Biochemical Pharmacology, Vol. 28, pp. 991–994.

0006-2952/79/0401-0991 $02.00/0

REVERSAL OF THE CYTOTOXICITY OF 3′-AMINO-3′-DEOXYTHYMIDINE BY PYRIMIDINE DEOXYRIBONUCLEOSIDES

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(*Received* 23 *August* 1978; *accepted* 6 *October* 1978)

Abstract—Mammalian cell replication is strongly inhibited by 3′-amino-3′deoxythymidine (3′-aminothymidine). This cytotoxicity can be specifically prevented or reversed by pyrimidine 2′-deoxyribonucleosides. The addition of 50 μM 2′-deoxycytidine to L1210 cells treated with 10 μM 3′-aminothymidine for 24 hr reduced the population doubling time from about 38 hr to 17 hr. The control cells doubled every 13 hr. Another cytotoxic effect produced by 3′-aminothymidine is a dose- and time-dependent increase in cell volume. 2′-Deoxycytidine can effectively prevent and reverse this increase. 3′-Aminothymidine appears to be a potent selective inhibitor of DNA synthesis in L1210 cells. The incorporation of [^{3}H] thymidine into DNA was inhibited by 50 per cent at 1 μM 3′-aminothymidine, a concentration which reduced L1210 replication by about 65 per cent. The rate of incorporation of [^{3}H] adenine into DNA, another measure of DNA synthesis, was reduced similarly by 3′-aminothymidine, and 2′-deoxycytidine eliminated this inhibition as well. An effect on RNA or protein synthesis was not detected. The incorporation of [^{3}H] uridine or [^{3}H] adenine into RNA, or of tritiated amino acids into protein, was not reduced by 25 μM 3′-aminothymidine. These results suggest that selective disruption of DNA metabolism may account for the cytotoxicity of 3′-aminothymidine.

Minor modification of chemotherapeutic agents may produce marked changes in their spectrum of activity and degree of selective toxicity. We have found that substitution of thymidine and its analogs at the 3′- or the 5′- position with an amino function yields compounds with striking differences in biological activity [1–3]. For example, 5′-amino-5′-deoxythymidine and 5-iodo-5′-amino-2′,5′-dideoxyuridine (AIdUrd) strongly inhibit herpes simplex type 1 replication in the absence of detectable host cell toxicity [2, 3]. Conversely, 3′-amino-3′-deoxythymidine (3′-aminothymidine) potently inhibits mammalian cell growth but is not a good antiviral agent [1], whereas the 3′,5′-diamino analog lacks both activities [1]. Phosphorylation of AIdUrd, which is required for biological activity, occurs only in herpes simplex infected cells and, thus, accounts for the selective toxicity of the drug [4]. Although the biochemical basis of the cytotoxicity produced by 3′-aminothymidine is unknown, the findings reported in this paper suggest that interference with DNA synthesis is involved.

MATERIALS AND METHODS

Materials. 3′-Aminothymidine was first synthesized by Horwitz *et al.* [5] and Miller and Fox [6] and was prepared as described previously [1]. [Methyl-^{3}H] thymidine (20 Ci/m-mole), [5-^{3}H] uridine (25 Ci/m-mole) and G-^{3}H-labeled amino acids from a reconstituted protein hydrolysate were obtained from Schwarz/Mann, Orangeburg, NY, and [2-^{3}H]adenine (14 Ci/m-mole) was purchased from Moravek Biochemicals, City of Industry, CA, The Sigma Chemical Co., St. Louis, MO, supplied the thymidine (dThd), 2′-deoxyuridine (dUrd) and 2′-deoxycytidine (dCyd).

Cell culture. Suspension cultures of L1210 cells were grown at 37° in Fischer's medium supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, NY). Growth was exponential for at least 72 hr after 5×10^5 cells were plated at a density of 1×10^4 cells/ml in 15-ml culture tubes. Three separate tubes were prepared for each experimental point. Cell counts were done with a model ZBI Coulter counter (Hialeah, FL.).

Cell volume. Mean and modal cell volumes were determined with an electronic multichannel particle size analyzer (Coulter Electronics, Hialeah, FL) calibrated with polystyrene microspheres (diameter = 18.04 μ). The samples were triturated before sizing to assure single cell suspensions.

Macromolecular synthesis. The incorporation of radiolabeled precursors into protein, RNA and DNA was used as an estimation of the rates of macromolecular synthesis. L1210 cells (approximately 2×10^5 cells/ml) were pulse labeled for 30 min with 1.5 μCi/ml of either the tritiated amino acids, uridine or thymidine at 0, 1, 2, 3 and 4 hr after treatment with different concentrations of 3′-aminothymidine. After labeling, the cells were collected by centrifugation (200 *g*) in ice-cold phosphate buffered saline (PBS), resuspended in 0.1 ml PBS containing 2 mM EDTA, and then spotted on Whatman no. 1 paper discs. The filters were batch washed twice with 5% trichloroacetic acid, once with 95% ethanol, and dried. Radioactivity was quantified by liquid scintillation spectrometry.

In other experiments, [2-^{3}H]adenine was used as a precursor, and its incorporation into both RNA and DNA was determined. The adenine (1 μCi/ml) was added 4 hr after drug treatment, samples were removed 20, 40 and 60 min later, and then the cells were collected by centrifugation in ice-cold PBS. The nucleic acids were precipitated and washed with 0.7 M $HClO_4$, and the RNA was then hydrolyzed at 60° for 30 min in 0.3 M KOH. After acidification with 0.7 M $HClO_4$ and centrifugation, portions were removed for the determination of [2-^{3}H]adenine incorporation into RNA by

liquid scintillation spectrometry. The precipitate of each sample was washed twice in 0.7 M $HClO_4$, hydrolyzed for 30 min at 70° in 0.7 M $HClO_4$, and the amount of radioactivity in the supernatant fraction determined.

RESULTS

Effects on cell growth. In a previous study, we showed that the inhibition of L1210 cell growth by 3′-aminothymidine could be prevented by pyrimidine 2′-deoxyribonucleosides (dThd, dUrd and dCyd), but not by other nucleosides [1]. As shown in Fig. 1, this effect is dependent on the concentrations of both the protecting and toxic agents. Approximately 40 times more 3′-aminothymidine was required to inhibit L1210 cell growth by 50 per cent if 50 μM dUrd was also present. Intermediate shifts in the dose–response curve resulted from the addition of 5 μM and 25 μM dUrd. The degree of protection achieved was limited by the toxicity exerted by dUrd (Fig. 1) and dThd at concentrations greater than 50 μM. We observed that dCyd effectively antagonized the action of 3′-aminothymidine and is not toxic at 100 μM; thus it was used in the following experiments.

The inhibition of L1210 cell growth caused by 3′-aminothymidine can be reversed as well as prevented by pyrimidine deoxyribonucleosides. The effect of adding 50 μM dCyd to cells at 0, 8 and 24 hr after treatment with 10 μM 3′-aminothymidine is shown in Fig. 2. The rates of growth, estimated from the population doubling times between 24 and 72 hr, were similar (approximately 13 hr) for control cells and cells rescued at 8 hr. If no dCyd was added, the cells exposed to 3′-aminothymidine had a doubling time of about 38 hr as compared to 17 hr for cells rescued at 24 hr. This experiment has been repeated several times and the results in Fig. 2 represent the minimum degree of reversal seen. In two experiments, there was no difference in the growth rate or the cell number of untreated cells and cells rescued after 8 hr. The magnitude of reversal is dependent on the concentration of both 3′-aminothymidine and the added pyrimidine deoxyribonucleosides in a manner similar to that for protection (Fig. 1).

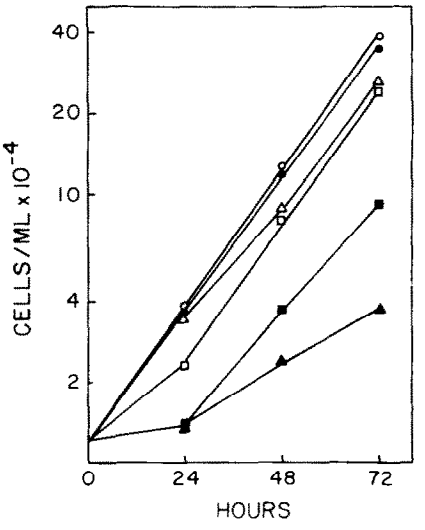

Fig. 2. Reversal with dCyd of the inhibition of L1210 cell growth caused by 3′-aminothymidine. L1210 cells were treated at 0 hr with PBS (●), 50 μM dCyd (○) and 10 μM 3′-aminothymidine alone (▲), or in combination with 50 μM dCyd added at 0 hr (△), 8 hr (...) or 24 hr (■). Cells were counted after 24, 48 and 72 hr of incubation.

Effects on cell volume. A cytotoxic effect caused by a number of anticancer drugs is cellular enlargement [7]. We have found that treatment of L1210 cells with 3′-aminothymidine produces a dose-dependent and time-dependent increase in their volume. The ability of dCyd to prevent and reverse this effect is shown in Fig. 3. After the addition of 10 μM 3′-aminothymidine to exponentially growing L1210 cells, an increase in the modal cell volume of the population was evident within 4 hr. Cells treated for 8 hr and 24 hr had volumes 50 and 100 per cent larger, respectively, than cells which had been exposed to PBS. If dCyd and 3′-aminothymidine were added simultaneously, the volume increase

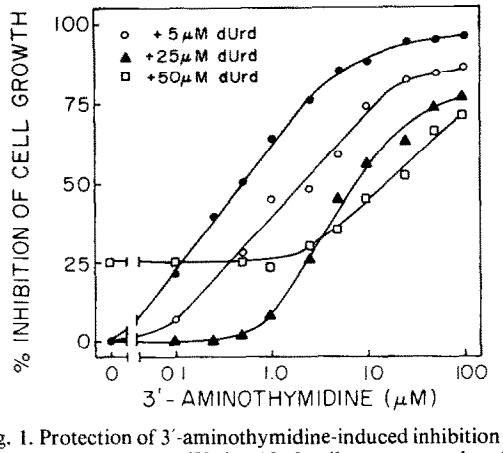

Fig. 1. Protection of 3′-aminothymidine-induced inhibition of L1210 cell growth by dUrd. L1210 cells were treated at the same time with concentrations of 3′-aminothymidine, varying from 0.1 μM to 100 μM, and either PBS (●), 5 μM dUrd (○), 25 μM dUrd (▲) or 50 μM dUrd ( ). The cultures were incubated for 72 hr and the number of cells was determined.

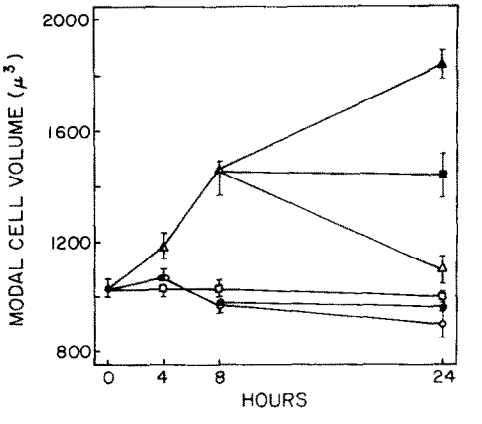

Fig. 3. Effects of 3′-aminothymidine alone and in combination with dCyd on the volume of L1210 cells. Exponentially growing L1210 cells were treated at 0 hr with PBS (●), 50 μM dCyd (○), 10 μM 3′-aminothymidine alone (▲), or in combination with 50 μM dCyd added at 0 hr ( ), 4 hr (△) or 8 hr (■). Cell volumes (mean ± S. D., n = 3) were determined at 0, 4, 8 and 24 hr.

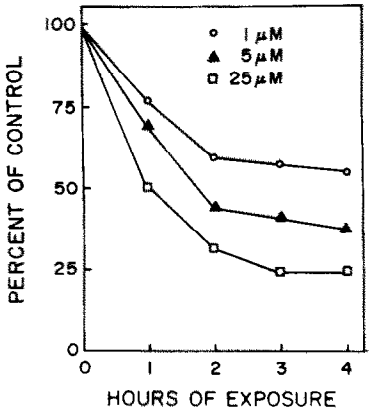

Fig. 4. Effects of 3′-aminothymidine on the incorporation of [methyl-^{3}H] thymidine (7.5×10^{-8} M) into the DNA of L1210 cells. L1210 cells were exposed to 1 μM (○), 5 μM (▲) or 25 μM (□) 3′-aminothymidine and pulsed with [methyl-^{3}H] thymidine for 30 min at 1 hr intervals. These data are the average of two separate experiments. Control values averaged 17,900 cpm/10^5 cells.

was prevented. Reversal of the effect is clearly shown by the addition of dCyd 4 hr after 3′-aminothymidine. In this case, the volume increase continued for another 4 hr but by 24 hr the cell volume approached control values.

Effects on macromolecular synthesis. Interference with DNA metabolism by 3′-aminothymidine seemed likely since the compound is a dThd analog. Rates of DNA synthesis in L1210 cells were estimated from the amount of [methyl-^{3}H]thymidine incorporated into DNA during 30-min pulses (Fig. 4.). Inhibition increased with the amount of 3′-aminothymidine added and with the time of exposure. A 40 per cent decrease in incorporation was evident 2 hr after the addition of 1 μM 3′-aminothymidine, a dose which inhibits cell growth by about 65 per cent. Since the drug differs from dThd only at the 3′-position and might alter [methyl-^{3}H]thymidine metabolism and dTTP pool sizes, [2-^{3}H]adenine was also used as a precursor for DNA and RNA biosynthesis. In the experiment shown in Fig. 5, the incorporation of [2-^{3}H]adenine into DNA and RNA was determined from the same sample following separation of the nucleic acids. L1210 cells were exposed to 3′-aminothymidine (10 μM) for 4 hr in the presence or absence of dCyd (50 μM) prior to labeling. DNA synthesis was inhibited about 75 per cent by the aminonucleoside, an effect which was prevented fully by the addition of dCyd. The incorporation of [2-^{3}H]adenine in RNA was unaltered under these experimental conditions (Fig. 5B). The lack of effect on RNA synthesis was confirmed in experiments measuring the rate of [5-^{3}H]uridine incorporation into RNA. The synthesis of proteins in L1210 cells, estimated by the incorporation of tritiated amino acids into protein, was also not inhibited by 25 μM 3′-aminothymidine (data not shown) even after 4 hr of exposure. These data suggest that 3′-aminothymidine is a potent selective inhibitor of DNA synthesis.

DISCUSSION

The results of this study suggest that 3′-aminothymidine is a selective inhibitor of DNA metabolism in L1210 cells. Inhibition of DNA biosynthesis measured by the incorporation rates of two nucleoside precursors ([methyl-^{3}H]-thymidine and [2-^{3}H]adenine) was marked at concentrations which inhibit cell growth by about 65 per cent. Neither RNA nor protein synthesis was altered detectably at a 25-fold greater drug concentration. A related compound, 3′-deoxy-3′-fluorothymidine, has been shown to be a selective inhibitor of DNA synthesis [8]. The fluoro derivative is metabolized to the 5′-triphosphate and subsequently incorporated into DNA, where it is likely to terminate chain growth [9]. 3′-Aminothymidine may be acting in a similar fashion and its metabolism is under investigation. It is also possible that the compound may be incorporated internally into DNA via the formation of a 3′-phosphoramidate bond. The presence of a 5′-phosphoramidate bond resulting from the incorporation of AIdUrd into herpes simplex DNA has been demonstrated in this laboratory [4]. The triphosphates of two other nucleosides modified at the 3′-position, 2′,3′-dideoxyadenosine and 2′,3′-dideoxythymidine, terminate DNA chain growth in cell-free systems [10, 11], and the latter has been shown to be phosphorylated and produce chain termination in replicating mammalian cells [12].

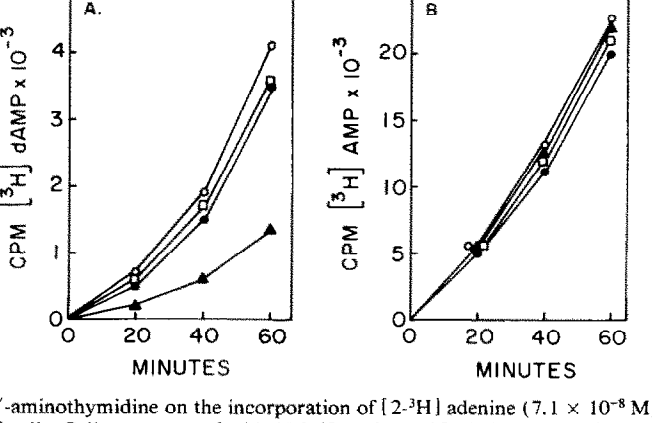

Fig. 5. Effects of 3′-aminothymidine on the incorporation of [2-^{3}H] adenine (7.1×10^{-8} M) into the DNA and RNA of L1210 cells. Cells were treated with PBS (●), 50 μM dCyd (○), 10 μM 3′-aminothymidine (▲) or with both 10 μM 3′-aminothymidine and 50 μM dCyd (□), for 4 hr prior to labeling with [2-^{3}H] adenine. The radioactivity associated with DNA (panel A) and RNA (panel B) in each sample was determined as described in Materials and Methods.

Other metabolic effects, mediated through interaction with enzymes involved in DNA biosynthesis, may be produced by 3′-aminothymidine or one of its potential metabolites. Deoxythymidine kinase obtained from mouse Sarcoma 180 cells is inhibited by 3′-aminothymidine [13].

The increase in cell volume caused by 3′-aminothymidine is consistent with a selective effect on DNA synthesis. Several investigators have observed a similar increase in the volume of cells treated with compounds which inhibit DNA but not protein synthesis [7, 14, 15]. Such agents primarily affect cells synthesizing DNA and block the progression of cells through the S phase. Cells will accumulate at the G1/S border, and the resulting condition of unbalanced growth will lead to abnormally large cells [16]. 3′-Aminothymidine may be acting in this manner.

The site of the protective interaction between 3′-aminothymidine and the pyrimidine deoxyribonucleosides has not been elucidated. Nor is it known whether metabolism of dCyd, dUrd or thymidine to any of the nucleotides is a prerequisite for the prevention of toxicity. However, the ability of the pyrimidine deoxyribonucleosides to reverse 3′-aminothymidine-induced toxicity, even 24 hr after drug treatment, indicates that competition at the level of cellular transport is not of major importance. Inhibition of metabolic activation (e.g. phosphorylation), prevention of incorporation into DNA, or competition at a site of enzyme inhibition could be involved in the protective interaction. Perhaps this *in vitro* phenomenon can be extended to preferential rescue of host tissues *in vivo*.

Acknowledgements—This investigation was supported by the United States Public Health Service Grant CA-05262 from the National Cancer Institute. P. H. F. is supported by Public Health Service Postdoctoral Fellowship CA-05604 from the National Cancer Institute and T. S. L. is supported, in part, by a grant from the Bristol-Myers Co.

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