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SYNERGISTIC INTERACTION BETWEEN ACIVICIN (AT-125) AND 6-THIOGUANINE IN THE MURINE LEUKEMIA L1210

BIOCHEMICAL AND CYTOKINETIC CONSIDERATIONS

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Abstract—The effect of the purine antagonist acivicin (AT-125) on the metabolism and cytotoxicity of 6-thioguanine was examined in the murine leukemia L1210. Cells exposed to 5×10^{-6} M acivicin for 18 hr followed by 10^{-5} M [^{14}C]-6-thioguanine for 2 hr accumulated 2.90 ± 0.17 nmoles 6-thioguanine/10^6 cells compared to 0.69 ± 0.07 nmoles 6-thioguanine/10^6 cells in untreated controls. Intracellular accumulation of 6-thioguanine monophosphate, a lethal 6-thioguanine metabolite, increased from 0.27 ± 0.05 to 1.08 ± 0.13 nmoles 6-thioguanine monophosphate/10^6 cells following the same acivicin exposure. A similar increment was observed for the formation of 6-thioguanine triphosphate. These alterations in 6-thioguanine metabolism were associated with an increase in the intracellular level of 5-phosphoribosyl-1-pyrophosphate, an obligatory substrate in 6-thioguanine activation (57.9 ± 7.6 vs 13.4 ± 2.3 ng 5-phosphoribosyl-1-pyrophosphate/10^6 cells). In contrast, there was a 50% reduction in the amount of 6-thioguanine incorporated into RNA and DNA following acivicin pretreatment. Cytofluorometric analysis revealed that an 18-hr exposure to 5×10^6 M acivicin increased the population S-phase cells, which are more sensitive to the actions of 6-thioguanine, by 50% relative to untreated controls. In both suspension culture growth and soft agar studies, the sequential administration of acivicin followed by 6-thioguanine resulted in substantial growth inhibitory activity; in contrast, the effects of the reverse sequence were subadditive. Pretreatment of L1210 cells with acivicin potentiates the action of subsequently administered 6-thioguanine, and the mechanism may involve both biochemical as well as cytokinetic factors. *In vivo* studies involving the sequential administration of these agents appear warranted.

Acivicin (AT-125, α-amino-3-chloro-2-isoxazoline-5-acetic acid; NSC 163501) is a fermentation-derived amino acid antimetabolite with significant antitumor activity against a variety of experimental tumors, both *in vitro* and *in vivo* [1]. In mammalian cells, it is believed to act as a glutamine antagonist since its toxic effects may be reversed by coadministration of L-glutamine in cell lines so tested [2]. Acivicin interferes with a variety of L-glutamine amidotransferases involved in *de novo* purine and pyrimidine synthetic pathways. For example, it has been shown to inhibit CTP synthetase [3], XMP aminase [3] and pyrophosphate phosphoribosyltransferase [2] in several murine tumor cell lines. Acivicin has demonstrated synergistic biochemical and antitumor effects when administered in conjunction with PALA (*N*-[phosphonacetyl-L-aspartate]) in a PALA-resistant variant of the Lewis lung carcinoma [4]. Based on these biochemical studies, it has been suggested that acivicin might be a logical agent to combine with pyrimidine analogs such as cytosine arabinoside and 5-azacytidine [5].

6-Thioguanine (6-TG) is a purine analog which is an effective agent in the treatment of acute leukemia in man [6]. It is converted to its monophosphate form, 6-TGMP, by the enzyme hypoxanthine-guanine phosphoribolsytransferase [7]. This metabolite interferes with tumor cell growth by inhibiting a variety of *de novo* purine synthetic enzymes, including L-glutamine amidotransferase [8], guanylate kinase [9] and inosinic acid dehydrogenase [10]. 6-TG may also be converted to the deoxyribonucleoside triphosphate derivative and subsequently incorporated into tumor cell DNA [11]. This latter effect has been felt to account for the antineoplastic activity and delayed cytotoxity of 6-TG in some tumor cell lines [12, 13], although this has not been a universal finding [14].

Previous studies in the sarcoma 180 ascites tumor have demonstrated therapeutic synergism between 6-TG and agents which inhibit L-glutamine amidotransferase such as 6-methylmercaptopurine riboside (MeMPR) [15]. This synergism is believed to result from increased availability of 5′-phosphoribosyl 1-pyrophosphate (PRPP) in MeMPR-treated cells and enhanced 6-TGMP formation. A similar mechanism has been invoked to explain the interaction in L1210 cells between methotrexate and 5-fluorouracil, a

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pyrimidine analog which utilizes PRPP for its conversion to the nucleotide form [16]. The purpose of the present study was to determine whether acivicin, through its inhibitory effects on *de novo* purine synthesis, might augment the metabolism and nucleotide formation of subsequently administered 6-TG in the murine leukemia L1210. In addition, an attempt was made to correlate enhanced toxicity of 6-TG with increased PRPP availability, enhanced 6-TGMP formation, and increased incorporation of 6-TG into tumor cell DNA. Finally, the effect of acivicin exposure on the cell cycle traverse of L1210 cells was also examined, since cytokinetic factors have been shown previously to play an important role in the expression of 6-TG cytotoxicity [17].

MATERIALS AND METHODS

Cells. L1210 cells were maintained in suspension culture in Fischer's medium supplemented with 10% heat-inactivated horse serum (GIBCO, Grand Island, NY). They were kept in 75 cm^2 sterile tissue culture flasks (Corning, Corning, NY) in a 37°, 5% CO_2 water-jacketed incubator. Cells were passed twice weekly and were routinely tested for mycoplasma contamination. Under these conditions, cells had a doubling time of 14–16 hr and reached plateau phase growth at a concentration of 8×10^5 cells/ml. For all experiments, cells in logarithmic phase growth (concentration 2–4 10^5 cells/ml) were used. A model ZBI Coulter counter (Coulter Electronics, Hialeah, FL) was utilized to enumerate cells. Viability of cells was determined by trypan blue exclusion.

Drugs and radiochemicals. Acivicin was supplied by the Upjohn Co. (Kalamazoo, MI). It was stored as a dry powder at −20° and dissolved in sterile saline prior to use. 6-TG and GMP were purchased from the Sigma Chemicals Co. (St. Louis, MO). [^{3}H]-Adenine (24.5 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). [^{14}C]-6-TG (56 mCi/mmole) was purchased from Moravek Biochemicals (Brea, CA). It was reconstituted in sterile Fischer's medium at neutral pH and stored at −20° until use. Purity of material, determined by high-pressure liquid chromatographic analysis, was greater than 98%.

Intracellular accumulation of 6-thioguanine. The effect of acivicin exposure on the total intracellular accumulation of [^{14}C]-6-TG in L1210 cells was determined utilizing a rapid centrifugation method [18]. Briefly, L1210 cells in exponential phase growth were placed in 25 cm^2 tissue flasks and exposed to various concentrations of acivicin (1×10^{-7} to 5×10^{-5} M). The flasks were then placed in a 37°, 5% CO_2 water-jacketed incubator for periods of 6–18 hr, after which the cells were centrifuged for 6 min at 1000 rpm, the drug-containing medium was discarded, and the cell pellet was resuspended in 2 ml of fresh medium. The samples were then pipetted into reaction flasks to which were added 0.02 ml of 4-2-(hydroxyethyl)-1-piperazine-ethonesulfonic acid buffer (pH 7.4) and [^{14}C]-6-TG (final concentration, 10^{-5} M). The intracellular accumulation of [^{14}C]-6-TG in control and acivicin-treated cells at various time points was determined by a previously described method [18] and was expressed as nmoles 6-thioguanine/10^6 viable cells.

Incorporation of [^{14}C]*-6-thioguanine into RNA and DNA.* Incorporation of 6-TG into L1210 cell nucleic acid was determined by separating RNA and DNA under nondegrading conditions utilizing $CsSO_4$ gradient centrifugation [19]. Approximately 5×10^7 logarithmically growing cells were exposed to acivicin as previously described, washed, and incubated for 2 hr with 10^{-5} M [^{14}C]-6-TG at 37°. The cells were then washed twice with 5 ml of Dulbecco's phosphate-buffered saline (PBS), resuspended in PBS at 10^7 cells/ml, and digested by the addition of 2.5 mg of pronase B (Calbiochem-Behring Corp., La Jolla, CA) in 2 ml of 0.01 M Tris (pH 7.4), 0.01 M EDTA and 5% sodium dodecyl sulfate. Subsequent purification was accomplished by phenol extraction. The nucleic acids were precipitated by the addition of 0.1 vol. of 4 M NaCl and 2 vol. of absolute methanol. Samples were then centrifuged at 10,000 *g* for 30 min, and the pellets were resuspended in 0.005 M EDTA. The suspension was then made 50% (v/v) with formamide and heated at 80° for 5 min. Samples (0.5 ml) were then added to 5.4 ml of 0.005 M EDTA mixed with an equal volume of saturated $CsSO_4$, to yield a starting density of 1.52 g/ml and centrifuged at 44,000 rpm in a Beckman Ti-50 rotor for 60 hr at 20°. Fractions of 0.4 ml were collected from the bottom of each tube and assayed for acid-precipitable radioactivity. [^{14}C]-6-TG counts eluting in the RNA region (density 1.62 to 1.68 g/ml) and DNA region (density 1.42 to 1.48 g/ml) were determined and used as a measure of incorporation of 6-TG (or its metabolites) into L1210 cell RNA and DNA respectively.

Determination of 6-thioguanine monophosphate formation. The formation of 6-TGMP, a lethal 6-TG metabolite, was determined in control cells, as well as in cells exposed to various concentrations of acivicin. Cells were incubated with acivicin as described in the previous section, washed, and resuspended in 2 ml of fresh Fischer's medium with 10% horse serum containing 10^{-5} M [^{14}C]-6-TG. After a 2-hr incubation in a 37° shaking water bath, the cell pellet was washed twice in cold PBS, the supernatant fraction was discarded, and the cells were precipitated with 0.75 ml of cold 5 N $HClO_4$. The supernatant fraction was then neutralized with cold 4 N KOH, and the precipitated $KClO_4$ was removed. 6-TGMP in the supernatant fraction was determined by a previously described [20] high pressure liquid chromatographic method utilizing an Altex model 332 system. A Whatman anion-exchange column (Partisil SAX: 4.6 mm inside diameter × 25 cm) was used to separate the components. A linear gradient from 10 mM to 1 M NaH_2PO_4 (pH 3.5) was formed over a 30-min period; the flow rate was 1.8 ml/min. Radioactivity eluted in a single peak in the monophosphate region and was quantitated by combining 0.5-ml samples with 10 ml of aqueous scintillation mixture and counting in a liquid scintillation spectrometer. 6-TGMP formation was expressed as nmoles 6-TGMP/10^6 viable cells.

Determination of 6-thioguanine triphosphate. The effect of acivicin pretreatment on the formation of 6-TGTP was determined by high-pressure liquid chromatographic separation of nucleotides in acid-

soluble extracts. L1210 cells were exposed to acivicin and [^{14}C]-6-TG as described in the previous section, and cold perchlorate extracts were obtained. Following neutralization with KOH, the extracts were separated isocratically on a Partisil SAX column utilizing a 0.4 M NaH_2PO_4, pH 3.3 buffer with a flow rate of 2 ml/min. Radioactivity eluting in the GTP region (½ peak retention time 30.5 min) was quantitated, and the level of 6-TGTP was expressed as nmoles 6-TGTP/10^6 cells. Comparisons of 6-TGTP levels were made between control cells and cells exposed to acivicin.

PRPP assay. L1210 cells (50 ml) in logarithmic cell growth were exposed to acivicin as in the preceding section, and alterations in intracellular levels of PRPP were determined by a method which measures the conversion of adenine to AMP in the presence of adenine phosphoribolsyltransferase. The details of this procedure have been described previously [21]. PRPP levels in control and acivicin-treated cells were expressed as ng PRPP/10^6 cells.

Cytofluorometric studies. The effect of acivicin exposure on the cell cycle traverse of L1210 cells was studied utilizing an Epics V flow cytometer (Coulter Electronics). Cells were stained according to a minor modification [22] of the propidium iodide technique of Krishan [23]. Logarithmically-growing cells were incubated with various concentrations of acivicin for 18 hr, after which they were centrifuged at 1200 *g* for 8 min at room temperature. The supernatants fractions were removed with a sterile pipette, and the cell pellets were resuspended in a solution containing 50 μg/ml propidium iodide in 10^{-2} M Tris (pH 7), containing 5 mM $MgCl_2$ and 30 μg/ml RNAase (Sigma Chemical Co.). The suspension was incubated at 37° for 2 hr, or longer, and subsequently stored without fixation at 4° prior to analysis. Under these conditions, samples remained stable for 2-week periods or longer. Histograms of 50,000 cells were obtained with an Epics V flow cytometer utilizing a 488-nm argon ion laser line to induce fluorescence. Ten-micrometer diameter ¼ bright fluorespheres (Coulter) were used to standardize fluorescence intensity. DNA content distribution histograms were recorded on an X-Y recorder and analyzed by the Epics B integration routine utilizing user setting channel limits to yield the percentage of the cell population in G_1, S and G_2 phases.

Suspension culture studies. The effect of sequential administration of acivicin and 6-TG was assessed on the growth of L1210 cells in suspension culture. L1210 cells in logarithmic phase growth (concentration 2–4 × 10^5 cells/ml) were placed in 25 cm^2 sterile flasks containing various concentrations of acivicin. The flasks were maintained in a 37°, 5% CO_2 incubator for a period of 18 hr, after which the cells were washed twice with fresh Fischer's medium and resuspended in tubes containing 5 ml of medium with 10% horse serum. Various concentrations of 6-TG were added to the tubes, which were then placed in the incubator for 2 hr. After this period, the cells were washed twice with fresh medium, resuspended in medium containing 10% horse serum, and the cell density was adjusted to 5 × 10^4 cells/ml. Samples (5 ml) for each condition were pipetted into 6-well tissue culture plates (Costar, Cambridge, MA), and the plates were placed in the incubator. In some experiments, cells were exposed to the drugs in the reverse sequence, e.g. a 2-hr exposure to 6-TG followed by an 18-hr exposure to acivicin. At 24-hr intervals, 1-ml aliquots of the cell suspension were removed for cell density determinations. The increment in cell number was expressed as $N/N_o \times 100\%$ where N represents the cell density at 72 hr and N_o represents the initial seeding concentrations (5 × 10^4 cells/ml). Comparisons were made between the growth of control cells, and cells exposed to the drugs alone and in sequence.

Cloning studies. The growth of L1210 cells in soft agar following single and sequential drug exposure was assessed utilizing a slight modification of a previously described soft agar cloning technique [18]. L1210 cells were exposed to acivicin and 6-TG as described in the preceding section. After washing and resuspension in fresh Fischer's medium, cells were plated in soft agar in 18-mm 12-well plates (Costar). For each condition, a bottom layer was prepared consisting of 0.5 ml Fischer's medium containing 20% fetal calf serum and 0.5% Bacto agar (Difco, Detroit, MI). The top layer, which contained 200 cells/condition, consisted of 0.5 ml Fischer's medium containing 20% fetal calf serum and 0.3% Bacto agar. The plates were then placed in a 100% humidified, 37°, 5% CO_2 incubator for 8 days. At the end of this period, colonies, consisting of groups of 50 or more cells, were scored with the aid of an Olympus model CK inverted microscope. Cloning efficiency of cells under these conditions was approximately 60%. Inhibition of colony formation was expressed as $N/N_c \times 100\%$, where N = number of colonies formed for each condition and N_c = number of colonies formed by control cells. Comparisons were made between colony formation by control cells, and cells exposed separately and sequentially to acivicin; 6-TG was defined according to the *in vitro* criteria previously described by Valeriote and Lin [24] and Momparler [25].

RESULTS

Total intracellular accumulation of [^{14}C]-6-*thioguanine.* Table 1 illustrates alterations in 6-TG intracellular accumulation in L1210 cells following acivicin exposure. There was a 4-fold increase in the total intracellular accumulation of 6-TG, both at 45 and 120 min, in cells exposed to 5 × 10^{-6} M acivicin for 18 hr compared to control cells. Both lower (10^{-6} M) and higher (10^{-5} M) concentrations of acivicin resulted in smaller increments in 6-TG accumulation. Shorter incubation intervals (6 and 12 hr) with 5 × 10^{-6} M also produced increments in 6-TG intracellular accumulation, but these were smaller than those observed following an 18-hr exposure. Similar increments were obtained when lower 6-TG concentrations (10^{-7} and 10^{-6} M) were utilized (data not shown).

6-*Thioguanine monophosphate formation.* Intracellular accumulation of 6-TGMP in L1210 cells was also increased following an 18-hr exposure to acivicin (Table 2). Exposure of cells to 5 × 10^{-6} M acivicin for 18 hr resulted in a 4-fold increase in the intracellular generation of 6-TGMP and 6-TGTP relative

Table 1. Total intracellular accumulation of 6-TG in L1210 cells following exposure to acivicin*

Exposure interval (hr)	Acivicin concn (M)	6-TG intracellular accumulation (nmoles 6-TG/10^6 cells) 45 min	2 hr
Control		0.33 ± 0.05	0.69 ± 0.07
18	5×10^{-6}	1.44 ± 0.14	2.90 ± 0.17
18	10^{-6}	0.49 ± 0.05	0.97 ± 0.08
18	10^{-5}	0.50 ± 0.06	1.21 ± 0.09
12	5×10^{-6}	0.82 ± 0.10	ND†
6	5×10^{-6}	0.46 ± 0.04	ND

* Logarithmically-growing L1210 cells were incubated with acivicin at various concentrations for the interval shown, resuspended in fresh medium, and exposed to 10^{-5} M [^{14}C]-6-TG. The intracellular accumulation of 6-TG was determined after 45 and 120 min as described in Materials and Methods. values represent the mean of three experiments performed in duplicate ± one S.D.

† Not done.

to untreated control cells. In general, elevations of both the 6-TGMP level as well as the total intracellular accumulation of 6-TG and its metabolites were similar for each acivicin concentration (Table 1). The maximal increase in 6-TGMP level (1.08 ± 0.13 vs 0.27 ± 0.05 nmoles 6-TGMP/10^6 cells) occurred in cells exposed to 5×10^{-6} M acivicin for 18 hr.

6-Thioguanine triphosphate formation. Increments in 6-TGTP formation following acivicin exposure were similar for each acivicin concentration (Table 1). The maximal increase in 6-TGMP level (1.08 ± 0.13 vs 0.27 ± 0.05 nmoles 6-TGMP/10^6 cells) 5×10^{-6} M acivicin for 18 hr accumulated 0.78 ± 0.12 nmoles 6-TGTP/10^6 cells vs 0.17 ± 0.06 nmoles 6-TGTP/10^6 cells in control cells.

Incorporation of [^{14}C]-6-thioguanine into L1210 cell RNA and DNA. Representative $CsSO_4$ gradient nucleic acid profiles of L1210 cells exposed for 2 hr to [^{14}C]-6-TG are illustrated in Fig. 1. In both control and acivicin-treated cells, approximately 80% of the labeled 6-TG was found in the RNA fraction and 20% in the DNA fraction. Cells exposed to 5×10^{-6} M acivicin for 18 hr experienced a 50% reduction in [^{14}C]-6-TG incorporated into both RNA and DNA fractions relative to untreated control cells. A similar reduction in nucleic acid incorporation of 6-TG was noted following exposure of cells to both higher (10^{-5} M) as well as lower (10^{-6} M) acivicin concentrations (not shown).

Intracellular PRPP levels. Alterations in intracellular levels of PRPP in L1210 cells following an 18-hr acivicin exposure are illustrated in Table 3. Cells exposed to 5×10^{-6} M acivicin contained 57.9 ± 7.6 ng PRPP/10^6 cells compared to 13.4 ± 2.3 ng PRPP/10^6 cells in untreated controls. Small increments in PRPP levels were observed in cells exposed to acivicin concentrations of 10^{-6} M or lower. As the acivicin concentration increased further (e.g. 10^{-5} M), PRPP levels began to decline.

Cytofluorometric analysis. Exposure of L1210 cells to acivicin for 18 hr produced a net increase in the percentage of cells in S-phase (Table 4). In logarithmically-growing control cells, 43.3% were in S-phase; the number increased to 66.4% following an 18-hr exposure to 5×10^{-6} M acivicin, representing a relative increment of 53.3%. The population of G_1 phase cells declined under these conditions

Table 2. Effect of acivicin exposure on the formation of 6-TGMP and 6-TGTP in L1210 cells*

Acivicin concn (M)	Intracellular 6-TGMP formation (nmoles 6-TGMP/10^6 cells)	Intracellular 6-TGTP formation (nmoles 6-TGTP/10^6 cells)
Control	0.27 ± 0.05	0.17 ± 0.06
1×10^{-6}	0.48 ± 0.07	0.29 ± 0.04
2×10^{-6}	0.58 ± 0.06	0.32 ± 0.08
5×10^{-6}	1.08 ± 0.13	0.78 ± 0.12

* Logarithmically-growing L1210 cells were exposed for 18 hr to the indicated concentration of acivicin and incubated for 2 hr with 10^{-5} M [^{14}C]-6-TG. At the end of this period, the intracellular levels of 6-TGMP and 6-TGTP were determined by high-pressure liquid chromatographic analysis of the acid cell extracts as described in Materials and Methods. Values represent the mean of three experiments performed in duplicate ± one S.D.

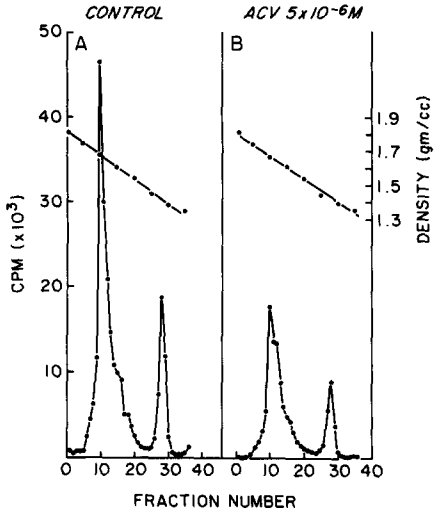

Fig. 1. Incorporation of [^{14}C]-6-TG into RNA and DNA of L1210 cells following acivicin treatment. Cells were grown in 5×10^{-6} M acivicin for 18 hr followed by a 2-hr exposure to 10^{-5} M [^{14}C]-6-TG, after which RNA and DNA were separated by $CsSO_4$ gradient centrifugation. Representative nucleic acid profiles are shown.

(44.2 vs 24.8%). A lower acivicin concentration (10^{-6} M) produced a slightly smaller increase in the population of S-phase cells (47.3%); as the concentration of acivicin was reduced further, the increment in S-phase cells declined.

Suspension culture growth studies. Inhibition of L1210 cell growth in suspension culture following exposure to 5×10^{-6} M acivicin for 18 hr, followed by 1×10^{-6} M 6-TG for 2 hr, was considerably greater than the effects of either agent administered alone (Fig. 2). Exposure to these concentrations of acivicin and 6-TG resulted in 37 and 26% inhibition of cell growth, respectively; sequential exposure of cells to acivicin followed by 6-TG produced a 94% reduction in cell growth. When lower concentrations of acivicin were utilized (e.g. 10^{-6} M) in conjunction with 6-TG, additive inhibitory effects were noted. When the 6-TG exposure preceded the acivicin

Table 3. Intracellular levels of PRPP in L1210 cells following acivicin exposure*

Acivicin concn (M)	PRPP level (ng/10^6 cells)
Control	13.4 ± 2.3
1×10^{-7}	14.8 ± 2.8
5×10^{-7}	17.8 ± 2.7
1×10^{-6}	18.9 ± 3.6
5×10^{-6}	57.9 ± 7.6
1×10^{-5}	42.8 ± 6.4

* Logarithmically-growing cells were exposed to the indicated acivicin concentration for 18 hr, and the amount of PRPP present in the cell extract was assessed by measuring the conversion of adenine to AMP in the presence of adenine phosphoribosyltransferase. Values represent the mean of duplicate experiments ± one S.D.

Table 4. Cell cycle traverse of L1210 following an 18-hr exposure to various concentrations of acivicin*

Acivicin concn (M)	% G_1	% S	% G_2	% Control S
Control	44.2	43.3	11.7	100.0
5×10^{-7}	37.3	56.2	8.7	129.8
10^{-6}	28.3	62.8	14.5	147.3
5×10^{-6}	24.8	66.4	6.6	153.3

* DNA histograms were obtained with a Coulter Epics V flow cytometer and user setting channel limits utilized to estimate the percentage of cells in the appropriate phase of the cell cycle.

administration, growth inhibitory effects were subadditive in nature. Exposure of cells to higher concentrations of acivicin (e.g. 10^{-5} M) alone for 18 hr resulted in substantial growth inhibitory effects (>90% reduction in growth); potentiation of 6-TG cyctotoxic effects was not noted at this concentration (data not shown).

Cloning studies. Synergistic inhibitory effects on the growth of L1210 cells in soft agar following sequential exposure to 5×10^{-6} M acivicin (18 hr), followed by 10^{-6} M 6-TG (2 hr), are illustrated in Fig. 3. The results are similar to those observed with

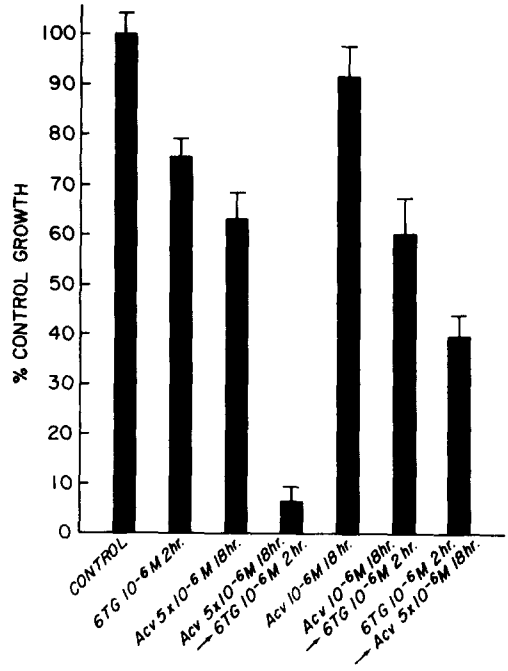

Fig. 2. Effect of sequential administration of acivicin and 6-TG on the growth of L1210 cells in suspension culture. L1210 cells were exposed to acivicin and 6-TG as shown, washed, resuspended in fresh medium, and seeded in 25 cm^2 tissue culture flasks at an initial concentration of 5×10^4 cells/ml. The height of the bar graph corresponds to the percent increment in cell number at 72 hr relative to untreated control cells. Experiments were performed in triplicate; values represent the mean for three separate experiments ± one S.D.

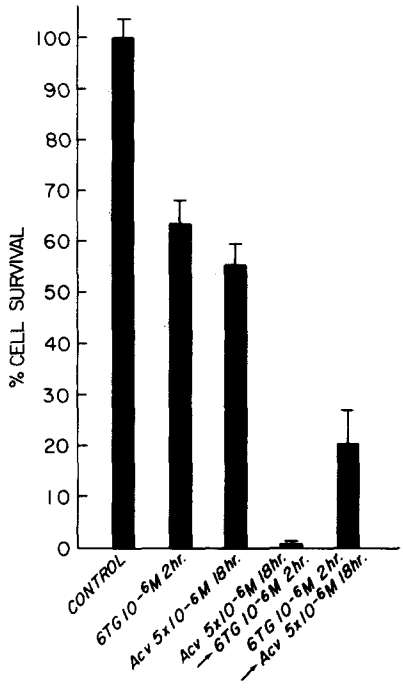

Fig. 3. Inhibition of L1210 formation in soft agar following sequential acivicin and 6-TG exposure. L1210 cells were exposed to acivicin and 6-TG as shown, washed, resuspended in fresh medium and plated in soft agar as described in Materials and Methods. The heights of the bars correspond to the percentage of colonies formed for each condition relative to untreated control cells ± one S.D. Experiments were performed in triplicate; values represent the mean for four separate experiments.

respect to the growth of L1210 cells in suspension culture, except that these drug concentrations and exposure intervals were slightly more inhibitory to L1210 colony formation. An 18-hr exposure to 5×10^{-6} M acivicin or a 2-hr exposure to 10^{-6} M 6-TG produced 55 and 36% reductions in L1210 colony formation respectively. In comparison, sequential exposure to acivicin followed by 6-TG resulted in a 97% reduction in colony formation. As with inhibition of growth of cells in suspension culture, the sequence 6-TG followed by acivicin resulted in subadditive inhibitory effects on colony formation by L1210 cells.

DISCUSSION

These studies demonstrate a dose- and sequence-dependent synergism between acivicin and 6-TG in the murine leukemia L1210 and suggest that both biochemical and cytokinetic factors may contribute to this interaction. Early investigations indicated that a number of purine antagonists, including azaserine, 6-diazo-5-oxo-L-norleucine (DON) and MeMPR, interact in a synergistic fashion with purine analogs such as 6-mercaptopurine and 6-TG [26, 27]. These and other studies suggest that enhanced availability of PRPP, an obligatory substrate in 6-mercaptopurine and 6-TG metabolism, may account for the synergistic interaction [15]. A similar mechanism has been invoked to explain antitumor synergism exhibited by methotrexate and MeMPR in conjunction with 5-fluorouracil, a pyrimidine analog converted to its nucleotide form in the presence of PRPP [16, 28]. More recently, acivicin, an inhibitor of several mammalian amidotransferases, has been added to the list of purine antagonists capable of augmenting intratumoral levels of PRPP [29]. It would, therefore, appear to be a logical candidate agent to combine with antimetabolites such as 6-TG, which require PRPP for conversion to active derivatives.

Our study demonstrates that pretreatment of L1210 cells with a minimally-inhibitory acivicin dose (5×10^{-6} M) is capable of inducing greater than a 5-fold increase in the intracellular level of PRPP, and that this increment was associated with a smaller increase in both the total intracellular accumulation of 6-TG as well as one of its lethal metabolites, 6-TGMP. Studies in other cell lines have demonstrated a K_m for PRPP with respect to hypoxanthine-guanine phosphoribosyltransferase of greater than 2×10^{-5} M [30]. It is noteworthy that these kinetic investigations have been carried out in cell-free systems; it is possible that, in the intact cell, compartmentalization may reduce the net availability of PRPP [31]. If, as has been suggested, PRPP is the rate-limiting factor in such transferase reactions [32], the increments in the concentration of this sugar phosphate may account for augmented metabolism of cosubstrates such as 6-TG.

In addition to these considerations, a purine antagonist such as acivicin might also augment the cytotoxic effects of 6-TG through reduction in the intracellular levels of competing normal purine nucleotides. For example, by interfering with *de novo* purine synthesis, acivicin might reduce intracellular concentrations of GMP, thereby potentiating the inhibitory effects of 6-TGMP on enzymes such as guanylate kinase [9]. Similarly, reductions in intracellular levels of GTP and dGTP might favor misincorporation of 6-TGTP and 6T-dGTP into L1210 cell RNA and DNA respectively. The potential ability of acivicin to augment 6-TG nucleotide formation and reduce levels of competing metabolites might contribute to the striking cytotoxic effects of this drug combination.

We have found that potentiation of 6-TG metabolism and lethal effects by acivicin was associated with a 50% reduction in the incorporation of this purine analog into L1210 cell RNA and DNA. This result is at variance with several earlier studies demonstrating a correlation between 6-TG cytotoxicity and incorporation into DNA [33–35]; it is in agreement with the conclusions of other investigators who did not find nucleic acid incorporation of substituted thiopurines to correlate with cytotoxicity in adenocarcinoma 755 [36] and L1210 cells *in vivo* [37] or cultured P388 cells *in vitro* [38]. Our findings may reflect a decrease in total nucleic acid synthesis in cells exposed to an agent capable of inhibiting both purine and pyrimidine biosynthetic pathways. In an analogous setting, Kufe and Egan [28] found that exposure of human lymphoblasts to MeMPR had little effect on 5-fluorouracil incorporation into total cellular RNA. In contrast, performance of simul-

taneous [32] P-incorporation studies revealed a 5-fold increase in the incorporation of 5-fluorouracil into newly-synthesized RNA. It is possible that acivicin may be capable of increasing 6-TG incorporation into newly-synthesized L1210 cell DNA, and that this event may be obscured by a net decrease in DNA synthesis. If the increased frequency of 6-TG misincorporation into new DNA is a critical determinant of cell death, then potentiation of 6-TG cytotoxicity might occur despite a net decrease in the absolute amount of 6-TG incorporated into nucleic acids. In view of these considerations, the present data do not permit definitive conclusions to be drawn concerning the relative contribution of 6-TG incorporation into DNA to its lethal effects. Efforts to define the effects of various purine antagonists on 6-TG incorporation into RNA and DNA, taking into account perturbations in nucleic acid synthetic rates, are currently in progress.

Previous studies have demonstrated that factors affecting DNA synthesis may have a profound effect on the expression of 6-TG cytotoxicity. For example, Schabel *et al.* [39] demonstrated that prior administration of ara-C, whose active metabolite ara-CTP is an inhibitor of DNA polymerase, may protect normal mice from the effects of subsequently administered 6-TG. More recently, Lee and Sartorelli [40] have shown that a variety of DNA synthesis inhibitors may antagonize the lethal actions of previously administered 6-TG in L1210 cells. Since 6-TG, like ara-C, is primarily active against cells engaged in DNA synthesis [41], agents capable of synchronizing cells might be expected to potentiate 6-TG cytotoxicity. Cytofluorometric analysis revealed that exposure of L1210 cells to 5×10^{-6} M acivicin for 18 hr increased the percentage of S-phase cells from approximately 43 to 66% and this could, in part, account for the synergistic interaction between acivicin and 6-TG when administered in a sequential fashion. Moreover, the sequence dependence of the synergistic interaction between acivicin and 6-TG suggests that additional mechanisms may be operative. Removal of acivicin might be associated with reinitiation of DNA synthesis in an expanded and synchronized population of S-phase cells. Such cells might be particularly vulnerable to the cytotoxic effects of a purine antagonist such as 6-TG. Acivicin may, therefore, potentiate 6-TG cytotoxicity by two potential mechanisms: it may enhance its metabolism by increasing the availability of PRPP, and it may augment the population of susceptible cells. We have found that exposure of L1210 cells to progressively higher concentrations of 6-TG (e.g. 10^{-4} M) for 2 hr, followed by thorough washing, results in a maximal cytotoxic effect of approximately 90% (unpublished observation). Enhanced cell killing resulting from acivicin-induced augmentation of 6-TG nucleotide formation may, therefore, approach a plateau level; further cytocidal effects may depend upon cytokinetic factors.

In addition to their effects on the expression of 6-TG-mediated cytotoxicity, cytokinetic factors may also affect the metabolism of purine analogs such as 6-TG. The activities of several purine and pyrimidine salvage pathway enzymes have been shown to be increased in the S-phase cell fraction. For example, the pyrimidine salvage pathway enzyme deoxycytidine kinase has been shown to be more active in cells actively engaged in DNA replication [42]. It is possible that the activity of the purine salvage pathway enzyme hypoxanthine-guanine phosphoribosyltransferase may similarly be increased in the S-phase population. Moreover, in our studies, perturbations in 6-TG metabolism were recorded for the total cell population. It is theoretically posible that acivicin-induced metabolic alterations in the subset of S-phase cells might be greater than those observed for the population of cells as a whole. To address this question, it would be necessary to perform simultaneous biochemical studies on actively cycling cells, which might be separated from noncycling cells through centrifugal elutriation [43]. Since actively cycling stem cells may be a more relevant population from the standpoint of clonogenicity, examination of 6-TG metabolism in these cells might clarify the role of acivicin in potentiating 6-TG cytotoxicity.

These studies suggest both a dose- and sequence-dependent synergistic interaction between acivicin and 6-TG which may have implications for the design of *in vivo* trials employing these agents. It would appear logical to administer acivicin prior to 6-TG based on the biochemical and cytokinetic considerations described above. It must be noted that factors relevant to the *in vivo* setting may not be reflected by the *in vitro* model. For example, drug pharmacokinetic characteristics, as well as circulating and intratumoral levels of competing purine bases (e.g. hypoxanthine), may have an important bearing on the therapeutic efficacy of a regimen employing acivicin and 6-TG in the intact animal. Nevertheless, the sequential administration of these agents has a rational biochemical and cytokinetic basis, and the initiation of *in vivo* studies appears warranted.

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