DNA fragmentation, dATP pool elevation and potentiation of antifolate cytotoxicity in L1210 cells by hypoxanthine

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Summary Exogenous purines (≥ 10^{-11}M) can modulate the cytotoxicity of methotrexate (MTX) in cultured cells, protecting cells at low MTX concentrations (≤ 8 × 10^{-8}M) and markedly potentiating its effect at higher concentrations. The ability of hypoxanthine (HX) to modulate the effects of two antifolates—ICI 198583 (an inhibitor of thymidylate synthetase) and piritrexim (PTX, a lipophilic inhibitor of DHFR)—was investigated using cultured mouse leukaemic cells, L1210. HX (10^{-4}M) was found to potentiate only the cytotoxicity of DHFR inhibitors (MTS and PTX), increasing cell kill by 20–70 fold to the level achieved by an equivalent concentration (10^{-3}M) of ICI 198583 alone. Agarose gel electrophoresis of DNA extracted from cells exposed to antifolates for 24 h demonstrated that the chromatin was cleaved into multimers of 200 base pairs. This pattern of DNA cleavage indicates cell death via apoptosis. The degree of DNA fragmentation was found to be closely linked to cytotoxicity. DNA fragmentation increased from 50% in cells treated with 10^{-5}M MTX or PTX to 70% when HX was added with the drugs, a level achieved by 10^{-1}M ICI 198583 alone. HX potentiation of cytotoxicity was correlated with a substantial increase in dATP in conjunction with low dTTP pools. The specific potentiation of DHFR inhibitors by HX may be due to their inhibition of purge synthesis with a concurrent rise in PRPP levels. Addition of HX with MTX substantially raised intracellular purine levels via the salvage pathway as indicated by ribonucleotide pool measurements. ICI 198583, on the other hand, stimulated de novo purine synthesis with or without added HX. Treatment with MTX plus HX or ICI 198583 (with or without HX) caused a reduction of dTTP pools to 8% of untreated control and excess dATP accumulation. The subsequent elevation (to 300% of control) of the dATP pool may provide a signal for endonucleolytic fragmentation of DNA and subsequent cell death.

Biochemical studies have traditionally focused on the role of methotrexate (MTX) as an inhibitor of the dihydrofolate reductase (DHFR) enzyme, causing a depletion of the reduced folate pool and subsequent inhibition of thymidylate synthesis (Cadman, 1983). However, the polyglutamylated forms of MTX have also been potent inhibitors of some other folate-dependent enzymes involved in purine synthesis and folate conversions (Allegra et al., 1984). The sites of action of MTX and its polyglutamylate derivatives on purine and thymidylate synthesis are illustrated in Figure 1.

Exogenous purines only rescued cultured cells from the cytotoxic effects of low concentrations (below 8 × 10^{-8}M) of MTX. At higher MTX concentrations, exogenous purines markedly potentiated the cytotoxicity of MTX (Taylor et al., 1982). The purines were thought to either increase the cytotoxicity of MTX (Borsa & Whitmore, 1969; Fairchild et al., 1988) or else become toxic in themselves (Taylor et al., 1982; Yoshioka et al., 1987a). Recent studies have reported that MTX-treated NIH/3T3 cells accumulated single-stranded and double-stranded DNA breaks preceding cell death and that the DNA damage was prevented by the inhibition of protein synthesis by cycloheximide (Lorico et al., 1988). DNA extracted from MTX-treated HL-60 cells and electrophoresed on an agarose gel has been reported to be fragmented into multimers of approximately 200 base pairs (bp) (Kaufmann, 1989). This is a characteristic biochemical marker for a mode of cell death known as apoptosis or programmed cell death (Wyllie et al., 1984).

In this study, we compared the effects of HX on the cytotoxicity of two non-classical antifolates with MTX in murine leukaemic L1210 cells. HX (10^{-4}M) was used because it was reported to be the highest non-toxic concentration which elicited the maximum potentiation of MTX cytotoxicity in L1210 cells (Taylor et al., 1982). Piritrexim (PTX) is a small lipid soluble diaminopyrido-pyrimidine inhibitor of DHFR (Figure 1) which is not polyglutamylated in cells (Duch et al., 1982). ICI 198583 (2-desamino-2-methyl-10-propargyl-5,8-diazaflavonic acid) is a quinazoline-based inhibitor of thymidylate synthetase (Figure 1) and is the 2-desamino, 7-methyl derivative of the prototype drug, N10-propargyl-5,8-diazaflavolic acid CB3717. This modification increased the solubility of the drug and the cytotoxicity by 40-fold (IC50 = 0.09 mM) (Hughes et al., 1988).

Materials and methods

Chemicals

Methotrexate was obtained as a solution (25 mg ml^{-1}) from David Bull laboratories (Lexia Place, Victoria, Australia). Piritrexim was obtained from Burroughs Wellcome Co (Research Triangle Park, NC, USA) and ICI 198583 was a gift from Dr A. Jackman (Institute of Cancer Research, Surrey, UK). Piritrexim and ICI 198583 were made up as 1 mM stock solutions. ICI 198583 was dissolved in 0.15 M NaHCO3 and PTX 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. 3H-labelled deoxyribonucleosides were purchased from Radiolabelled Centre (Ameresham, Buckinghamshire, UK). Unlabelled deoxyribonucleotides were purchased from Sigma Chemical Co. (St Louis, MO, USA) and PL Biochemicals Inc. (Milwaukee, WI, USA). DNA polymerase (Klenow fragment) was obtained from Pharmacia, USA. The poly deoxyadenylate-deoxythymidylate template was purchased from Miles laboratories (Elkhart, IN, USA).

Cell culture

Mouse leukaemia L1210 cells were grown in suspension culture in Roswell Park Memorial Institute Medium (RPMI) 1640 supplemented with 10% non-dialysed fetal calf serum (FCS), L-glutamine and gentamicin (32 mg ml^{-1}). The doubling time of the cells was approximately 11–12 h. In all experiments, cells were set up at 5 × 10^6 cells ml^{-1} and allowed to grow undisturbed for 24 h before addition of drugs. All treatments were carried out with exponentially growing cell cultures. Cell counts were made by phase-
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The effect of DNA fragmentation was quantified using centrifugation to separate intact chromatin from fragmented DNA (Sellins & Cohen, 1987). A total of 3 x 10^6 cells were washed once in PBS (Dulbecco's phosphate buffered saline) (Cytosystems, Castle Hill, NSW) and lysed with 0.4 M hypotonic lysing buffer (pH 7.5) containing 10 mM Tris.Cl, 1 mM EDTA and 0.2% Triton X-100. The lysate was incubated on ice for 15 min and then centrifuged at 13000 g for 10 min. Both the supernatant and the pellet was precipitated separately in 12.5% trichloroacetic acid (TCA) at 4°C overnight. The precipitate was pelleted at 11000 g for 4 min.

The DNA in the precipitate was hydrolysed by heating to 90°C for 10 min in 80 ml 5% TCA and quantified using a modification of the diphenylamine method (Sellins & Cohen, 1987). The degree of DNA fragmentation refers to the percentage of DNA in the 13000 g supernatant divided by the total DNA from the pellet and supernatant.

**Figure 1** Pathway of purine and thymidine synthesis and sites of action of antifolates. Methotrexate (MTX) inhibits the dihydrofolate reductase (DHFR) enzyme, but its polyglutamated derivatives (MTX/Glu) can also directly inhibit de novo purine synthesis and thymidylate synthase. Pteropterin (PTX) is a specific inhibitor of DHFR while ICI 198583 is a potent inhibitor of thymidylate synthetase. Inhibition of purine synthesis leads to an elevation of phosphoribosylpyrophosphate (PRPP) level which enhances hypoxanthine (HX) conversion to inosine monophosphate (IMP). N-glycinamide ribonucleotide (GAR); 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR); N-succino-AICAR (SAICAR); dihydrofolate (FH2); tetrahydrofolate (FH4); 5,10-methenyl-tetrahydrofolate (CHFH); 10-formyl-tetrahydrofolate (CHOF1); 5,10-methyltetrahydrofolate (CH2FH4).

**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 ml of growth medium per well into 96 well round-bottom plates (Crown Corning, Liverpool, NSW) 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 48 wells for each dilution. If drug treatment resulted in a high number of negative wells, the cells were plated at 10 x higher concentration. The cultures were incubated in a humidified 10% CO2, 5% O2 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 minimisation (Taswell, 1981). Cloning results were expressed as colony forming units (c.f.u.ml^-1) 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 100%.

**DNA extraction**

A total of 1 x 10^6 cells were washed once in PBS and lysed in a 0.05 M Tris.Cl buffer (pH 8), containing 10 mM EDTA, 0.1 M NaCl, 0.5% SDS and 200 mg ml^-1 protease K (Sigma Chemical Co.). The lysate was incubated at 50°C for 3 h before being extracted with phenol (twice), chloroform/isoamyl alcohol (24:1) (twice) and ether (twice). The sample was then treated with 100 mg ml^-1 RNase A (Sigma Chemical Co.) for 1 h at 37°C and then with 200 mg ml^-1 proteinase K for 1 h at 37°C. The sample was extracted again with phenol and chloroform and the DNA concentrated by Centricon centrifugation (Amicon, Danvers, MA, USA) to prevent loss of fragmented DNA. An amount of 10 mg of DNA from each sample was analysed by electrophoresis on a 1% agarose gel containing ethidium bromide (0.3 μg ml^-1) using 1 x TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA).

**DNA fragmentation assay**

Approximately 1 x 10^6 cells were washed twice in cold PBS and extracted with cold 0.6 M perchloric acid (Kemp et al., 1986). The neutralised extracts were subjected to HPLC analysis (Sant et al., 1989) to determine the relative quantity of purine and pyrimidine nucleotides. The eluted metabolites were monitored by an LK2140 rapid spectral detector connected to an IBM XT microcomputer (Sant et al., 1989; Lyons & Christopherson, 1990). The area under each peak was calculated using the Nelson Analytical 3000 series chromatography data system (Version 5.0). The results were expressed as percentage area of the untreated control (derived from the mean of the 0, 6 and 12 h samples).

**Deoxyribonucleoside triphosphate pool assay**

A total of 5 x 10^6 viable cells were washed once in cold PBS (with 2 mM EDTA) and extracted with ice cold 60% ethanol. The extract was lyophilised and resuspended in 500 ml of 10 mM Tris buffer (pH 7.85). The sample was then centrifuged at 11000 g for 15 min at 4°C and the supernatants stored at -20°C. The deoxyribonucleotides were measured by a modification of the DNA polymerase assay (Mann & Fox, 1986). 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 HX on MTX and the results shown in Figure 2a are similar to those using a soft agar cloning assay (Taylor et al., 1982). Using the same microtitration assay two other antifolates, PTX and ICI 198583 were also tested. The effects of 24 h exposure to ICI 198583 (the inhibitor of thymidylate synthetase) in the presence or absence of exogenous HX is shown in Figure 2b. For the three different concentrations of the drug used (10^-5, 10^-4 and 10^-3 M), the addition of 10^-4 M HX had no significant effect on the cytotoxicity of ICI 198583. On the other hand, when PTX (an inhibitor of DHFR) was used, there was a marked potentiation of
cytotoxicity at concentrations at or greater than 10⁻⁴ M. There was no protection at a lower drug concentration (10⁻⁵ M) as shown in Figure 2c.

Electrophoretic analysis of DNA extracted from untreated and drug-treated cells is shown in Figure 3. The DNA from the untreated control cells was unfragmented high molecular weight DNA (Figure 3, lane 2). The addition of 10⁻⁴ M HX alone did not result in fragmented DNA (Figure 3, lane 3), but the addition of 10⁻³ M MTX, PTX or ICI 198583 for 24 h resulted in DNA which was extensively cleaved into the distinctive multimers of approximately 200 bp (Figure 3, lane 4, 6 and 8 respectively). The addition of 10⁻³ M HX did not alter the appearance of the bands (Figure 3, lane 5, 7 and 9).

The degree of DNA fragmentation was determined by separating the cleaved DNA from the intact chromatin by centrifugation and measuring the amount of DNA present in the supernatant and pellet using the diphenylamine method (Sellins & Cohen, 1987). The degree of DNA fragmentation refers to the ratio of DNA in the supernatant to the total DNA in the supernatant and pellet. The degree of DNA fragmentation in the untreated control and in cells exposed to the antifolates for 24 h is shown in Figure 4. The background fragmentation in the untreated control was approximately 5%. The addition of 10⁻⁴ M HX alone did not significantly (P > 0.5; Student’s t-test) increase the degree of fragmentation. However, when 10⁻³ M MTX or PTX was added together with HX, there was a 20% (P < 0.01) increase in fragmentation. This was associated with the potentiation of the cytotoxicity of MTX and PTX by HX (Figure 2a and c). In contrast, the addition of HX with 10⁻³ M ICI 198583 made little difference (3%) to the degree of fragmentation compared with ICI 198583 alone. This finding is in agreement with the lack of potentiation of ICI 198583 cytotoxicity by HX (Figure 2b).

To explore the possible biochemical mechanisms underlying purine potentiation, the perturbations of the ribonucleotide and deoxyribonucleoside triphosphate pools were measured in cells treated with 10⁻³ M MTX or ICI 198583 in the absence or presence of 10⁻³ M HX.

Since MTX depletes folate pools and its polyglutamylated derivatives inhibit specific enzymes involved in the purine synthesis pathway, the pools of the four ribonucleotides (ATP, GTP, CTP and UTP) as well as two purine intermediates (SAICAR and IMP) were measured over a 24 h period following the addition of the drugs. As shown in Figure 5a, 10⁻³ M MTX tended to reduce particularly after 12 h the purine precursor pools and ATP and GTP levels (Figure 5b). This was accompanied by an increase in the pyrimidine pools (UTP and CTP) after 12 h as shown in Figure 5c. When 10⁻⁴ M HX was added with MTX, there was an increase in all four ribonucleotide pools (1.5–2-fold) as well as a sharp rise (three-fold) in the IMP pool when compared with the untreated control. In cells treated with 10⁻³ M ICI 198583, there was a rise in all the ribonucleotide and precursor pools (Figure 5d, e and f). The addition of HX with ICI 198583 made little difference in the ribonucleotide pools, but there was an increase in the IMP pools.

Previous reports have shown that purine potentiation of MTX cytotoxicity was closely associated with a marked increase (three-fold) in intracellular dATP pool (Taylor et al.,
1982). Our experiments using the same DNA polymerase assay to determine the deoxyribonucleoside triphosphate pool perturbations have shown a similar increase in dATP pool after 12 h as shown in Figure 6a. However, in cells treated with 10^{-5} M ICI 198583 alone, there was also a substantial increase (three-fold) in the dATP pool after 12 h drug exposure as shown in Figure 6b. The addition of 10^{-4} M HX with ICI 198583 did not increase the dATP pool further. Both MTX with HX and ICI 198583 with or without exogenous HX, depressed the dTTP pool levels to a similar extent as shown in Figure 6a and b. HX by itself caused minor perturbations in dATP (111 ± 1.47% (mean ± s.d.) and dTTP (79 ± 11%) after 12 h exposure.

Discussion

The cytotoxicity of MTX has been shown to be modulated by the presence of exogenous purines (Taylor et al., 1982). This study compares the ability of a preformed purine (hypoxanthine) to modulate the cytotoxicity of two other antifolates: PTX, an inhibitor of DHFR which is not polyglutamylated in cells (Duch et al., 1982) and ICI 198583, a potent inhibitor of thymidylate synthetase (Hughes et al., 1988; Jackman et al., 1988; 1990). Using a microtitration cloning assay, 10^{-5} M ICI 198583 was found to be more toxic (reducing the number of c.f.u. ml^{-1} to 0.02% of the untreated control) than 10^{-4} M MTX (1.1%) or 10^{-5} M PTX (0.6%). The addition of 100 mM HX potentiated the cytotoxicity of the two inhibitors of DHFR (MTX and PTX), increasing their cytotoxicity to the same level as ICI 198583 alone. This potentiation of the cytotoxicity of the DHFR inhibitors by HX was also observed in cells treated with metoprine, a lipophilic inhibitor of DHFR (data not shown). As with ICI 198583, HX did not affect the cytotoxicity of another thymidylate synthetase inhibitor, 5-fluorodeoxyuridine (FUdR) (data not shown).

Two hypotheses may explain the purine potentiation of the DHFR inhibitors. Firstly, Fairchild et al. (1988), after performing tritiated uridine uptake and cell cycle studies, concluded that the potentiation of MTX cytotoxicity in L1210 cells by HX was due to the restoration of normal RNA
Dinucleotides (dNTPs) can be blocked by the enzyme dNTPase, which is involved in single-stranded DNA synthesis. The inhibition of this enzyme by dATP increases the dNTP pool balance in cells treated with MTX or ICI 198583.

**Figure 6** Changes in intracellular levels of dATP and dTTP with time in L1210 cells treated with either (a) 10^{-7}M MTX or (b) 10^{-5}M ICI 198583. (●, ○) dATP; (▲, △) dTTP. (●, ▲) drug alone; (○, △) drug plus 10^{-5}M HX. Means ± S.D. were obtained from a total of four replicates from two separate experiments. All results expressed as a percentage of the zero-hour untreated control. Control levels (pmol/10^6 cells) of dATP and dTTP in were 10 ± 2 (mean ± S.D.) and 19 ± 4 respectively.

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