-half. A graphic presentation of the potentiation of ACV by A1110U is shown in the isobologram (24) of Fig. 4, where the displacement of the curve to the left indicates synergistic interaction.

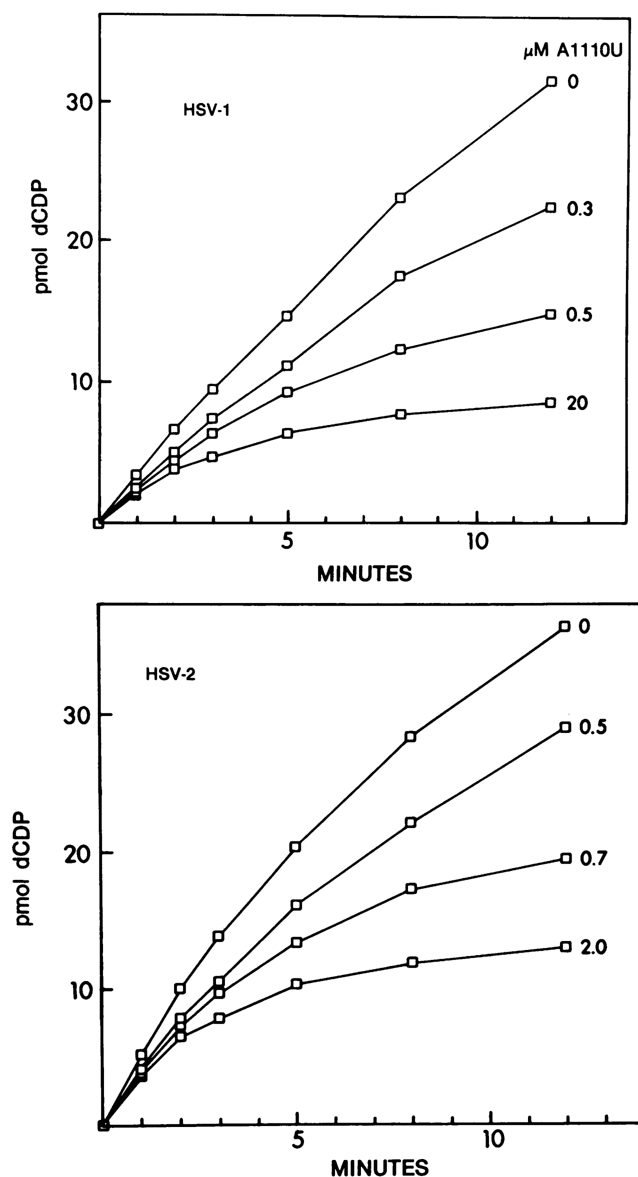

FIG. 1. Inhibition of HSV-1 (*Upper*) and HSV-2 (*Lower*) ribonucleotide reductases by A1110U. Ribonucleotide reductase was assayed in the presence of 2 μM [^{14}C]CDP, 10 mM dithiothreitol, 200 mM Hepes (pH 7.7) buffer, and A1110U at the indicated concentrations. Reactions were initiated with enzyme and sampled at the indicated times.

Analysis of the synergistic inhibition of HSV-1 plaque formation was complicated by the steepness of the inhibition curve produced by A1110U alone. An IC_{50} of 1.4 ± 0.13 μM was obtained for A1110U. However, there was no inhibition at concentrations below 0.8 μM, while there was 100% inhibition at concentrations above 2 μM. Potentiation of ACV was detectable only within a narrow window. For example, 0.6 μM A1110U neither inhibited plaque formation nor potentiated ACV. However, 1 μM A1110U inhibited plaque formation by 17% and potentiated ACV by 5-fold, and 1.2 μM A1110U inhibited plaque formation by 37% and potentiated ACV by 30-fold. Here, the degree of potentiation is the ratio of the theoretical IC_{50} to the actual IC_{50} for ACV in the presence of a given concentration of A1110U. The former was calculated from a curve generated by adjusting the ACV curve by a factor accounting for the independent inhibition by A1110U and assuming additive inhibition between the two agents.

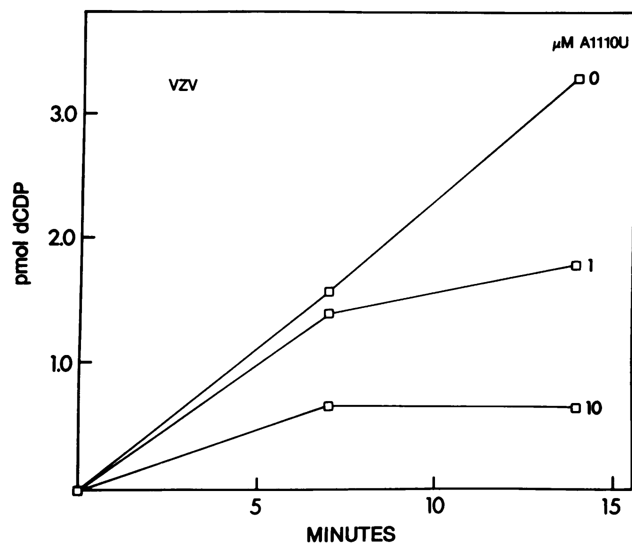

FIG. 2. Inhibition of VZV ribonucleotide reductase by A1110U. VZV ribonucleotide reductase was assayed in the presence of 2 μM [^{14}C]CDP, 10 mM dithiothreitol, 200 mM Hepes (pH 7.7) buffer, and A1110U at the indicated concentrations. Reactions were initiated with enzyme and sampled at the indicated times.

Antiviral activities were also determined by monitoring the ability of ACV, A1110U, or both to prevent the cytopathic effects of HSV-1 and HSV-2. Cell viability was assessed by the dye-uptake method (25). In these experiments, submicromolar (noninhibitory) concentrations of A1110U significantly decreased the IC_{50} of ACV for both types of virus (J. Hill, personal communication). The reason for the apparent difference between the dye-uptake and plaque reduction methods for assessing synergy against HSV-1 is not known.

A1110U also potentiated the ability of ACV to inhibit replication of VZV in tissue culture. Although the sensitivity of the replicating host fibroblasts to the toxicity of A1110U prevented a full-range study, potentiation of the inhibitory potency of ACV was demonstrated at a subtoxic, subinhibitory concentration of A1110U. It can be seen in Fig. 5 that 1.5 μM A1110U significantly decreased the concentration of ACV required to inhibit VZV replication. Virus replication had decreased susceptibility to ACV alone due to the thymidine present in the fetal calf serum.

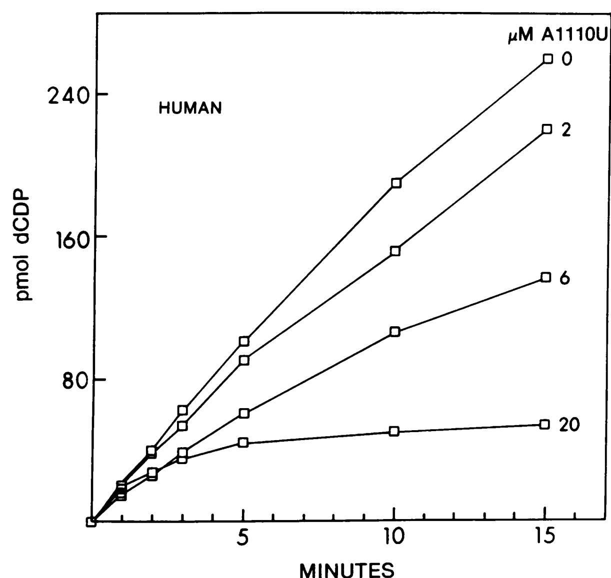

FIG. 3. Inhibition of human ribonucleotide reductase by A1110U. Human ribonucleotide reductase was assayed in the presence of 12 μM [^{14}C]CDP, 5 mM ATP, 6 mM $MgCl_2$, 5 mM dithiothreitol, 100 mM Hepes (pH 7.4) buffer, and A1110U at the indicated concentrations. Reactions were initiated with enzyme and sampled at the indicated times.

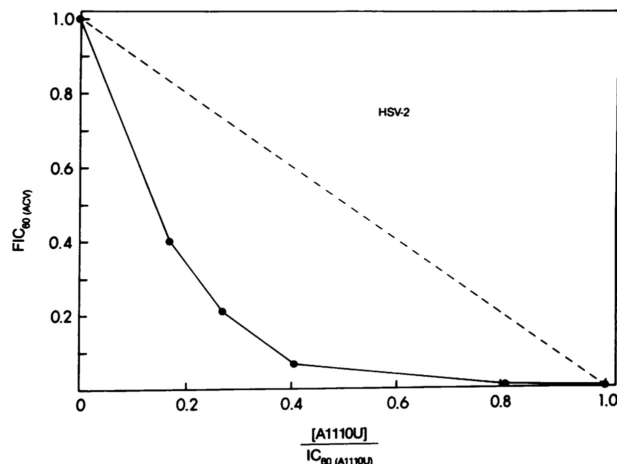

FIG. 4. Synergistic inhibition of HSV-2 replication by A1110U and ACV. The concentration of each inhibitor was covaried and the inhibition of plaque formation was assessed. The $FIC_{60(ACV)}$ is the ratio of the concentration of ACV required to inhibit plaque formation by 60% in the presence of a fixed concentration of A1110U to the concentration required in the absence of A1110U. The x axis is the ratio of the fixed concentration of A1110U to the concentration of A1110U that produced 60% inhibition of plaque formation in the absence of ACV. IC_{60} values were used because >50% inhibition was observed at some of the combination doses. The broken line shows the theoretical plot for independent inhibitors.

A1110U/ACV Interactions in Uninfected Cells. Interestingly, ACV had no effect on the toxicity of A1110U toward uninfected mammalian cells. As shown in Fig. 6, although A1110U was toxic to rapidly growing Vero cells (50% effective dose = 3.2 μM), its toxicity was not modified by ACV at concentrations up to 200 μM. A1110U was not toxic to confluent (static) Vero cells at concentrations up to 10 μM. It should be restated here that all antiviral studies were performed under conditions where the host cells were not subject to any observable toxicity.

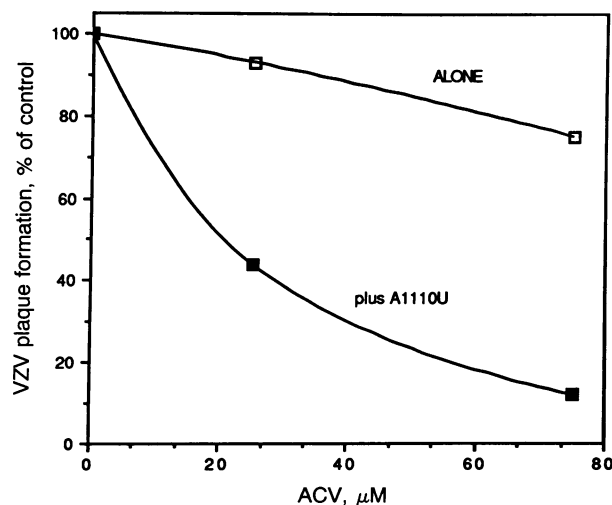

FIG. 5. Potentiation by A1110U of the inhibition of VZV replication by ACV. The ability of ACV to inhibit VZV replication by ACV was assessed in the presence and absence of 1.5 μM A1110U. All values are compared to the average control value obtained in the absence of added drugs.

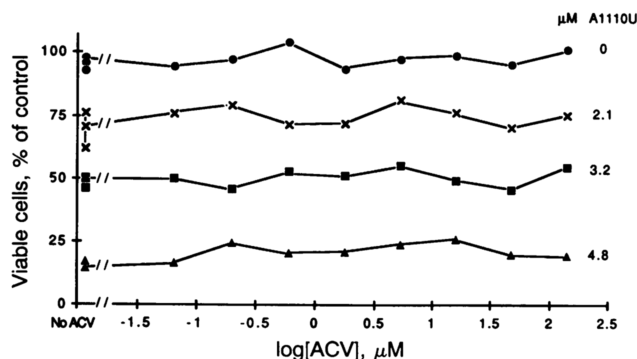

FIG. 6. ACV does not modify the cellular toxicity of A1110U. The ability of A1110U to inhibit the growth of Vero cells was monitored in the presence of ACV at the indicated concentrations.

Effects of A1110U on Cellular Deoxynucleoside Triphosphate and ACVTP Levels. In confirmation of earlier reports (17, 26), Table 1 shows that ACV caused the dGTP pool to increase in HSV-1 infected cells. The addition of A1110U, as expected, decreased the dGTP pool by 90%. It also promoted a large increase in the pool of ACVTP. The present finding that inhibition of ribonucleotide reductase results in the selective depletion of purine deoxynucleotide pools has been reported for other ribonucleotide reductase inhibitors (27–29).

DISCUSSION

A1110U was found to be a potent inhibitor of HSV and VZV ribonucleotide reductases. Submicromolar concentrations caused the rates of these enzymes to decrease rapidly in a manner that was indicative of enzyme inactivation. Further evidence that the time-dependent decrease in the rate of product formation was due to inactivation of the ribonucleotide reductases and not to the modification of another reagent in the reaction mixture was obtained when additions of fresh enzyme to totally inhibited reactions produced a second cycle of decelerating activities. The apparent inactivation of these enzymes is similar to that observed for A723U with HSV-1 and VZV ribonucleotide reductases (9, 17). However, A1110U is about 30-fold more potent than A723U and is among the most potent inactivators of HSV ribonucleotide reductase reported to date (6, 20, 30).

It is of interest that A1110U also inactivates human ribonucleotide reductase. However, approximately 10- to 20-fold higher concentrations are required to bring about rates of inactivation that are similar to those with the herpes virus enzymes. A recent study (D. J. T. Porter, J.A.H., and T.S., unpublished results) of the mechanism by which A1110U inactivates these enzymes has determined the basis for the selectivity. The ability of A1110U to chelate iron plays a central role. Both human and HSV ribonucleotide reductases are rapidly inactivated in the presence of A1110U plus $[A1110U]_2Fe$. However, the second-order rate constant for the inactivation of viral ribonucleotide reductase by $[A1110U]_2Fe$ (in the presence of low amounts of A1110U) is about 10-fold larger than the corresponding constant for the inactivation of the human enzyme. Furthermore, while HSV ribonucleotide reductase is inactivated by either uncomplexed A1110U or $[A1110U]_2Fe$, human ribonucleotide reductase is not inhibited by uncomplexed A1110U, and is inhibited, but not inactivated, by $[A1110U]_2Fe$. It can be concluded from those observations and the data of this report that there is negligible iron present in the HSV ribonucleotide reductase reaction mixture and significant iron in the mixture for the human enzyme. The addition of submicromolar concentrations of iron to produce a combination of A1110U and iron-complexed A1110U greatly increases the rates of inactivation of HSV ribonucleotide reductase. The weak inhibition and lack of inactivation of human ribonucleotide reductase by 2 μM A1110U (Fig. 3) indicates that all the inhibitor in that reaction mixture is in the iron-complexed form. It was shown (D. J. T. Porter, J.A.H., and T.S., unpublished results) that the source of iron is the commercial ATP, which is not required by HSV enzymes. Furthermore, when the concentration of A1110U was high enough to titrate all the iron and also provide uncomplexed A1110U, rapid inactivation of the human enzyme was observed. The complexities of these mechanisms emphasize the importance of studies that carefully control for the presence of iron. Although the data reported here fortuitously reflect the true differences in potency of A1110U for viral and human ribonucleotide reductases, the absence of contaminating iron in the reaction mixture of the human enzyme would have greatly exaggerated the difference.

In any case, the selectivity of A1110U toward the viral enzymes was adequate to allow the demonstration of synergistic potentiation of the activity of ACV against HSV-1, HSV-2, and VZV replication. Modest concentrations of A1110U greatly reduced the IC_{50} values of ACV without significant toxicity to the host cells. Although A1110U was toxic to rapidly replicating mammalian cells, it was considerably less deleterious to confluent cells, and most importantly, the cytotoxicity of A1110U was not augmented by ACV. Thus, the synergistic potentiation of ACV by A1110U is selective for the replicating viruses. The finding that topical preparations of A1110U and ACV are well tolerated on mammalian skin while producing synergistic antiviral activity *in vivo* (§, ¶) further attests to the practical selectivity of this combination.

The mechanism by which A1110U potentiates ACV is similar to that of A723U (17). Both ribonucleotide reductase inactivators prevent the build-up of dGTP, which in their absence would be concomitant to the inhibition of viral DNA synthesis by ACVTP. It should be pointed out that the increase in dGTP pools is probably a direct consequence of the absence of feedback inhibition of these viral ribonucleotide reductases. A similar increase would not be expected in uninfected cells because of the elaborate regulation of cellular ribonucleotide reductase (reviewed in refs. 7 and 8).

The second aspect to the synergistic interaction is the ability of these ribonucleotide reductase inactivators to elevate greatly the pools of ACVTP. The combination of the decrease in dGTP and the increase in ACVTP confers a 100-fold increase in the ratio of ACVTP to dGTP and certainly increases the probability for the binding of ACVTP to the herpes virus DNA polymerases. Although the mech-

Table 1. Effects of A1110U on deoxynucleoside triphosphate and ACVTP levels

Treatment		Intracellular pools, pmol/10^6 cells					Ratio
A1110U, μM	ACV, μM	dCTP	dTTP	dATP	dGTP	ACVTP	ACVTP/ dGTP
0	0	20	225	20	16	—	—
0	10	21	240	82	68	2.6	0.038
2	0	16	260	20	8.0	—	—
2	10	16	260	33	6.4	23	3.6

Confluent cultures of Vero cells were infected with HSV-1 (Patton strain) at 10 plaque-forming units per cell. After 1-hr incubation at 37°C, the medium was replaced with fresh medium and A1110U and [2-^{3}H]ACV where indicated. After an additional incubation of 8 hr, the cells were collected by centrifugation and extracted with 3.5% perchloric acid. Supernatants were neutralized with KOH and treated with periodate and methylamine to remove ribonucleotides. Treated extracts were chromatographed on a Whatman Partisil PXS10 column with a linear gradient of 0.3–0.8 M KH_2PO_4, pH 3.5. Pools of deoxynucleotides were quantitated by their UV absorbances and ACVTP by its specific activity.

anism for elevating the concentrations of ACVTP is not yet understood, as described before (17), it is most likely related to the inhibition of ribonucleotide reductase. Recently, Karlsson and Harmenberg (31) reported that inhibition of HSV ribonucleotide reductase by A723U decreases the excretion of thymidine by infected cells. If this reflects a decrease in intracellular thymidine, then the reduced ability of thymidine to compete with ACV for phosphorylation by HSV thymidine kinase should promote the formation of ACVTP.

The potency of A1110U as an inhibitor of herpes virus ribonucleotide reductase is probably a major factor for its efficacy *in vivo*. Recent studies show that while A1110U and ACV produce synergistic therapy for infected mice, A723U and ACV do not (M. N. Ellis and T.S., unpublished results). The experiments showing that topical A1110U potentiates the antiherpetic activity of ACV and that this combination therapy provides synergistic treatment for animals infected with either wild-type or ACV-resistant viruses (§, ¶) offer encouragement for the development of a similar treatment for human disease.

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