Biochemical Pharmacology, Vol. 42, No. 3, pp. 507–513, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITION OF 2-DESAMINO-2-METHYL-10-PROPAGYL-5,8-DIDEAZAFOLIC ACID CYTOTOXICITY BY 5,10-DIDEAZATETRAHYDROFOLATE IN L1210 CELLS WITH DECREASE IN DNA FRAGMENTATION AND DEOXYADENOSINE TRIPHOSPHATE POOLS

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(*Received* 15 *October* 1990; *accepted* 15 *March* 1991)

Abstract—5,10-Dideazatetrahydrofolate (DDATHF) is an antifolate drug, the cytotoxic effects of which can be fully reversed by hypoxanthine, suggesting that DDATHF exerts its effects by inhibiting *de novo* purine biosynthesis. ICI198583 is a quinazoline based inhibitor of thymidylate synthase. In this study we examine the interaction between treatment of mouse leukaemic L1210 cells with these drugs. The addition of DDATHF with ICI198583 was correlated with a decrease in ICI198583 cytotoxicity in a dose dependent manner. This protection was associated with a decrease in DNA fragmentation, and a drop in intracellular dATP pools. These results support the hypothesis that inhibitory effects on *de novo* purine biosynthesis by inhibitors of dihydrofolate reductase may limit cytotoxicity, and indicate that a rise in dATP pools may be an important cytotoxic signal.

The mechanisms of antifolate cytotoxicity have been widely investigated over the past decades. Methotrexate (MTX†) is one of the most clinically important antifolates. Methotrexate and its polyglutamylated derivatives directly inhibit dihydrofolate reductase (DHFR) and other folate dependent enzymes. This results in a sharp decrease in thymidylate and *de novo* purine synthesis [1, 2]. Recently, molecular analysis of DNA from MTX treated cells has shown that the chromatin was fragmented into multimers of approximately 200 base pairs [3]. This is a distinctive biochemical marker for a form of cell death known as apoptosis [4]. It is an active process whereby an endogenous endonuclease cleaves the chromatin between the nucleosomes to produce the characteristic DNA fragments [4]. Mouse L1210 cells treated with 2-desamino-2-methyl-10-propagyl-5,8-dideazofolic acid (ICI198583)—a quinazoline based inhibitor of thymidylate synthase [5], also die via apoptosis.‡ Addition of preformed purines has been shown to markedly increase the cytotoxicity of a high concentration of MTX (10^{-5} M) and piritrexim—a lipophilic inhibitor of DHFR [6] to the level achieved by an equivalent concentration of ICI198583.‡ Exogenous purines have no effect on ICI198583 treatment.‡ Purine potentiation of the two DHFR inhibitors, and ICI198583 cytotoxicity are associated with extensive DNA fragmentation and elevation of intracellular dATP pools. A rise in dATP levels has been shown to be correlated with cytotoxicity in a variety of experimental systems [7–11] and to be an important signal for apoptosis [8, 9, 12].

If the DHFR inhibitors are relatively inefficient in killing cells at a high drug concentration (10^{-5} M) compared with the thymidylate synthase inhibitor (ICI198583) because of their inhibitory effects on *de novo* purine synthesis, then an agent which specifically inhibits the purine pathway should decrease the cytotoxicity of thymidylate synthase inhibitors 5,10-Dideazatetrahydrofolate (DDATHF) is such an antifolate. DDATHF is a structural analog of tetrahydrofolate whose effects can be fully reversed by hypoxanthine (HX) suggesting that DDATHF exerts its effects solely by inhibiting *de novo* purine synthesis [13]. A previous study has already shown that DDATHF and the less potent CB3717 drug (N^{10}-propagyl-5,8-dideazafolate) are antagonistic in their interactions, although the reason for this interaction is not known [14]. In this study, we examined the interactions between ICI198583 and DDATHF in mouse leukaemic L1210 cells and speculate on the possible biochemical basis for their activity. The addition of DDATHF with ICI198583 was correlated with a decrease in ICI198583 cytotoxicity in a dose dependent manner. The protection by DDATHF was also associated with a decrease in DNA fragmentation and a drop in intracellular dATP pools. These findings support the hypothesis that MTX cytotoxicity may be inefficient because of its inhibitory effects on *de novo* purine synthesis and indicate that a rise in dATP pools may be an important signal for apoptosis.

MATERIALS AND METHODS

Chemicals. DDATHF was obtained from Lilly Research Laboratories (Indianapolis, IN, U.S.A.) and ICI198583 was a gift from Dr A. Jackman

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† Abbreviations: MTX, methotrexate; ICI198583, 2-desamino-2-methyl,N^{10}-propagyl-5,8-dideazafolic acid; DDATHF, 5,10-dideazatetrahydrofolate; PRPP, phosphoribosyl pyrophosphate; HX, hypoxanthine.

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(Institute of Cancer Research, Surrey, U.K.) DDATHF and ICI198583 were made up as 1 mM stock solutions. ICI198583 was dissolved in 0.15 M $NaHCO_3$ and DDATHF in water. Hypoxanthine was made up freshly as a 10 mM solution and 10 M NaOH solution was added dropwise until the drug was dissolved. ^{3}H-labelled deoxynucleosides were purchased from the Radiochemical Center (Amersham, U.K.). Unlabelled deoxynucleotides were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and P.L. Biochemicals Inc. (Milwaukee, WI, U.S.A.). DNA polymerase (klenow fragment) was obtained from Pharmacia, (Piscataway, NJ, U.S.A.). The polydeoxyadenylate–deoxythymidylate template was purchased from Miles Laboratories (Elkhart, IN, U.S.A.).

Cell culture. Mouse leukaemia L1210 cells were grown in suspension culture in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% non-dialysed foetal calf serum (FCS), L-glutamine (2 mM) and gentamycin (32 μg/mL). The doubling time of the cells was approximately 11–12 hr. In all experiments, cells were set up at 5×10^4 cells/mL and allowed to grow undisturbed for 24 hr before addition of drugs. All treatments were carried out with exponentially growing cell cultures. Cell counts were made by phase-contrast microscopy which was used to discriminate between live (phase-positive) and dead (phase-negative) cells.

Microtitration cloning assay. Cells were washed once and resuspended in drug-free medium. A viable cell count was made and the culture diluted to the required cell number. The cells were distributed in 200 μL of drug-free medium per well, into 96 well round-bottom plates (Crown Corning, Liverpool, N.S.W.) using a Titertek multichannel pipette (Flow Laboratories, Irvine, U.K.). Cloning efficiency was determined by plating doubling dilutions of viable cells ranging from 5 to 0.625 cells/well, with 48 wells for each dilution. If drug treatment resulted in a high number of negative wells, the cells were plated at 10× higher concentration.

The plates were incubated in a humidified 10% CO_2 atmosphere and the wells were inspected for positive colonies after 14 days. The cloning efficiency of the cells was calculated from the proportion of negative wells using Poisson statistics and χ^2 minimization [15]. Cloning results were expressed as colony forming units/mL which were calculated from percentage cloning efficiency times viable cell concentration of cultures at time of cloning. The cloning efficiency of the control culture of L1210 cells was 90%.

DNA extraction. The cells (1×10^7) were washed once in Dulbecco's phosphate buffered saline (PBS) and lysed in a 0.05 M Tris–Cl buffer (pH 8), containing 10 mM EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulphate (SDS) and 200 mg/mL proteinase K (Sigma Chemical Co.). The lysate was incubated at 50° for 3 hr before being extracted with phenol (twice), chloroform/isoamyl alcohol (24:1) (twice). The DNA was then precipitated overnight at −20° with 0.1 vol. 3 M sodium acetate and 2 vol. of absolute ethanol. The DNA was pelleted by centrifugation at 13,000 *g* for 10 min before being resuspended in TE buffer (10 mM Tris–HCl (pH 8), 1 mM EDTA). The sample was then treated with 100 μg/mL RNase A (Sigma Chemical Co.) for 1 hr at 37° and then with 200 μg/mL proteinase K for another hour at 37°. The sample was extracted again with phenol and chloroform and the DNA precipitated again. DNA (10 μg) from each sample was analysed by electrophoresis on a 1% agarose gel containing ethidium bromide (0.3 μg/mL).

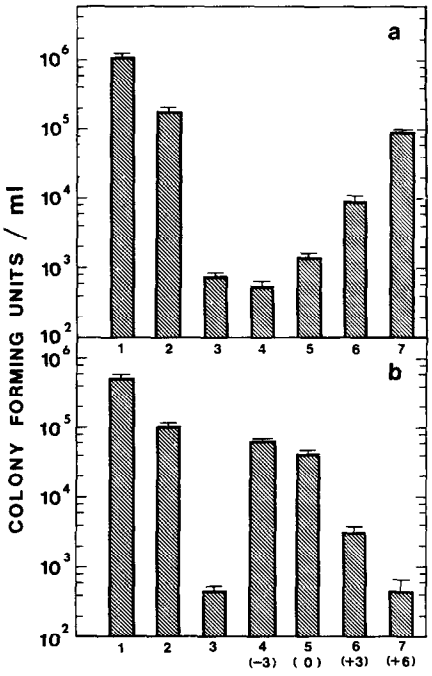

Fig. 1. Inhibition of ICI198583 cytotoxicity by DDATHF in L1210 cells. Columns 1, 2 and 3 in (a) and (b) represent the values of the untreated control, 10^{-5} M DDATHF and 10^{-5} M ICI198583 respectively. (a) Columns 4–7 represent values for drug combinations of 10^{-5} M ICI198583 plus 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M DDATHF, respectively. (b) Columns 4–7 represent values for drug combination of 10^{-5} M DDATHF and ICI198583 with DDATHF added 3 hr before (−3), simultaneously (0) and 3 (+3) and 6 (+6) hr after the addition of ICI198583. Cytotoxicity was determined using a microtitration assay (as described in methods) 24 hr after the addition of ICI198583. SE of means derived from plating four serial dilutions (48 wells/dilution) for each drug treatment.

DNA Fragmentation assay. The cells (3×10^6) were washed once in PBS (Cytosystems, Castle Hill, N.S.W.) and lysed with 0.4 mL hypotonic lysing buffer (pH 7.5) containing 10 mM Tris–Cl, 1 mM EDTA and 0.2% (v/v) Triton X-100. The lysate was incubated on ice for 15 min and then centrifuged at 13,000 *g* for 10 min to separate intact chromatin from fragmented DNA. Both the supernatant and the pellet were precipitated separately in 12.5% (w/v) trichloroacetic acid (TCA) at 4° overnight. The precipitate was pelleted at 11,000 *g* for 4 min.

The DNA in the precipitate was hydrolysed by heating to 90° for 10 min in 80 μL 5% TCA and quantified using a modification of the diphenylamine method [16]. The degree of DNA fragmentation

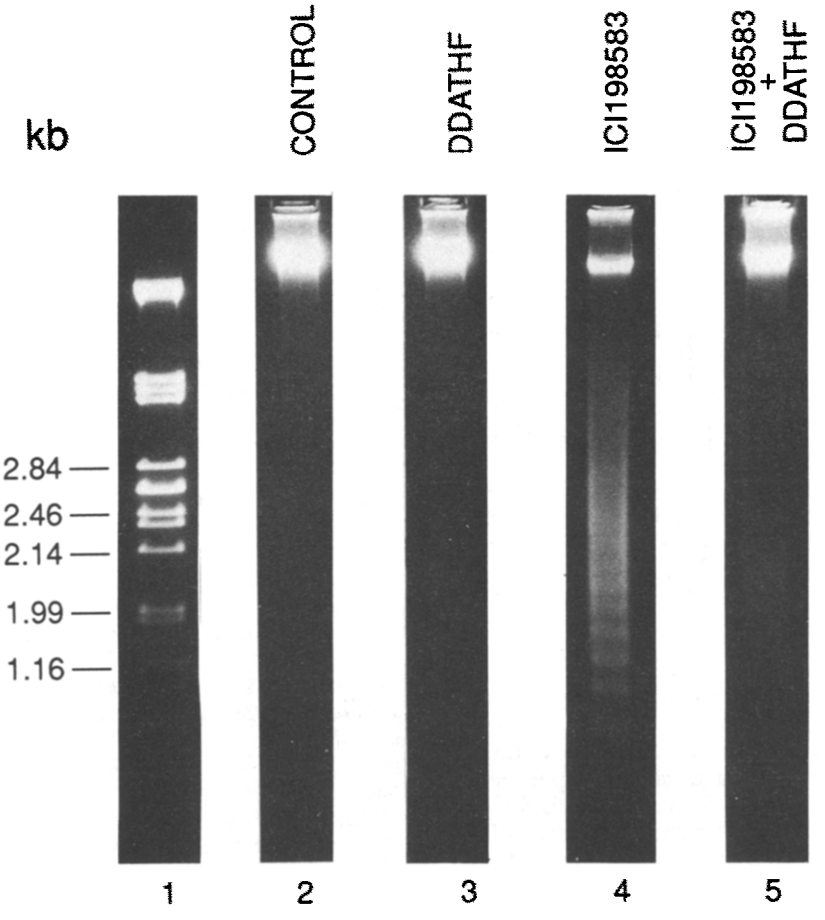

Fig. 2. Agarose gell electrophoresis of DNA from L1210 cells exposed to antifolates. A 10^{-5} M concentration of the antifolates was used. Drug exposure time was 24 hr. DDATHF and ICI198583 were added simultaneously. Approximately 10 μg of DNA from each sample was analysed on a 1% agarose gel. DNA size markers (lane 1) were derived from a *Pst* 1 restriction enzyme digestion of λ phage DNA (Sigma).

refers to the percentage of DNA in the 13,000 *g* supernatant divided by the total DNA from the pellet and supernatant.

Deoxyribonucleoside triphosphate pool assay. The viable cells (5×10^6) were washed once in cold PBS (with 2 mM EDTA) and extracted with ice cold 60% (v/v) ethanol. The extract was lyophilised and resuspended in 500 μL of 10 mM Tris buffer (pH 7.85). The sample was then centrifuged at 11,000 *g* for 15 min at 4° and the supernatants stored at −20°. The deoxyribonucleotides were measured by a modification of the DNA polymerase assay [11]. The concentrations of the deoxyribonucleoside triphosphates were determined from calibration curves of picomole amounts of pure standards.

RESULTS

A microtitration cloning assay was used to determine the effects of DDATHF on the cytotoxicity of ICI198583 as shown in Fig. 1. Addition of DDATHF at concentrations higher than 10^{-8} M produced marked protection of cells exposed to 10^{-5} M ICI198583 for 24 hr as shown in Fig. 1a. The cytotoxicity was decreased by a 100-fold when 10^{-5} M DDATHF was added with ICI198583. The degree of protection also varied depending on whether DDATHF was added before or after ICI198583 as shown in Fig. 1b. DDATHF (10^{-5} M) was most protective when the drug was added 3 hr before ICI198583 and its effect decreased rapidly as DDATHF was added 3 and 6 hr after ICI198583.

Electrophoretic analysis of DNA extracted from the untreated and drug treated L1210 cells is shown in Fig. 2. The DNA from the untreated control culture remained as unfragmented high molecular weight DNA. DNA from cells exposed to 10^{-5} M DDATHF for 24 hr showed only faint apoptotic bands (the multimers of approximately 200 base pairs) compared with an equivalent concentration of ICI198583. When 10^{-5} M DDATHF was added with 10^{-5} M ICI198583, the apoptotic bands were again fainter than ICI198583 alone, suggesting that DDATHF was preventing cells from dying by apoptosis.

The degree of DNA fragmentation was determined

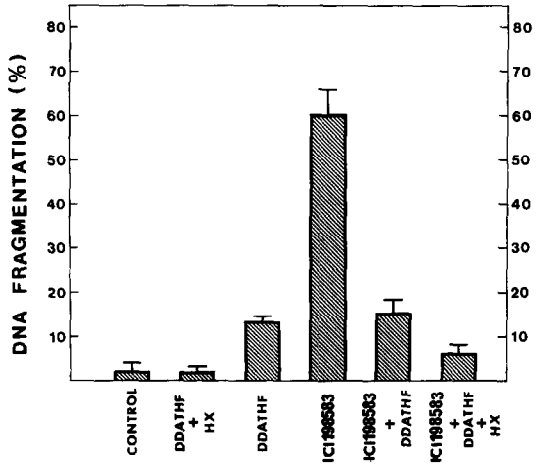

Fig. 3. Degree of DNA fragmentation in L1210 cells treated with antifolates. Drug exposure time was 24 hr. A 10^{-5} M concentration of each antifolate and 10^{-4} M HX were used. Combinations of drugs were added simultaneously. Means ± SD were derived from a total of nine replicates from three separate experiments.

by centrifugation of cell lysate to separate intact from fragmented DNA and using the diphenylamine method [16] to quantify the amount of DNA in the supernatant and pellet fraction. As shown in Fig. 3, the background fragmentation in the untreated control was approximate 2%. In cells exposed to 10^{-5} M ICI198583 for 24 hr, the degree of DNA fragmentation was 60% (*t*-test 21.1; $P < 0.01$). When 10^{-5} M DDATHF was added with 10^{-5} M ICI198583, the amount of fragmentation was significantly reduced to 15% (*t*-test 15.5; $P < 0.01$), a level comparable to 10^{-5} M DDATHF alone (*t*-test 1.4; $P < 0.19$).

Purine potentiation of MTX cytotoxicity has been demonstrated to be closely correlated with the rise in intracellular levels dATP levels [7–10]. To determine whether the protection from ICI198583 cytotoxicity by DDATHF was correlated with a drop in dATP levels, the changes in dATP and dTTP pools in cells treated with either 10^{-5} M DDATHF or ICI198583 alone or in combination was followed for 24 hr as shown in Fig. 4. Cells treated with ICI198583 showed a substantial peak (3-fold) in dATP levels after 12 hr exposure (Fig. 4a) in conjunction with low dTTP (Fig. 4b) as previously observed and discussed elsewhere.* The addition of DDATHF with ICI198583 prevented the rise in dATP levels, lowering it to approximately 23% of the control after 6 hr (Fig. 4a). The dTTP pools remained at 6–20% without recovering to higher levels as seen in ICI198583 treated cells (Fig. 4b). Cells treated with DDATHF alone had reduced dATP pools (to approximately 14% of the untreated control) after 6 hr, which remained near that level for the rest of the time course (Fig. 4a). The dTTP pool showed a sharp increase (>2-fold) after 6 hr exposure before decreasing to 10% as shown in Fig. 4b.

To test whether the ICI198583–DDATHF drug combination behaved similarly to MTX in being potentiated by preformed purines, 10^{-4} M HX was added with 10^{-5} M concentrations of the two drugs. When the amount of DNA fragmentation was assayed, the addition of HX significantly decreased the DNA fragmentation to 10% (Fig. 3) (*t*-test 6.3; $P < 0.01$). To explore whether the concentration of DDATHF may affect the degree of purine potentiation, a viable cell count was made at 24 and 48 hr after cells had been exposed to 10^{-5} M ICI198583 with either 10^{-6} or 10^{-5} M DDATHF. The addition of 10^{-4} M HX alone was capable of rescuing cells exposed to the two different concentrations of DDATHF. DDATHF alone (10^{-6} and 10^{-5} M) inhibited cell proliferation to 35% of control with little difference in cell kill through the 48 hr period. The concentration of DDATHF appeared to be important in determining how HX interacted with the ICI198583–DDATHF drug combination. Hypoxanthine made little difference to the cytotoxicity of ICI198583 with 10^{-6} M DDATHF while at 10^{-5} M DDATHF, HX strongly protected ICI198583 treated cells, allowing them to proliferate at half the rate of the untreated control.

DISCUSSION

The study of the complex mechanisms of classical antifolate cytotoxicity (notably MTX) has been greatly advanced by the development of folate analogues which directly inhibit folate utilising enzymes involved in purine and pyrimidine synthesis. One such antifolate is ICI198583, a desamino derivative of folic acid which is a potent inhibitor of thymidylate synthase [5]. Another is DDATHF, a tetrahydrofolate analogue which has been demonstrated to inhibit glycinamide ribonucleotide transformylase, dramatically lowering the purine (ATP and GTP) pools and arresting cells at the late G_1/S phase of the cell cycle [13]. The effects of ICI198583 and DDATHF can be reversed using thymidine and HX respectively [5, 13], compared with MTX which requires both purines and pyrimidines to completely rescue many cell types from the effect of the drug [17].

Flow cytometry studies have shown that a high concentration of MTX (10^{-4} M) resulted in growth arrested cells which died after a few hours [17]. Analysis of the DNA from the MTX treated cells indicated that they died via a process known as apoptosis [3]. Apoptotic cells are characterized molecularly by the activation of an endonuclease which cleaves chromatin in the linker region between the nucleosomes, producing lengths of DNA fragments in multimers of approximately 200 base pairs [4]. The relative importance of inhibiting thymidylate or *de novo* purine synthesis in inducing apoptotic cells in MTX treated cells can be examined using the two novel antifolates. Comparisons of the relative cytotoxicity of ICI198583 and MTX have shown that MTX was approximately 60 times less toxic than ICI198583 at an equivalent concentration (10^{-5} M). Moreover, MTX cytotoxicity (as well as

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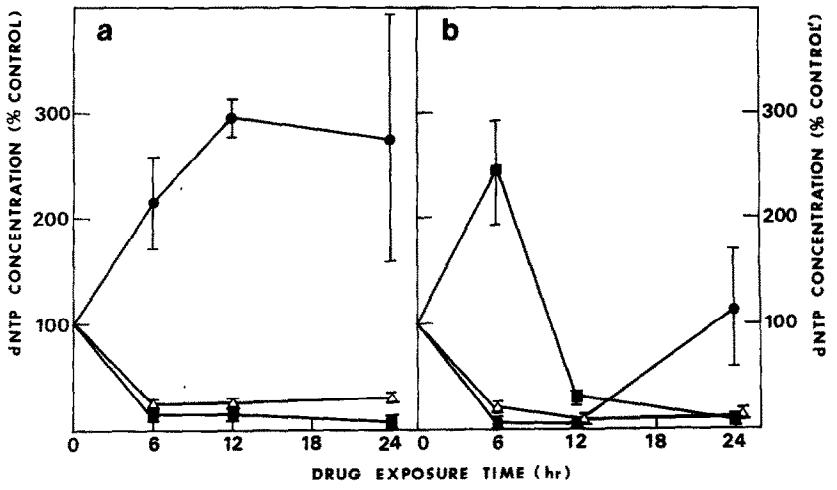

Fig. 4. Changes in intracellular levels of (a) dATP and (b) dTTP with time in L1210 cells treated with either 10^{-5} M ICI198583 (●) or 10^{-5} M DDATHF (■) alone or in combination (△). Means ± SD were obtained from a total of four replicates from two separate experiments. All results are expressed as a percentage of the zero hour untreated control. Control levels (pmol/10^{-6} cells) of dATP and dTTP were 10.7 ± 3 (mean ± SD) and 27.3 ± 7, respectively.

another DHFR inhibitor, piritrexim) can be potentiated by the addition of 10^{-4} M HX to the level achieved by ICI198583 alone.* Thus, the inhibition of *de novo* purine synthesis appeared to be an important limit to MTX cytotoxicity.

By adding DDATHF (a purine inhibitor) to ICI198583, the cytotoxicity of the pure thymidylate synthesis inhibitor should be diminished in the same manner as MTX. This was verified by a previous study which demonstrated that interactions between DDATHF and CB3717 (a less soluble form of ICI198583) was either non-interactive or antagonistic using H35 hepatoma cells [13].

As shown in Fig. 1a, the degree of protection from 10^{-5} M ICI198583 cytotoxicity by DDATHF increased in a dose dependent manner. Moreover, the time at which 10^{-5} M DDATHF was added to 10^{-5} M ICI198583 treated cells was important as well. The greatest protection of the first part studied was seen when cells were exposed to DDATHF for 3 hr prior to the addition of ICI198583. If added 6 hr later than ICI198583, the protection was low to non-existent (Fig. 1b). As yet, the mechanism of protection is still unclear. Galivan *et al.* [14] suggest that the protection they reported between DDATHF and CB3717 may be due to direct interaction between the two drugs. They ruled out competition for transport into the cell since these two drugs utilised different uptake routes and suggested instead that the site of antagonism may be for the folypolyglutamate synthetase where the two drugs can compete as substrate for the enzyme. This is unlikely since the level of dTTP (which reflects the degree of thymidylate synthase inhibition by ICI198583) in cells exposed to both DDATHF and ICI198583 remained at the same level as ICI198583 treated cells after 12 hr exposure (Fig. 4) even though there was a 100-fold decrease in cell death (Fig. 1a). However, a direct measurement of thymidylate synthetase activity is needed to clarify this issue. The protection of ICI198583 toxicity by DDATHF may have a similar mechanism to CB3717 and DDATHF interactions but competition for transport remains a possibility in the former, because the two agents use the same transport protein.

Our findings indicate that the mechanism of protection by DDATHF may be via the apoptotic pathway. Electrophoresis of the DNA from L1210 cells treated with the drugs alone or in combination for 24 hr suggests that the number of apoptotic cells (as defined by the relative brightness of the apoptotic bands or the multimers of 200 base pairs) decreased when 10^{-5} M DDATHF was added with 10^{-5} M ICI198583 compared with ICI198583 by itself. A concentration of 10^{-5} M DDATHF alone was also capable of inducing apoptosis. These findings are supported by quantitative studies on the degree of DNA fragmentation as shown in Fig. 3. Cells exposed to 10^{-5} M ICI198583 had 60% of their DNA fragmented. The addition of 10^{-5} M DDATHF reduced the fragmentation to 15%, a level achieved by 10^{-5} M DDATHF alone.

Previous studies on the perturbations of dNTP pools induced by antifolate treatment have demonstrated a strong correlation between a rise in intracellular dATP levels and cytotoxicity of the drugs [7–10]. Other studies have found that the elevated dATP pools may act as a signal for apoptosis [8, 9, 12]. The strong inhibition of ICI198583 cytotoxicity by DDATHF was also correlated with a drop in dATP levels to 20% of the untreated control (Fig. 4a). The importance of elevated dATP pools as a signal for apoptosis was strengthened by the observation that cells exposed to 10^{-5} M DDATHF had their dATP levels reduced to 25% of the control (Fig. 4a) and remained growth arrested

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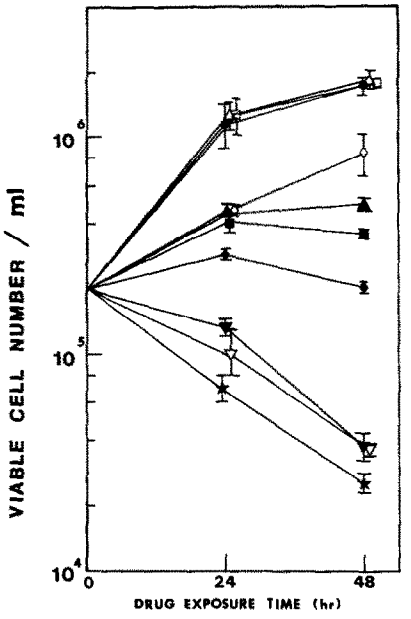

Fig. 5. Growth curves of L1210 cells exposed to combinations of ICI198583, DDATHF and HX over a 48 hr period. (●) untreated control, (□) 10^{-6} M DDATHF plus 10^{-4} M HX, (△) 10^{-5} M DDATHF plus 10^{-4} M HX, (▲) 10^{-6} M DDATHF, (■) 10^{-5} M DDATHF, (▼) 10^{-5} M ICI198583 plus 10^{-6} M DDATHF, (▽) 10^{-5} M ICI198583 plus 10^{-6} M DDATHF and 10^{-4} M HX, (◆) 10^{-5} M ICI198583 plus 10^{-5} M DDATHF, (◇) 10^{-5} M CB3819 plus 10^{-5} M DDATHF and 10^{-4} M HX, (★) 10^{-5} M ICI198583. Means ± SD were obtained from a total of four replicates from two separate experiments.

for 24–48 hr with little increase in cell death (Fig. 5). This contrasted with ICI198583 treated cells which resulted in large cell death after 24 hr exposure to the drug (Fig. 5).

To examine whether the CB3819–DDATHF drug combination can be potentiated by HX in the same manner as MTX, 10^{-4} M HX was added with 10^{-5} M concentration of the two drugs. As shown in Fig. 3, the DNA fragmentation studies and viable cell counts (Fig. 5) suggest that HX actually decreased further the cytotoxicity of the drug combination even though the concentration of HX used was sufficient to completely rescue cells from the effects of 10^{-5} M DDATHF (Figs. 3 and 5). The reasons for this protection may be quite complicated, involving changes in the folate pools or PRPP levels. Measurements of the dATP and dTTP pools of cells treated with the drug combination plus HX should help determine whether the exogenous HX was being efficiently metabolised.

Our findings, however, do not preclude the hypothesis proposed by Fairchild *et al.* [18], that the negative effect on purine synthesis by MTX prevents drug treated cells from progressing to the more toxic S phase and that the addition of HX enables more cells to be exposed to the cytotoxic mechanisms of MTX rather than the purines themselves becoming toxic. Studies using synchronised cells and the addition of DDATHF at various points in the cell cycle may clarify whether it is the block in late G1/S phase induced by DDATHF or whether it is a drop in dATP levels which is the main mechanism of protection of ICI198583 cytotoxicity.

In summary, recent reports have shown that the interactions between a purine inhibitor, DDATHF and a thymidylate synthase inhibitor, CB3717 were either non-interactive or antagonistic, although the reasons for it are not known. Our studies using DDATHF and a more potent thymidylate synthase inhibitor, ICI198583 support those findings. The protection of ICI198583 cytotoxicity by DDATHF increased in a dose dependent manner and at 10^{-5} M DDATHF, the cytotoxicity was a 100-fold less than 10^{-5} M ICI198583 alone. The protection was correlated with a marked drop in intracellular dATP levels to 20% of the control as well as a decrease in the distinctive pattern of DNA fragmentation characteristic of apoptotic cells, suggesting that the mode of protection by DDATHF is in the reduction of the apoptotic signal. Moreover, the observations that the dTTP pools remained equally depressed in cells treated with ICI198583 in the absence or presence of DDATHF suggest that the mode of antagonism may not be direct competition between the two drugs for activating enzymes. The data presented in this study support the hypothesis that the inhibition of *de novo* purine synthesis by MTX may be an important part in limiting the cytotoxicity of this antifolate and that elevated dATP levels may be a strong signal for apoptosis.

Acknowledgement—This study was supported by the University of Sydney Cancer Research Fund.

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