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5,10-DIDEAZATETRAHYDROFOLIC ACID REDUCES TOXICITY AND DEOXYADENOSINE TRIPHOSPHATE POOL EXPANSION IN CULTURED L1210 CELLS TREATED WITH INHIBITORS OF THYMIDYLATE SYNTHASE

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Abstract—5,10-Dideazatetrahydrofolic acid (DDATHF) reduces *de novo* purine biosynthesis by inhibiting glycinamide ribonucleotide transformylase. ICI D1694 and CB3717 are folate-based inhibitors of thymidylate synthase (TS). Fluorodeoxyuridine (FdUrd) following metabolism to FdUMP also inhibits TS. In cultured L1210 cells DDATHF reduced the toxicity of ICI D1694, CB3717 and FdUrd in a concentration-dependent manner. This protection correlated with a prevention of the increase in intracellular dATP pools seen in cells exposed to the TS inhibitors alone. The possibility that DDATHF protection might be due to competition for cell entry is not likely since CB3717 and FdUrd but not ICI D1694 enter the cell by independent transport processes. Exogenous hypoxanthine (HX) had no effect on the toxicity of TS inhibitors. However, HX increased the protective effect of DDATHF from ICI D1694 toxicity, had no effect on the CB3717–DDATHF interaction, and reduced the protective effect of DDATHF on FdUrd toxicity. HX prevented the fall in dATP levels caused by DDATHF addition to cells treated with TS inhibitors. HX had different effects on dTTP levels in cells treated with DDATHF and quinazoline TS inhibitors compared to FdUrd. Together these results support the hypothesis that imbalance in dTTP and dATP pools is an important determinant of cytotoxicity in antifolate-treated cells. In addition, these findings suggest that intracellular reduced folates interconvert to catalyse reactions in metabolically perturbed cells.

Key words: antifolates; deazafolate; quinazoline; fluorodeoxyuridine; modulation

The mechanisms of antifolate cytotoxicity have been studied for many years. MTX†, the most clinically important antifolate drug, directly inhibits DHFR, resulting in depletion of intracellular reduced folates. Lowering of reduced folate levels leads to inhibition of *de novo* purine and thymidylate synthesis [1, 2]. Moreover, MTX polyglutamates and an accumulation of FH_2 following MTX treatment further impair purine and thymidylate biosynthesis [3–5]. Exogenous thymidine prevents, to varying extents in different cell lines, the growth inhibitory and cytotoxic effects of MTX treatment. Exogenous purines together with thymidine totally prevent the effects of MTX treatment in cultured cells. However, exogenous purines alone have been shown to markedly potentiate the cytotoxicity of high concentrations of both MTX and PTX, a lipid soluble inhibitor of dihydrofolate reductase [6, 7]. The potentiation of MTX cytotoxicity by exogenous purines was noted to correlate with a rise in dATP pool levels [6, 7]. It has been postulated that inhibitors of dihydrofolate reductase which disrupt both thymidylate and *de novo* purine synthesis (e.g. MTX and PTX at high concentration) may be less cytotoxic than pure thymidylate synthase inhibitors because imbalance in deoxyribonucleoside triphosphate levels is less extreme [5–8].

DDATHF is an inhibitor of GAR and AICAR transformylase enzymes of *de novo* purine synthesis [9] (Fig. 1). CB3717, ICI 198583 and ICI D1694 are quinazoline inhibitors of thymidylate synthase [10–12]. DDATHF has been reported to reduce cytotoxicity caused by CB3717 treatment of cultured cells [13]. Previous studies in our laboratory have shown that DDATHF treatment reduced the cytotoxicity of ICI 198583, a derivative of CB3717. This protective effect correlated with a reduction in the dATP pool increase caused by ICI 198583 treatment alone [14]. It was proposed that the imbalance in dTTP and dATP levels in antifolate-treated cells might be an important trigger for cell death [7]. In this paper, further studies are reported investigating the interaction of DDATHF with inhibitors of thymidylate synthase namely ICI D1694, CB3717 and fluorodeoxyuridine (FdUrd). Our results add further support to the hypothesis that imbalance in dTTP and dATP pools is an important determinant of cytotoxicity in antimetabolite-treated cells.

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† Abbreviations: MTX, methotrexate; PTX, piritrexim; DDATHF, 5,10-dideazatetrahydrofolate; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; ICI 198583, 2-desamino-2-methyl, N^{10}-propargyl-5,8-dideazafolic acid; ICI D1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-methyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; FU, fluorouracil; FdUrd, fluorodeoxyuridine; HX, hypoxanthine; TS, thymidylate synthase; DHFR, dihydrofolate reductase; 5,10-CH_2FH_4, 5,10-methylene tetrahydrofolate; GAR, glycinamide ribonucleotide; AICAR, aminomidazolecarboxamide ribonucleotide; PRPP, phosphoribosyl pyrophosphate.

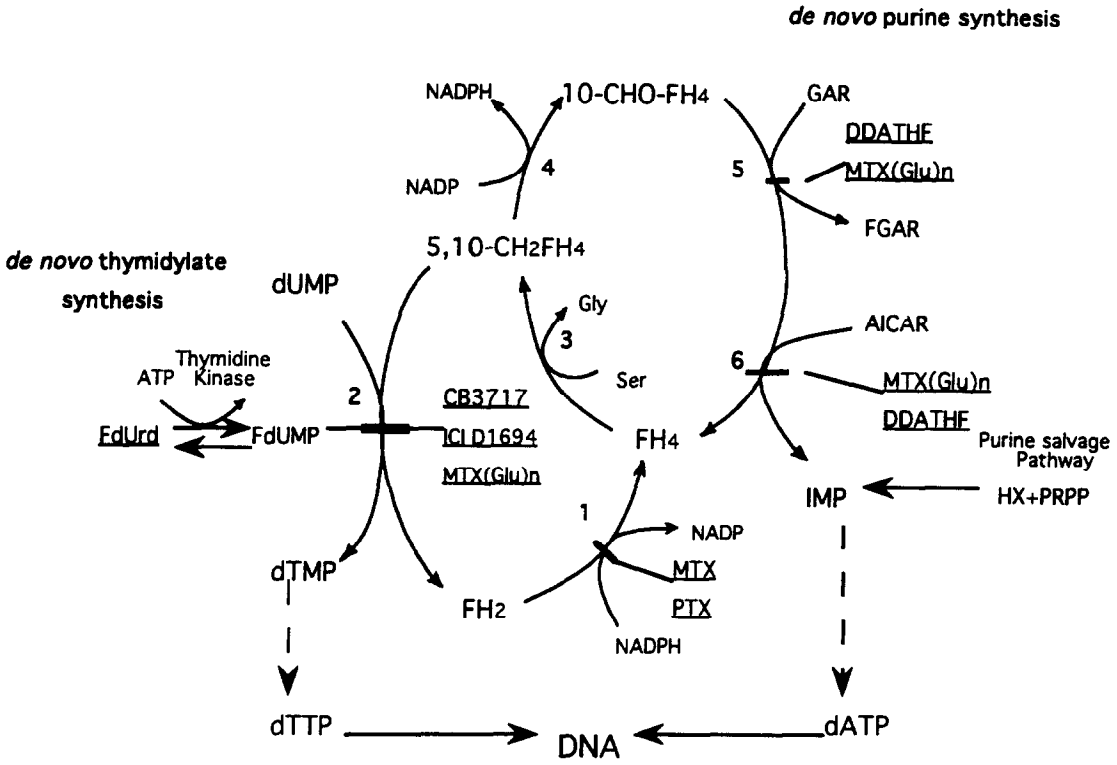

Fig. 1. Scheme of *de novo* purine and thymidylate synthesis. Enzymes: 1. DHFR; 2. TS; 3. serine hydroxymethyltransferase; 4. multifunctional protein possessing the activities of 5,10-methylene-tetrahydrofolate dehydrogenase plus 5,10-methenyltetrahydrofolate cyclohydrolase plus 10-formyl-tetrahydrofolate synthetase; 5. GAR formyltransferase; 6. AICAR formyltransferase.

MATERIALS AND METHODS

Chemicals. DDATHF was obtained from Lilly Research Laboratories (Indianapolis, IN, U.S.A.) and ICI D1694 from ICI Pharmaceuticals (Macclesfield, U.K.). FdUrd was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). CB3717 was a generous gift of Dr. A. L. Jackman, Department of Biochemical Pharmacology, Institute of Cancer Research, Sutton, U.K. DDATHF and ICI D1694 were dissolved in water and made as 10 mM stock solutions. These were stored at 4° in the case of ICI D1694 and −20° for DDATHF until use. [8-^{3}H]-dATP and [methyl-^{3}H]TTP were purchased from the Radiochemical Centre (Amersham, U.K.). Unlabelled deoxynucleosides were purchased from Sigma and P.L. Biochemicals Inc. (Milwaukee, WI, U.S.A.). DNA polymerase (Klenow fragment) was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Polydeoxyadenylate-deoxythymidylate template was purchased from Miles Laboratories (Elkhart, IN, U.S.A.) and Sigma.

Cell culture. The mouse leukaemia L1210 cell line has been maintained in continuous culture in this laboratory for many years. The cells are grown as suspension cultures at 37° in RPMI 1640 medium (Flow Laboratories, U.S.A.) supplemented with 10% non-dialysed foetal calf serum (FCS), L-glutamine (2 mM) and gentamycin (32 μg/mL). Cell population doubling time was approximately 11–12 hr.

Cell growth inhibition. Cells were set up at 5×10^4 cells/mL (2 mL) on Costar 24-well cluster plates (Cambridge, MA, U.S.A.). Cells were allowed to grow undisturbed for 24 hr before addition of drugs and hypoxanthine. All treatments were carried out with exponentially growing cell cultures. After 48 hr incubation with drugs, cell counts were made by phase-contrast microscopy which was used to discriminate live (phase-positive) and dead (phase-negative) cells.

Microtitration cloning assay. Cells were washed once after drug treatment and resuspended in drug-free medium. Cells were counted and the viable cells were 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, NSW, Australia) using a Titertek multichannel pipette (Flow Laboratories). Cloning efficiency was determined by plating doubling dilutions of viable cells ranging from 5 to 0.625 cells/well, with 24 wells for each dilution. If drug treatment resulted in a high number of negative wells, the cells were plated at 10× higher cell concentration (i.e. 50 to 6.25 cells/well). The plates were incubated in a humidified 10% CO_2, 5% O_2 atmosphere and the wells were inspected for positive colonies after 14 days. Positive colonies were scored

Table 1. Drug growth inhibitory effects for L1210 and L1210-MTX cells

Drug	IC_{50} in culture (at 48 hr) (μM)		Resistance (fold)
	L1210 cells	L1210-MTX	
MTX	0.011 ± 0.003*	64.7 ± 1.3	5881
DDATHF	0.78 ± 0.06	95.0 ± 7.07	122
CB3717	2.5 ± 0.5	14.2 ± 0.47	5.7
ICI D1694	0.0088 ± 0.0005	1.87 ± 0.26	212
FdUrd	0.006 ± 0.001	0.0063 ± 0.002	1

* Data are the means ± SE ($N \geq 3$).

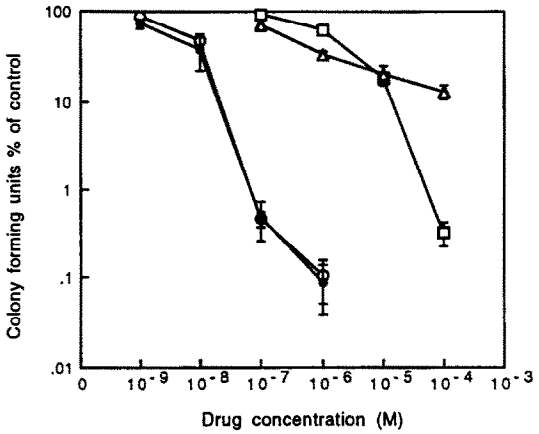

Fig. 2. Cytotoxicity of ICI D1694, CB3717, DDATHF and FdUrd in L1210 cells. (○) ICI D1694, (□) CB3717, (△) DDATHF, (●) FdUrd. Cytotoxicity was determined using a microtitration assay (as described in Materials and Methods). Drug exposure time was 24 hr. Data are the means ± SD (bars) from three experiments.

if wells contained 100 or more viable cells at 14 days. The cloning efficiency of the cells was calculated from the proportion of negative wells using Poisson statistic and χ^2 minimization [15]. Cloning results were expressed as colony forming units/mL which was calculated from the percentage cloning efficiency times viable cell concentration of cultures at the time of cloning. The cloning efficiency of the control culture of L1210 cells was 100%.

Deoxyribonucleoside triphosphate pool assay. The cells (5×10^6) were collected by centrifugation, washed once in cold PBS and nucleotides extracted with ice cold 60% (v/v) ethanol. The extract was lyophilized 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 supernatant stored at −20°. The deoxyribonucleotides were measured by a modification of the DNA polymerase assay [16]. The concentrations of deoxyribonucleoside triphosphates were determined from calibration curves of picomole amounts of pure standards.

RESULTS

Cytotoxicity of TS inhibitors and DDATHF

The IC_{50} of MTX, CB3717 and ICI D1694 at 48 hr were similar to the previous reports of studies with L1210 cells (Table 1) [12]. In the L1210-MTX cells, a cell line which was continuously exposed over 6 months to increasing MTX concentrations from 10 nM to 10 μM, the IC_{50} of MTX was increased over 5000-fold compared to the parent line. The IC_{50} for DDATHF and ICI D1694 in these latter cells was increased 122- and 212-fold, suggesting that both drugs utilize a common cell membrane transport system. In contrast, the IC_{50} for CB3717 was increased only 5.7-fold and for FdUrd was unchanged compared to the parent line suggesting drug uptake by different mechanisms.

A microtitration cloning assay was used to determine the cytotoxicity of drugs used in this study as shown in Fig. 2. In L1210 cells, the potency of ICI D1694 is similar to FdUrd, and both drugs are more cytotoxic than CB3717 and DDATHF. At low concentration CB3717 is less potent than DDATHF (Table 1) but at 100 μM CB3717 is more cytotoxic than DDATHF (100 μM) (Fig. 2).

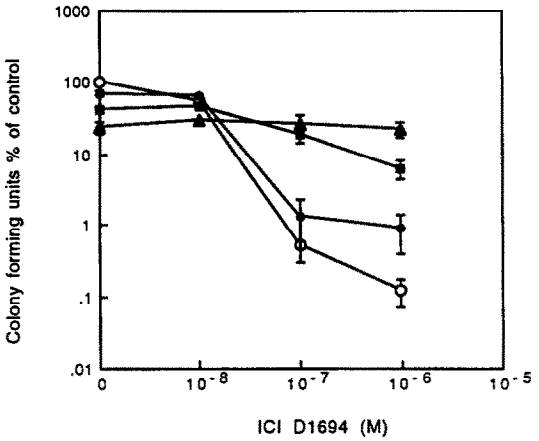

Fig. 3. Effect of DDATHF on the cytotoxicity of ICI D1694 in L1210 cells. (○) ICI D1694 alone, (●) ICI D1694 plus 0.1 μM DDATHF, (■) plus 1 μM DDATHF, (▲) plus 10 μM DDATHF. First block point is DDATHF alone. Cytotoxicity was determined using a microtitration assay. Drug exposure time was 24 hr. Data are the mean ± SD (bars) from at least three experiments.

Effects of DDATHF on ICI D1694 and CB3717 cytotoxicity

The effects of DDATHF on the cytotoxicity of ICI D1694 for 24 hr are shown in Fig. 3. DDATHF (0.1, 1 and 10 μM) protected ICI D1694 (0.1 and 1 μM) cytotoxicity in a concentration-dependent manner. At low ICI D1694 concentration (0.01 μM) the outcome was less than additive. The effects of DDATHF on the cytotoxicity of CB3717 are shown in Fig. 4. DDATHF 10 μM largely prevented the toxicity of equimolar or higher concentrations of CB3717 (Fig. 4a). The concentation dependence of the DDATHF protection was investigated by varying

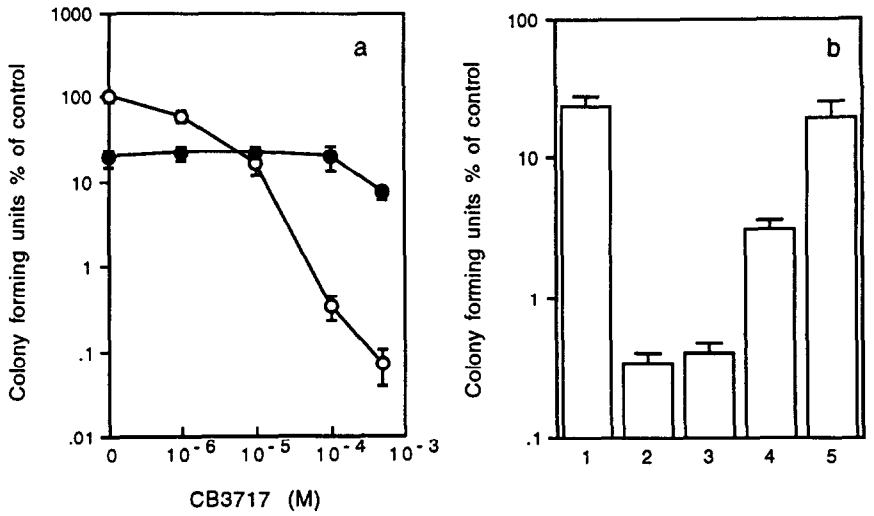

Fig. 4. (a) Effect of DDATHF on CB3717 cytotoxicity, (○) CB3717 alone; (●) CB3717 plus 10 μM DDATHF. The first block point is 10 μM DDATHF alone. (b) Effect of different concentrations of DDATHF on 100 μM CB3717 cytotoxicity. Columns 1 and 2 represent 10 μM DDATHF and 100 μM CB3717, respectively. Columns 3–5 represent values for 100 μM CB3717 plus 0.1, 1 and 10 μM DDATHF respectively. Data are the mean ± SD (bars) from two experiments.

the DDATHF concentrations (0.1–10 μM) and maintaining constant the concentration of CB3717 (100 μM). Figure 4b indicates that the degree of protection from toxicity due to CB3717 increased with increasing concentrations of DDATHF up to 10 μM. At equimolar concentrations, cytotoxicity was similar to DDATHF (100 μM) by itself (data not shown).

Earlier studies in our laboratory have demonstrated that purine potentiation of MTX cytotoxicity was closely correlated with a rise of intracellular dATP levels [6, 7]. To determine whether DDATHF protection from ICI D1694 and CB3717 cytotoxicity was mediated through effects on dNTP pools, dATP and dTTP pools were monitored in cells treated under the same conditions as shown in Fig. 5. DDATHF 10 μM alone reduced dATP pools to approximately 30% of control by 6 hr even though toxicity was minimal (Fig. 5). dTTP pools in DDATHF treated cells declined at late time points. These observations support the notion that DDATHF treatment primarily disrupts purine synthesis with delayed effects on dTTP levels.

dNTP pool changes after treatment with ICI D1694 and CB3717 showed a sustained reduction in dTTP and a time-dependent increase in dATP level up to 12 hr. Changes in dNTP level after 12 hr may be confounded by the effects of nucleosides and bases released into the culture medium by dying cells [17]. After ICI D1694 (0.1 μM) or FdUrd (0.1 μM) treatment, approximately 40–50% of cells were dead at 24 hr. Addition of DDATHF with ICI D1694 prevented the rise in dATP levels seen after ICI D1694 or CB3717 treatments alone. Indeed ATP levels were reduced to approximately 30% of control (Fig. 5a and b).

The possibility that competition for cell entry might contribute to the DDATHF interaction with ICI D1694 was investigated by studying the DDATHF interactions with CB3717 which utilises a different transport process [18], in part explaining the relative insensitivity of cells to this compound. DDATHF (10 μM) treatment had differing effects on dTTP pools in ICI D1694 (0.1 and 1 μM) and CB3717 (100 μM) treated cells. With ICI D1694 (0.1 μM), DDATHF (10 μM) partially prevented the fall in dTTP and a similar effect was seen at 1 μM ICI D1694 (data not shown). With CB3717, DDATHF had no effect on dTTP at least until after 12 hr (Fig. 5c and d). These results are consistent with the notion that competition for transport between DDATHF and ICI D1694 may complicate interpretation of their intracellular interactions. The effects of DDATHF on CB3717 toxicity, and intracellular dNTP pools may illuminate the cytotoxic mechanisms of thymidylate synthase inhibition, and our results are consistent with dNTP pool imbalance being important.

Effects of DDATHF on FdUrd cytotoxicity

FdUrd is a pyrimidine analogue which following metabolism to FdUMP inhibits thymidylate synthase. FdUrd enters cells by the nucleoside transport process distinct from the folate transport mechanisms [19]. The effects of different concentrations DDATHF on the cytotoxicity of FdUrd are shown in Fig. 6. DDATHF (1 and 10 μM) reduced the cytotoxicity of FdUrd (0.1 and 1 μM). This effect was DDATHF concentration dependent, though less so than the interaction with quinazoline thymidylate synthase inhibitors. The effects of DDATHF treatment on dATP and dTTP pools in FdUrd-treated cells are shown in Table 2. Cells exposed to FdUrd 0.1 μM alone had reduced dTTP and increased dATP levels similar to that of ICI D1694- and CB3717-treated cells. In FdUrd-treated

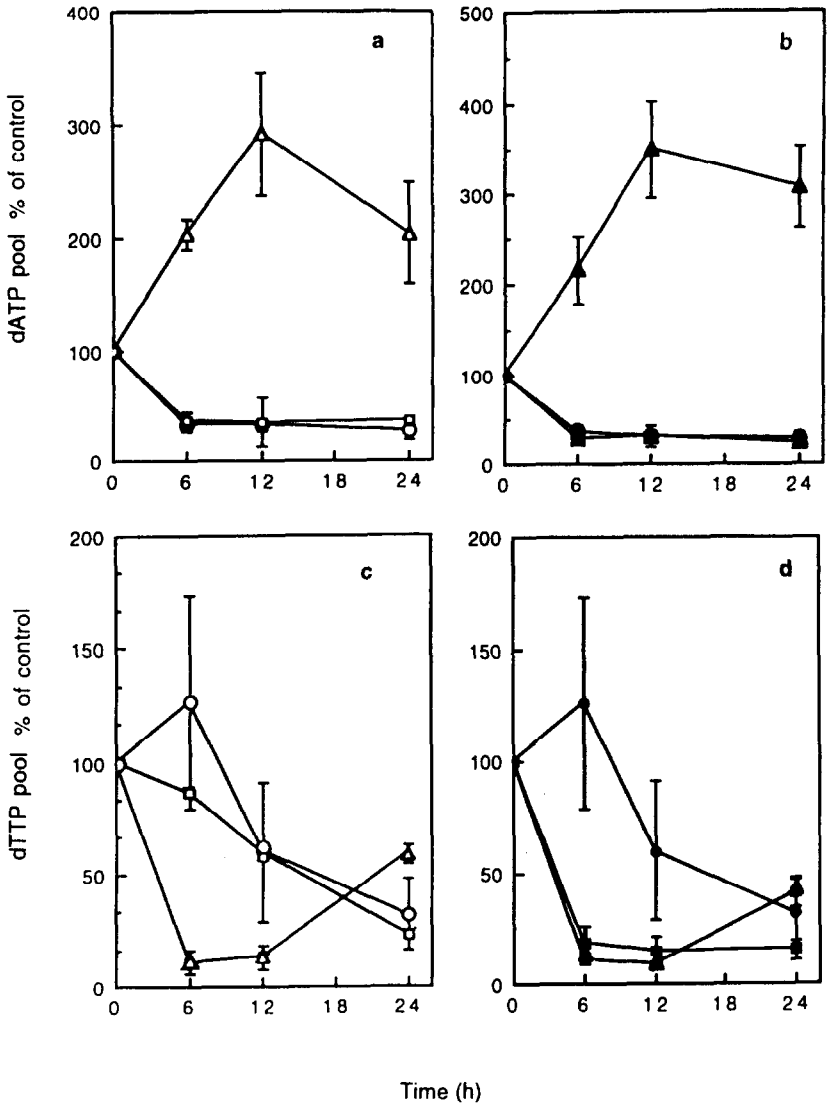

Fig. 5. Effects of DDATHF on dATP and dTTP levels in quinazoline antifolate-treated L1210 cells: (a) and (b) refer to dATP, and (c) and (d) to dTTP. (a) and (c) show results with time of treatment with 0.1 μM ICI D1694 (△), 10 μM DDATHF (○), or the combination (□); (b) and (d) show results with time of 100 μM CB3717 (▲), 100 μM DDATHF (●), or the combination (■). Means ± SD were obtained from four duplicates of two separate experiments. All results are expressed as a percentage of the zero hour untreated control.

cells, 10 μM DDATHF reduced dATP pools but did not influence dTTP levels for the first 12 hr.

Effects of exogenous hypoxanthine

The effect of exogenous hypoxanthine (100 μM) on the interaction of DDATHF with inhibitors of thymidylate synthase was investigated (Fig. 7). As expected, HX reduced the cytotoxicity of DDATHF alone. However, HX had no effect on the toxicity due to the inhibitors of thymidylate synthase alone, HX increased the protective effect of DDATHF from ICI D1694 toxicity as shown in Fig. 7a, but had no effect on CB3717 alone or CB3717 and DDATHF (Fig. 7b). HX had no effect on FdUrd toxicity and reduced the protective effect of DDATHF on FdUrd as shown in Fig. 7c.

The effects of HX on dNTP pool changes caused by ICI D1694, CB3717, FdUrd and DDATHF treatment are shown in Table 2. Cells exposed to 100 μM HX with 10 μM DDATHF had normal dATP and dTTP levels, indicating that exogenous HX can completely prevent the effects of DDATHF on dNTP levels. Similarly, HX had no effects at least for the first 12 hr on dATP or dTTP levels in cells exposed to ICI D1694, or CB3717 or FdUrd alone. However, HX substantially prevented the fall in dATP levels caused by DDATHF addition to cells treated with ICI D1694, CB3717 and FdUrd.

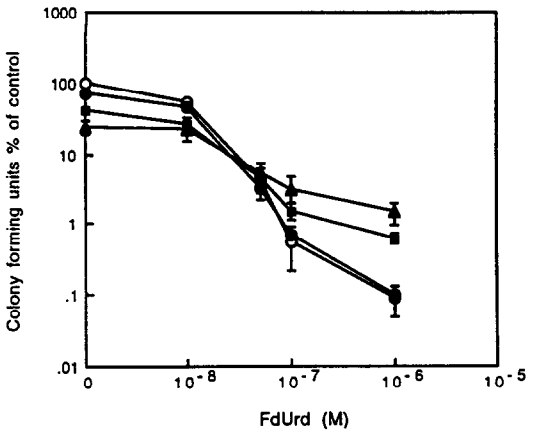

Fig. 6. Effect of DDATHF on the cytotoxicity of FdUrd in L1210 cells. (○) FdUrd alone, (●) FdUrd plus 0.1 μM DDATHF, (■) plus 1 μM DDATHF, (▲) plus 10 μM DDATHF, First block points are DDATHF (0.1, 1 and 10 μM) alone. Cytotoxicity was determined using a microtitration assay. Drug exposure time was 24 hr. Data are the mean ± SD (bars) from three experiments.

The effects of HX on dTTP levels in cells treated with DDATHF differed between quinazoline antifolate and fluoropyrimidine TS inhibitors. In ICI D1694-DDATHF-treated cells, HX completely restored dTTP pools to normal control level. In CB3717-DDATHF-treated cells, HX partly prevented dTTP pool reduction. In FdUrd-DDATHF-treated cells, HX did not significantly affect dTTP levels.

DISCUSSION

The development of new antifolate drugs which selectively inhibit different folate-metabolizing enzymes involved in purine and pyrimidine synthesis provides new tools for further investigating the complex perturbations of folate and nucleic acid metabolism caused by MTX treatment. CB3717, ICI 19853 and ICI D1694 are quinazoline derivatives of folate which are potent inhibitors of thymidylate synthase [10–12]. ICI D1694 is a more potent TS inhibitor and more soluble in water than CB3717 [12]. The mechanism of TS inhibition by ICI D1694 is non-competitive with respect to 5,10-CH_2FH_4 in L1210 cells [20]. ICI D1694 and its polyglutamates also inhibit DHFR, but the specificity is 100-fold greater for TS over DHFR [12]. DDATHF is a tetrahydrofolate analogue which inhibits GAR and AICAR transformylase (Fig. 1), leading to dramatically lowered purine ribonucleotide (ATP and GTP) pools and cell arrest at the late G1/S phase of the cell cycle [9, 21].

Deoxyribonucleoside triphosphates (dNTP) are the basic building block for the synthesis of DNA; a balanced supply of dNTP maintains the accuracy of DNA replication and also is important for DNA repair. Imbalance of dNTP can lead to mutation caused by nucleotide misincorporation and/or to cell death [22–24]. TS inhibitors (ICI D1694, CB3717 and FdUrd) reduce dTMP synthesis and hence dTTP levels, while *de novo* purine synthesis continues leading to a relative dATP increase. This dNTP imbalance contributes to growth arrest and cytotoxicity (Table 2). DDATHF inhibits *de novo* purine synthesis and reduces dATP pool. Inhibition of DHFR by MTX reduces the folate pools required for both purine and thymidylate synthesis. We have

Table 2. dATP and dTTP pool changes in drug-treated L1210 cells

Drug(s)*	dATP (% of control)†			dTTP (% of control)		
	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
HX	102 ± 17‡	115 ± 39	90 ± 15	108 ± 10	123 ± 11	91 ± 38
DDATHF	34 ± 7	30 ± 6	27 ± 7	126 ± 47	60 ± 31	32 ± 16
DDATHF + HX	88 ± 14	90 ± 36	101 ± 17	106 ± 33	94 ± 24	99 ± 21
ICI D1694	203 ± 14§	292 ± 54	203 ± 45	11 ± 5§	13 ± 5	59 ± 4
ICI D1694 + HX	209 ± 36	279 ± 40	258 ± 96	17 ± 3	14 ± 8	30 ± 13
ICI D1694 + DDATHF	32 ± 7	34 ± 22	30 ± 3	86 ± 5	59 ± 7	23 ± 2
ICI D1694 + DDATHF + HX	159 ± 35	129 ± 48	115 ± 7	94 ± 26	116 ± 39	113 ± 19
CB3717	216 ± 37§	349 ± 54	307 ± 45	11 ± 2	9 ± 7	41 ± 6
CB3717 + HX	210 ± 22	318 ± 57	185 ± 42	13 ± 8	8 ± 5	32 ± 2
CB3717 + DDATHF	29 ± 4	31 ± 12	24 ± 2	18 ± 8	14 ± 7	15 ± 4
CB3717 + DDATHF ± HX	148 ± 11	176 ± 17	ND	55 ± 10	64 ± 6	ND
FdUrd	257 ± 69§	287 ± 64	231 ± 31	11 ± 3	15 ± 8	33 ± 12
FdUrd + HX	216 ± 40	284 ± 64	274 ± 11	10 ± 5	11 ± 2	58 ± 29
FdUrd + DDATHF	61 ± 27	47 ± 16	45 ± 2	24 ± 6	19 ± 3	23 ± 9
FdUrd + DDATHF + HX	157 ± 47	303 ± 17	225 ± 76	10 ± 2	14 ± 2	40 ± 5

* 100 μM HX, 10 μM DDATHF, 0.1 μM ICI D1694, 100 μM CB3717 and 0.1 μM FdUrd were used.

† All results are expressed as a percentage of the zero hour untreated control. Control levels (pmol/10^6 cells) of dATP and dTTP were 19.1 ± 4.4 (means ± SD) and 30 ± 8.1, respectively.

‡ Means ± SD were obtained from a total of four duplicates from two separate experiments.

§ $P < 0.05$ (Student's *t*-test) for TS inhibitors compared with TS inhibitors plus DDATHF.

ND, not determined.

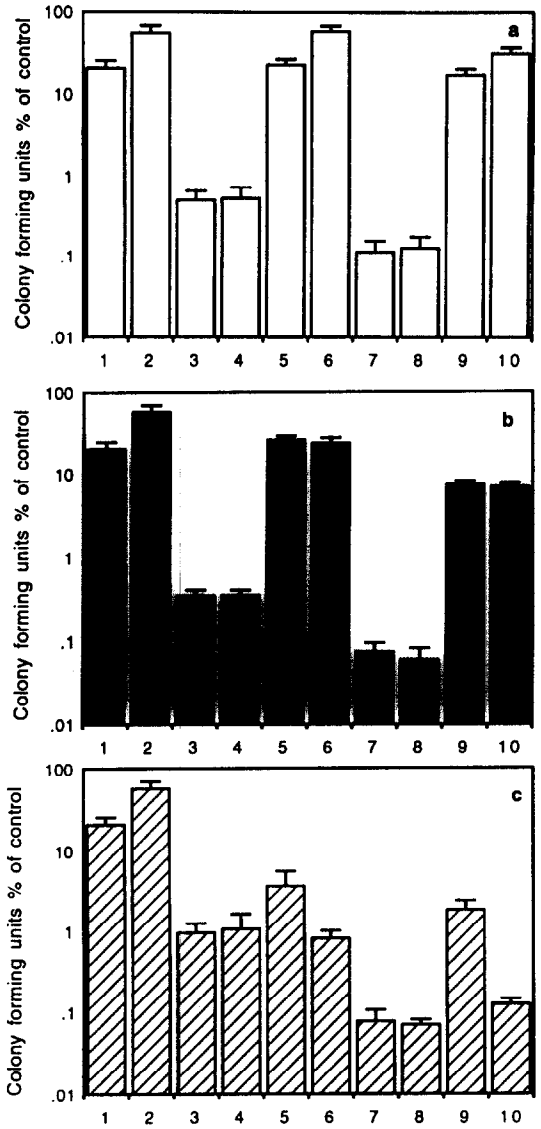

Fig. 7. Effects of hypoxanthine on cytotoxicity of TS inhibitors with or without DDATHF: (a) ICI D1694, (b) CB3717 and (c) FdUrd. Column 1. 10 μM DDATHF; Column 2. 10 μM DDATHF + 100 μM HX; Columns 3–6 represent treatment with TS inhibitors alone: (a) 0.1 μM ICI D1694, (b) 100 μM CB3717 and (c) 0.1 μM FdUrd, combined with 100 μM HX, 10 μM DDATHF and 10 μM DDATHF + 100 μM HX, respectively; Columns 7–10 represent TS inhibitor alone (1 μM ICI D1694, 500 μM CB3717 and 1 μM FdUrd), combined with 100 μM HX, 10 μM DDATHF and 10 μM DDATHF + 100 μM HX, respectively. Drug exposure time was 24 hr. Data are the mean ± SD (bars) from 2 (a) and (b), or from three (c) experiments.

previously reported that the inhibitor of TS ICI 198583 is more cytotoxic than MTX alone, but similar to MTX plus HX. HX potentiates MTX cytotoxicity and this is accompanied by dATP : dTTP pool imbalance [6, 7].

The observation that DDATHF treatment reduced the cytotoxicity and dATP levels increased in a concentration-dependent manner in ICI D1694 and FdUrd treated cells argues strongly against interference with drug uptake being the basis for protection. Moreover, the pattern of DDATHF interaction with CB3717 is similar to that with ICI D1694 yet CB3717 enters cell by a different transport mechanism [11, 18]. This observation suggests an interaction other than at the level of cell transport. Our L1210-MTX cells are cross-resistant to ICI D1694 (212-fold) and DDATHF (122-fold) but only 5.7-fold to CB3717 (Table 1). Our results are therefore compatible with those of Jackman *et al.* [12] who reported that cells resistant to ICI D1694 were also cross-resistant to MTX, DDATHF and ICI 198583. It seems that ICI D1694 enters cells via the reduced-folate carrier, which also transports DDATHF [25]. However, some studies report that DDATHF is a good substrate for membrane folate binding proteins [26, 27]. The transport mechanism for CB3717 is also not clear. Jansen *et al.* [28] reported that CB3717 may enter cells via both the reduced folate/MTX carrier and folate binding protein. CB3717 has poor growth inhibitory effects in L1210 cells (IC_{50} 2.5 μM) and less cross-resistance in L1210-MTX cells than MTX, ICI D1694 and DDATHF (Table 1). Folate binding protein may be the main route for CB3717 uptake in our cells. Competition for folate binding protein between DDATHF and CB3717 could contribute to the interaction between these two drugs [27].

DDATHF protection from CB3717 cytotoxicity is perhaps partly mediated at the cell membrane, but there are other potential bases for interaction within the cell. Increased levels of 5,10-CH_2FH_4 caused by "channelling" of intracellular folates after inhibition of FGAR by DDATHF could compete with CB3717 for TS binding (Fig. 1). In addition, Galivan *et al.* [13] suggested that DDATHF and CB3717 might compete as substrates for the folylpolyglutamate synthetase.

The effects of exogenous hypoxanthine on the DDATHF/thymidylate synthase inhibitor interactions indicate that in cells with reduced dTTP levels, an increase in dATP correlated with cytotoxicity (Table 2 and Fig. 5). These experiments also indicate, though indirectly, that exogenous hypoxanthine influences the intracellular utilisation of reduced folate co-factors in antifolate-treated cells. Hypoxanthine increased the dTTP level in cells exposed to both CB3717 and DDATHF, suggesting that HX reduced inhibition of thymidylate synthase by CB3717 treatment. Consideration of folate interactions (Fig. 1) reveals that increased 5,10-CH_2FH_4 in HX-treated cells may reduce CB3717 inhibitory effects on TS, perhaps by competition for the enzyme-binding site. Exogenous HX allows reduced folates which otherwise might be utilized as co-factors in *de novo* purine biosynthesis to accumulate and be interconverted to 5,10-CH_2FH_4. Previous studies which investigated the inhibition of thymidylate synthase by CB3717 have reported competitive and non-competitive inhibition with folate co-factors [9, 29, 30]. A recent study reported that CB3717 inhibition at low concentration was competitive with 5,10-CH_2-FH_4, while non-competitive at higher concentrations (80 μM and above) [10]. The effects of exogenous hypoxanthine

in the presence of DDATHF on the interaction of CB3717 but not ICI D1694 with thymidylate synthase suggest a difference in the mechanism of binding to the enzyme by the two quinazoline antifolates.

Inhibition of thymidylate synthase by FdUMP is dependent on adequate concentrations of 5,10-CH_2FH_4 which stabilizes the enzyme–inhibitor complex [31]. Treatment with FdUrd reduces dTTP and increases dATP pools and causes DNA fragmentation [22]. DDATHF (10 μM) reduced the cytotoxicity of FdUrd at both concentrations (0.1 and 1 μM) studied, and HX (100 μM) negated this protection. Protection by DDATHF from FdUrd toxicity was associated with a reduction below control level of dATP (in contrast to the 2-fold increase in FdUrd treated cells), and only a marginal effect in dTTP level which remained 25% below control levels at all time points. HX negation of DDATHF protection of FdUrd treated cells was associated with restoration of dATP levels to >1.5-fold control, and a restoration of dTTP level reduction (similar to that of FdUrd treatment alone). Since DDATHF, FdUrd and HX utilize different transport mechanisms, this interaction cannot be at the level of cell entry. HX had no effect on FdUrd toxicity or dATP/dTTP pool changes. HX bypasses the effects of DDATHF treatment on *de novo* purine biosynthesis (Fig. 1), and the dNTP pool effects of HX add further support to the hypothesis that dATP:dTTP pool imbalance contributes significantly to cytotoxicity.

The protective effect of DDATHF on FdUrd treated cells is less than the effect on quinazoline antifolates. DDATHF competition at the cell membrane level for quinazoline folate entry may contribute to these differences. The concentration differences (10–50-fold excess of CB3717 to DDATHF) complicate the interaction at the level of cell entry.

Other factors which might contribute to DDATHF protection from FdUrd toxicity being less than that against the quinazoline antifolates include the effect of FdUTP misincorporation into DNA following FdUrd but not quinazoline antifolate treatment. It is not plausible that DDATHF would affect FdUMP synthesis from FdUrd in a concentration-dependent manner; indeed at high DDATHF concentrations purine-ribonucleotide levels fall [9]. While hypoxanthine negated the DDATHF effect, it did not influence FdUrd toxicity or dNTP pool changes, and these results suggest that FdUrd conversion to FU with subsequent PRPP mediated anabolism to FU ribonucleotides is not a major pathway in these conditions.

The notion that in the presence of reduced dTTP, increased dATP is a major determinant of cytotoxicity in drug-treated cells is supported by our experiments. Possible mechanisms by which pool imbalance leads to cytotoxicity have not been studied here, but dNTP pool effects on DNA repair and misincorporation are possibilities [7, 22].

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