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ORIGINAL ARTICLE

Effects of extracellular purines on cytotoxicity of methotrexate

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Abstract
Purpose Nucleoside and base modulation of the cytotoxicity of nucleic acid and folate antimetabolite drugs has been widely discussed. Many investigators have observed reduced toxicity due to circumvention of drug-induced inhibition of de novo purine and pyrimidine synthesis. However, exogenous purine nucleosides and bases may also enhance the cytotoxicity of even moderate concentrations of antifolate drugs (MTX and PTX) which inhibit dihydrofolate reductase. In this study, the effects of nucleosides in the medium on the cytotoxicity and deoxyribonucleoside triphosphate pools after brief exposure of cultured cells to methotrexate have been studied in cultured L1210 murine leukaemia cells.
Methods Cell viability was determined by trypan blue exclusion assay. Colony formation was assessed by microtitration cloning assay. The deoxyribonucleotides were measured by a modification of the DNA polymerase assay. Purines were extracted with trioctylamine and 1,1,2-trichlorotrifluoroethane buffer and concentrations of purine bases were determined by HPLC.
Results Subculture of drug-treated cells in fresh medium containing 10% FCS led to greater toxicity than sub culture in 'conditioned' medium, i.e. fresh medium in which logarithmically growing cells had been cultured for 24 h before separation. Cells resuspended in fresh medium had increased dATP and sustained inhibition of dTTP levels, while cells subcultured in 'conditioned' medium had no elevation of dATP. Hypoxanthine concentration determined by HPLC in 'conditioned' medium was 0.9 μM compared to 6.7 μM in fresh medium. Resuspension of drug-treated cells in conditioned medium supplemented with 10 or 100 μM HX enhanced cytotoxicity and increased the dATP levels.
Conclusion These results add further evidence that purines present in normal culture conditions are important determinants of methotrexate cytotoxicity. Elevation of dATP levels after methotrexate treatment is an important modulator of cytotoxicity.

Keywords Methotrexate (MTX) · MTX modulation · dNTP imbalance · Purines · Cytotoxicity

Abbreviations

MTX	Methotrexate
HX	Hypoxanthine
PTX	Piritrexim
DHFR	Dihydrofolate reductase
DP	Dipyridamole
HPLC	High pressure liquid chromatography
FCS	Foetal calf serum
$HClO_4$	Perchloric acid
CMF	Cyclophosphamide methotrexate fluorouracil

Introduction

The modulating effects of exogenous purines and pyrimidines on the cytotoxicities of nucleic acid antimetabolite

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and antifolate drugs have been studied for many years [1–6]. These bases or nucleosides not only may disturb drug anabolism to cytotoxic metabolites, but also salvage may circumvent the inhibition of key enzymes of de novo purine or pyrimidine synthesis. The presence of nucleosides and bases in human and animal sera is well documented [7–11], and particularly high purine levels have been detected in bone marrow presumably derived in part from catabolism of nuclei during erythrocyte maturation [12]. Nucleotides, which are also present in human serum, may be catabolised by extracellular nucleotidases found in serum, and by ecto-enzymes bound to the external surface of the plasma membrane [13]. The hypoxic environment in tumour with or without the presence of necrosis is also likely to increase regional concentrations of nucleotides, nucleosides and bases [14].

Nucleoside and base modulation of the cytotoxicity of antimetabolite drugs has been widely discussed [6, 14, 15]. Many investigators have observed reduced toxicity due to circumvention of drug-induced inhibition of de novo purine and pyrimidine synthesis. However, exogenous purine nucleosides and bases may also enhance the cytotoxicity of even moderate concentrations of antifolate drugs (MTX and PTX) which inhibit dihydrofolate reductase [2, 6, 16, 17]. These studies have lead us to propose that when dTTP levels are reduced, elevation of dATP pools is the main cytotoxic mechanism of purine potentiation of cytotoxicity of DHFR inhibitors, and perhaps also an important determinant of antifolate toxicity per se. In present study, we investigate the effects of nucleosides in the medium on the cytotoxicity and deoxyribonucleoside triphosphate pools after brief exposure of cultured cells to methotrexate.

Materials and methods

Chemicals and reagents

Methotrexate (MTX) was obtained (in solution 25 mg/ml) from David Bull Laboratories (Lexia Place, VIC, Australia). Purine bases and nucleosides were purchased from Sigma and P.L. Biochemicals Inc (Milwaukee, WI, USA). Hypoxanthine (HX) was made up as a 10 mM solution and 10 M sodium hydroxide was added drop wise until the drug was dissolved. [8^{-3}H]dATP and [methyl^{-3}H]dATP were purchased from the Radiochemical Centre Amersham (UK). DNA polymerase (Klenow fragment) was obtained from Pharmacia (Piscataway, NJ, USA). Polydeoxyadenylate–deoxythymidylate template was purchased from Miles Laboratories (Elkhart, IN, USA) and Sigma. 0.2% trypan blue was purchased from Flow Laboratories, Ryde NSW, Australia.

Cell culture

L1210 murine leukaemia cell line was grown as suspension cultures at 37°C in RPMI 1640 media (Multicoil Bioscience, NSW, Australia) supplement with 10% non-dialysed foetal calf serum (FCS), L-glutamine (2 mg) and gentamicin and gentamicin (20 μg/mL). Cell population doubling time was approximately 11–12 h. Cells were set up at 5×10^4 cells/mL and allowed to grow undisturbed for 24 h addition of drugs. All observations were made by phase-contrast microscopy which was used to discriminate live (phase-positive) and dead (phase-negative) cells. 4 h after MTX treatment, L1210 cells were harvested by centrifugation at $200\times g$ for 5 min at room temperature and washed once with RPMI 1640 medium. The cells were suspended in fresh medium containing 10% foetal calf serum. In some experiments, conditioned medium was used for subculturing cells after drug-treatment.

Conditioned medium was obtained by setting up cells in fresh medium at a concentration of 5×10^4 cells/mL and allowing the cells to grow for 24 h. The medium was then harvested by centrifugation at $800\times g$ for 10 min. The supernatant was then filtered with a 0.2 μM sterile acrodisc (Gelman Science, USA), and was stored at 4°C. Dialysed FCS was made by dialysing FCS with dialysis membrane (Union Carbide Corporation, Chicago, USA) against five changes in 10× Hanks balanced salt solution over 3 days.

Microtitration cloning assay

Cells were washed once after drug-treatment and resuspended in drug-free medium. Cells were counted and the viable cells diluted to the required cell number. The cells were distributed into 96 well round-bottom plates (Crown Corrning, NY, USA) containing 200 μl of drug-free medium per well. Cloning efficiency was determined by plating doubling dilutions of viable cells ranging from 5 to 0.625 cells/well. Cells were distributed into 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. 50–6.25 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 if wells contained 100 or more viable cells at 14 days. The cloning efficiency of cells was calculated from the proportion of negative wells using Poisson statistic and X^2 minimisation [18]. Cloning results were expressed as colony forming units/ml, which was calculated from the percentage cloning efficiency multiplied by the viable cell concentration of cultures at the time of cloning.

Deoxyribonucleoside triphosphate pool assay

The cells (5×10^6) were collected by centrifugation ($200\times g$, 5 min), washed once in cold PBS and the nucleotides were 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 $11{,}000\times g$ for 15 min at 4°C and the supernatant was stored at −20°C. The deoxyribonucleotides were measured by a modification of the DNA polymerase assay [19]. 50 µL aliquots of supernatant, or appropriate standard solutions of dNTP, were assayed in duplicate in a reaction mixture containing 0.75 mM $MgCl_2$, 40 mM Tris buffer, pH 7.85, 2 mM dithiothreitol and 0.2 µL of DNA polymerase in a total volume of 200 µL. dATP and dTTP pools were assayed using a poly(dA–dT) template and [^{3}H]dATP and [^{3}H]dTTP, respectively (0.2 µCi/µmoL/assay). After 60 min incubation at 37°C, the reaction was stopped with 0.1 mL of 0.1 M sodium pyrophosphate. The trichloroacetic acid precipitates were filtered, and dried filter papers were counted in organic scintillant in an LKB1215 RackBeta scintillation counter (Wallac, Turku, Finland). Measured dNTP values were corrected for isotope dilution by complementary dNTPs from the cell extracts. The concentrations of deoxyribonucleoside triphosphates were determined from calibration curves of picomole amounts of pure standards.

Extraction of purines in culture media

Purines were extracted according to a modification of the method of Sherman et al. [20]. Briefly, 0.5 mL of medium was de-proteinized with 0.6 M $HClO_4$ and allowed to stand on ice for 15 min. The protein was pelleted by centrifugation ($9{,}000\times g$, 5 min) and the supernatant was neutralised by an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane. Phases were separated by centrifugation ($9{,}000\times g$, 5 min) and the upper aqueous phase was stored at −20°C for subsequent HPLC analysis. All chemicals were purchased from Sigma.

HPLC analysis of purines

Analysis was based on the method of Hull-Ryde et al. [21]. Separation was performed on a Waters HPLC system (Milford, MA, USA) with a Radial-PAK^{TM} C_{18} cartridge column. Peaks were identified by their retention times and absorbance at 254 nm. An aliquot of 50 µL of medium was eluted isocratically using 0.1 M ammonium dihydrogen phosphate buffer pH 5.5. Flow rate was 4.0 ml/min. The limit of detection of all compounds was 0.1 µM.

Statistical analysis

Student's t test was used to determine the significance of the differences between groups.

Results

Effects of subculture media on MTX cytotoxicity

A microtitration-cloning assay was used to determine the effects of subculture medium on the cloning efficiency of cells after exposure to MTX 10 µM for 4 h. Figure 1 shows the results according to time of incubation in drug free media for 2, 8, or 20 h after removal from MTX. Cells subcultured in drug free conditions for 2 h prior to cloning survived less well in both fresh and in conditioned media than cells exposed to MTX for 4 h and then immediately cloned. However, cells subcultured in fresh medium for 8 h after 4 h MTX exposure survived significantly less well than those subcultured in conditioned medium ($P < 0.01$). Similar results were observed at 20 h with 0.9 and 2.4% colony forming units, respectively, surviving ($P < 0.05$).

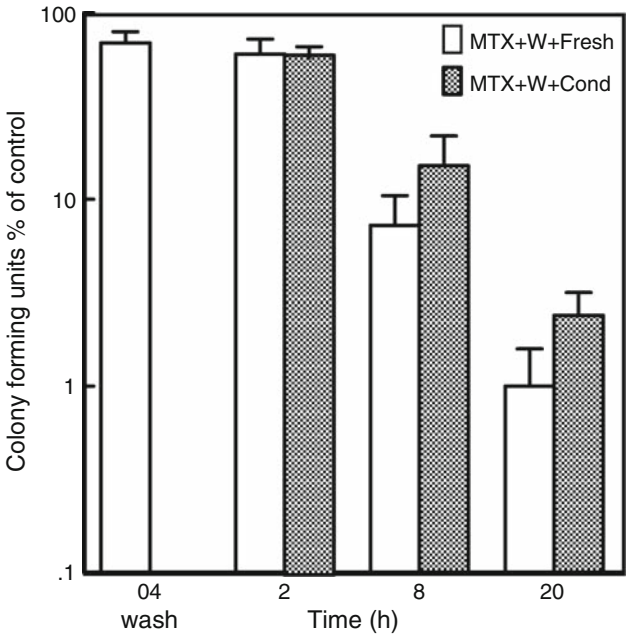

Fig. 1 The effects of subculture conditions for varying times on MTX cytotoxicity. L1210 cells were exposed to 10 µM MTX for 4 h and then washed (W). Cells were then cloned immediately (0) or subcultured in fresh medium (Fresh) or conditioned medium (Cond) for 2, 8, or 20 h prior to cloning. Data are the mean ± SD (*bars*) from at least six experiments

Effect of medium change on dNTP pool levels

Potentiation of MTX cytotoxicity by purines has been correlated with a rise in dATP level and a reduction in dTTP levels [2, 16]. The effects of subculture conditions after 4 h MTX treatment on deoxyribonucleoside triphosphate pools are shown in Table 1. In all conditions, MTX treatment reduced dTTP levels. However, in cells exposed for 4 h to MTX (10 μM), dATP levels fell to approximately 45% of control, but subsequent subculture in fresh medium lead to a rapid increase in dATP pool. In contrast, cells subcultured in conditioned medium after MTX treatment did not have elevated dATP, but dTTP remained reduced and similar to that of cells continuously exposed to MTX (10 μM) for up to 24 h. Similar results were obtained if cells were subcultured in fresh medium supplemented with dialysed FCS (Table 1). Cells subcultured in conditioned medium supplemented with HX (100 μM) had increased dATP similar to that in cells subcultured in fresh medium containing 10% FCS.

Continuous MTX 10 μM exposure reduced dATP and dTTP levels for the first 12 h, but at 24 h dATP level rose above control, but dTTP remained reduced. Table 1 also shows that dATP and dTTP change with time in culture in logarithmically growing L1210 cells. 24 h after establishing cultures at 5×10^4 cells/mL, cell numbers had increased to 2–2.5 $\times$ 10^5/mL, and dATP and dTTP levels were 21 and 39 pmol/10^6 cells, respectively. 24 h later, cell number was 8–10 $\times$ 10^5/mL, and dATP and dTTP levels had fallen by approximately 50%. dNTP pools changes during drug free cell culture are likely related to consumption via salvage pathway of nucleosides/bases in the medium.

Effect of exogenous HX on MTX cytotoxicity in conditioned medium

Figure 2 shows the effects of subculturing cells after 4 h exposure to MTX 10 μM in conditioned medium supplemented with HX. Exogenous HX (10 μM and 100 μM) enhanced cytotoxicity and increased dATP pools in a manner similar to that of subculture in fresh medium (Table 1). HX 1 μM added to the medium did not affect MTX cytotoxicity (data not shown).

Effect of dipyridamole on MTX cytotoxicity

To further explore the effect of nucleosides and bases in the medium after 4 h MTX exposure, we investigated the effects on cytotoxicity of subculturing cells in medium supplemented with dialysed FCS or containing dipyridamole, an inhibitor of nucleoside transport. Cells treated with MTX 10 μM for 4 h were washed and subcultured in fresh medium containing either 10% FCS or 10% dialysed

Table 1 dATP and dTTP concentration (pmol/10^6 cells) with time in culture and after drug treatment

	0 h[a]	4 h	6 h	12 h	24 h
dATP					
Control	21.2 ± 5.4[b]	22.1 ± 2.1	21.0 ± 4.8	18.8 ± 3.2	11.4 ± 4.3
MTX[c]		9.2 ± 3.1	7.4 ± 1.4	8.4 ± 2.4	17.2 ± 3.2
MTX + W + fresh			31.6 ± 9.1*	43.2 ± 9.0*	34.4 ± 10.2
MTX + W + Con			6.2 ± 2.4	7.2 ± 2.0	15.0 ± 2.4
MTX + W + Dial			4.2 ± 2.2	7.8 ± 2.6	10.2 ± 2.5
MTX + W + Con + HX			33.6 ± 7.2*	46.5 ± 6.2	30.1 ± 4.6
dTTP					
Control	39.2 ± 9.6	42.4 ± 7.6	37.4 ± 7.4	38.8 ± 7.4	19.4 ± 9.8
MTX		7.6 ± 2.4	4.0 ± 1.6	2.4 ± 0.8	5.0 ± 1.0
MTX + W + fres			6.4 ± 1.4	3.2 ± 0.8	8.9 ± 2.8
MTX + W + Con			6.2 ± 2.0	3.4 ± 1.2	5.4 ± 1.2
MTX + W + Dial			3.4 ± 0.5	3.3 ± 0.7	5.5 ± 2.0
MTX + W +Con + H X			4.8 ± 2.6	3.7 ± 2.3	6.5 ± 3.2

Time refers to total duration of cell culture after initial drug addition

* $P < 0.01$ (Student's t test) compare to other MTX treatment conditions

[a] Time refers to total duration of cell culture

[b] All results are expressed as a percentage of the zero hour untreated control. Mean ± SD were obtained from at least six duplicates from three separate experiments

[c] MTX + alone refers to cells exposed to MTX 10 μM for 4 h followed by washing and subculture in: *Fresh* Fresh medium containing 10% FCS, *Con* conditioned medium, *Dialy* fresh medium containing 10% dialysed FCS, *HX* hypoxanthine 100 μM

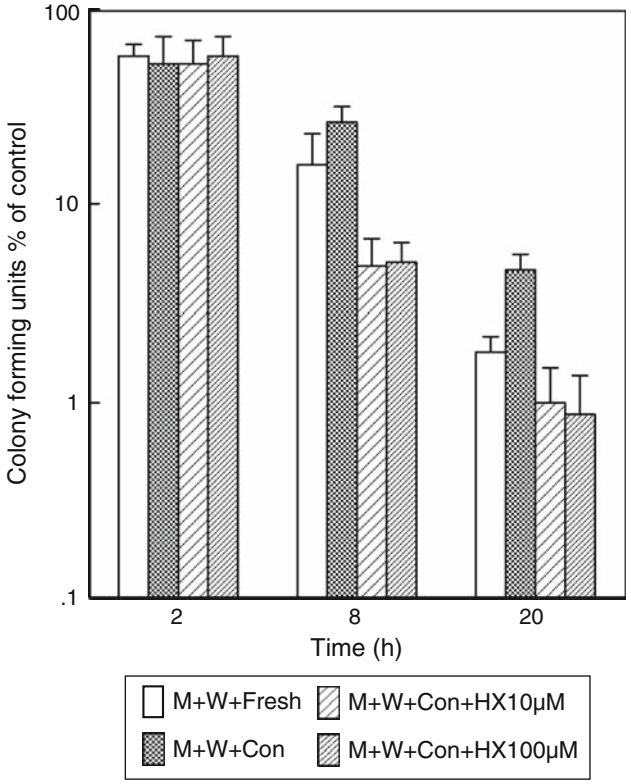

Fig. 2 Effects of exogenous HX in the subculture medium on MTX cytotoxicity. After 4 h 10 μM MTX exposure, cells were washed (W) and subcultured in fresh medium, conditioned medium, conditioned medium plus HX 10 μM, conditioned medium plus hypoxanthine 100 μM. for 2, 8, or 20 h prior to cloning. Data are mean ± SD (*bars*) from three experiments

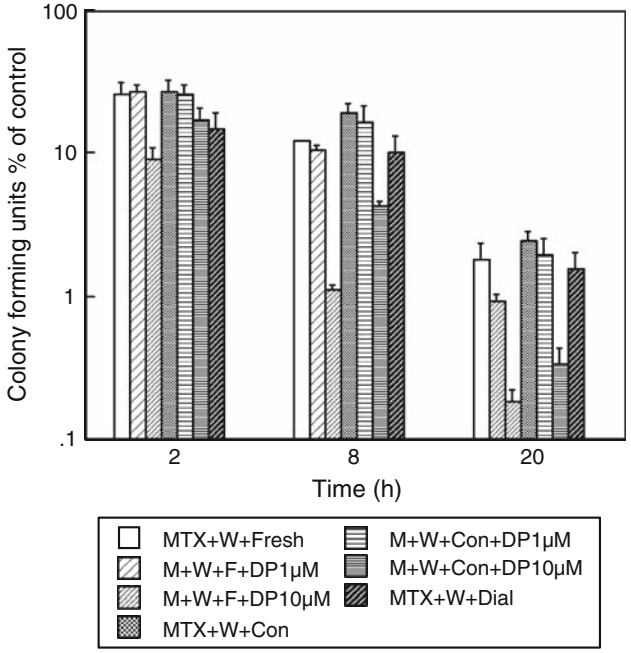

Fig. 3 Effect of DP in the subculture medium on MTX cytotoxicity. After 4 h MTX 10 μM exposure, cells were washed (W) and subcultured in fresh medium (F), fresh medium containing DP 1 μM, fresh medium containing DP 10 μM, conditioned medium (Con), conditioned medium containing DP 1 μM, conditioned medium containing DP 10 μM, medium supplemented with 10% dialysed (Dial) FCS for 2, 8 or 20 h prior to cloning. Data are mean ± SD (*bars*) from three experiments

FCS, or in conditioned medium, The effects of addition of 1 and 10 μM dipyridamole to the subculture medium were also investigated. Figure 3 shows that DP (1 μM) had minimal effects. However, 10 μM DP in fresh medium and to a lesser extent in conditioned medium enhanced cytotoxicity, suggesting that salvage of nucleosides was usually reducing cytotoxicity. Resuspension of drug-treated cells in medium containing DP (10 μM) or in dialysed FCS was more toxic than in conditioned medium ($P < 0.05$).

The observation that cellular dATP and dTTP levels of untreated cells fell by 50% after 24 h in culture (Table 1) prompted an experiment in which after 24 h culture, cells were exposed to 10 μM MTX without media change, after resuspension in fresh medium or with simultaneous addition of 10 μM HX (Fig. 4). The addition of HX or change to fresh medium at time of MTX addition enhanced cytotoxicity after 12 and 24 h treatment compared to simple addition of MTX without medium change ($P < 0.05$). These results further substantiate the view that consumption of exogenous purines in culture medium and prior to drug addition influences MTX cytotoxicity.

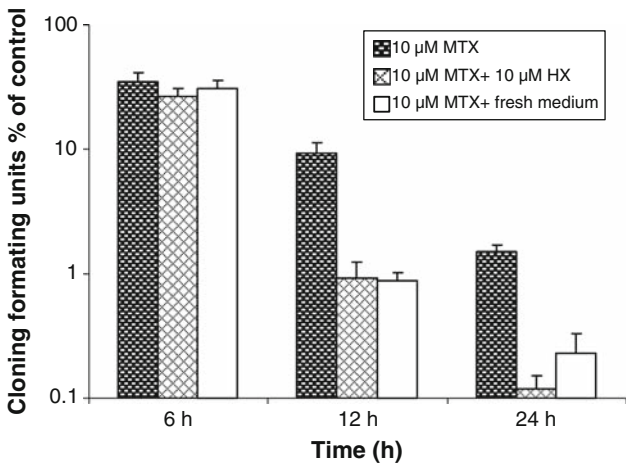

Fig. 4 Effect of culture conditions at the time of 10 μM MTX addition. The cells were cultured for 24 h. At that time cells were split into three groups, addition of MTX; addition of MTX and 10 μM HX; cells were spun down and resuspended in fresh medium (equal volume) with addition of MTX. Data are mean ± SD (*bars*) from three experiments

Purines in the culture medium

Concentrations of purine bases were determined by HPLC (Table 2). HX but not xanthine concentration was

Table 2 Concentrations of purine bases and nucleoside in different medium (μM)

	Fresh[a]	Conditioned	Dialysed[b]
Hypoxanthine	6.7 ± 0.5*	1.1 ± 0.2	0.9 ± 0.2
Xanthine	7.6 ± 1.3*	11.0 ± 0.4	<0.1
Inosine	0.8 ± 1.2	1.0 ± 0.4	<0.1

Data are the mean ± SD, $n = 6$ and conditioned medium samples were collected from six separate cell cultures

* $P < 0.01$ (Student t test) compared with conditioned medium

[a] Fresh medium containing 10% FCS

[b] Fresh medium containing 10% dialysed FCS

significantly higher in fresh medium than in conditioned medium ($P < 0.01$). Inosine concentration was similar in both media. Medium containing dialysed FCS had lower levels of purines. HX, xanthine and inosine did not change in fresh medium incubated without cells at 37°C for 48 h. HX recovery in water and in medium after extraction was 91 ± 6.0 and 127 ± 14%, respectively.

Discussion

MTX is an important agent in treatment of leukaemia and solid tumours, and as an immunosuppressant [22]. MTX inhibits dihydrofolate reductase, resulting in depletion of intracellular reduced folates. Lowered reduced folate levels inhibit both de novo purine and thymidylate synthesis [22–24]. The cytotoxicity of MTX (>0.1 μM) is increased by exogenous purines at least in cultured cells [2, 16, 17, 25, 26]. We have examined the effect of 'physiological purines' on the cytotoxicity of 4 h and longer exposure of L1210 cells to MTX 10 μM. This MTX concentration and exposure time was chosen to approximate the plasma pharmacokinetics, which may occur following standard MTX treatment regimens (e.g. CMF for breast cancer).

The culture conditions after MTX exposure were significant modulators of cytotoxicity. Cells subcultured in conditioned medium had reduced toxicity compared to those subcultured in fresh medium. Some human and rodent malignant cells including L1210 are methylthioadenosine phosphorylase (MTAP) deficient [27], though retaining inosine nucleosidase activity. MTAP is an essential enzyme in the salvage of adenine and methionine synthesis. Cancer cells lacking MTAP are consequently more dependent on the de novo synthesis of purines, and more susceptible to MTX treatment [28].

dTTP levels were reduced in both conditions, but subculture in fresh medium increased dATP levels, while conditioned medium reduced dATP. Experiments in which drug-treated cells were subcultured in medium supplemented with dialysed FCS gave similar results to those in conditioned medium. Addition of exogenous hypoxanthine to conditioned medium enhanced cytotoxicity, and dATP was similar to that in cells subcultured in fresh medium after MTX exposure. Taken together these results support the hypothesis that elevation of dATP with sustained reduction in dTTP is an important modulator of MTX cytotoxicity [2, 17, 35]. The mechanism of accumulation of intracellular dATP after MTX and exogenous HX exposure is not certain. In cells exposed to MTX plus HX, dTMP synthesis is reduced and dTTP levels fall while de novo purine synthesis continues leading to a relative dATP excess. Inhibition of AICAR transformylase by MTX causes a rise of AICAR, resulting in inhibition of AMP deaminase and adenosine deaminase [29]. This likely increases adenosine and leads to an accumulation of AMP [30], contributing to an increase in dATP in the presence of exogenous HX in medium. Our results indicate that the purine base levels in the culture medium are important determinants of MTX toxicity.

Plasma HX is 0.1–10 μM in normal subjects [8, 10, 15]. Increased HX and xanthine levels have been reported in bone marrow and plasma from patients with acute leukaemia and lymphoma (9.1–73 μM) [10, 12]. These 'physiological' concentrations of purines are clearly adequate to modulate MTX toxicity. MTX toxicity will be reduced or potentiated depending on the plasma MTX level. With MTX concentrations below 60 nM, purines tend to protect cells from MTX toxicity, but at MTX higher concentrations (above 80 nM), purines potentiate MTX cytotoxicity [2, 16].

HX and other purines are essential for fibroblast growth [31]. We found that HX concentrations fell over time in cell culture (Table 2), indicating purine consumption or catabolism. Moreover, dNTP levels in untreated cells changed over time in culture (Table 1). Many in vitro treatment protocols add drugs only after subcultured cells have been allowed to grow unperturbed for 24 h. This time lapse and ensuing changes in the medium may cloud interpretation of the importance of nucleic acid salvage pathways on treatment outcome [32, 33]. Figure 4 demonstrates this effect in regard to methotrexate cytotoxicity and furthermore shows that addition of HX to conditioned medium enhances toxicity.

Previous studies reported that dying cells release nucleosides and bases into their environment thereby complicating the analysis of cytotoxic mechanisms [3–5]. The effect of dipyridamole in the subculture medium (Fig. 3) illustrates the modulating effects of nucleosides/bases whose transport is impeded under these conditions.

The concentrations of nucleosides and bases in media identify the importance of salvage pathways in determining the outcome of antifolate and nucleic acid antimetabolite drug treatment in vitro. Surprisingly, few reports document

these levels in vitro or in vivo [5, 7–10]. Moreover, it has been recognised that extracellular nucleotides may be significant sources of nucleoside and bases for salvage as a result of cleavage by ectotriphosphatases and nucleotidases [12, 34].

References

1. Borsa J, Whitmore GF (1969) Cell killing studies on the mode of action methotrexate on L-cells in vitro. Cancer Res 29:737–744
2. Taylor IW, Slowiaczek P, Francis PR, Tattersall MHN (1982) Purine modulation of methotrexate cytotoxicity in mammalian cell lines. Cancer Res 45:5159–5164
3. Piper AA, Nott SE, MacKinnon WB, Tattersall MHN (1983) Critial modulation by thymidine and hypoxanthine of sequential methotrexate-5-fluorouracil synergism in murine L1210 cells. Cancer Res 43:5101–5105
4. Sobrero AF, Bertino JR (1986) Endogenous thymidine and hypoxanthine are a source of error in evaluating methotrexate cytotoxicity by clonogenic assay using undialysed fetal bovine serum. Int J Cell Cloning 4:51–62
5. Hughes JM, deFazio A, Tattersall MHN (1988) Modulation of antifolate cytotoxicity by metabolites from dying cells in a lymphocyte clonal assay. Brit J Cancer 57:459–463
6. Fox M, Boyle JM, Kinsella AR (1991) Nucleoside salvage and resistance to antimetabolite anticancer agents. Brit J Cancer 64:428–436
7. Holden L, Hoffbrand AV, Tattersall MHN (1980) Thymidine concentrations in human sera: variations in patients with leukaemia and megaloblastic anaemia. Eur J Cancer 16:115–121
8. Wung WE, Howell SB (1984) Hypoxanthine concentrations in normal subjects and patients with solid tumours and leukaemia. Cancer Res 44:3144–3148
9. Sinkeler S, Joosten E, Wevers R, Binkhorst R, Oei L (1986) Skeletal muscle adenosine, inosine and hypoxanthine release following ischaemic forearm exercise in myoadenylate deaminase deficiency and McArdle's disease. Adv Exptl Med Biol 195:517–523
10. Hashimoto H, Kubota M, Shimizu T, Kasai Y, Sano H, Adachi S, Akiyama Y, Mikawa H (1992) Effect of high-dose methotrexate on plasma hypoxanthine and uridine levels in patients with acute leukemia or non-Hodgkin lymphoma in childhood. Leukemia 6:1199–1202
11. Smolenska Z, Kaznowska ZD, Zarówny DHA, Simmonds HA, Smolenski RT (1999) Effect of methotrexate on blood purine and pyrimidine levels in patients with rheumatoid arthritis. Rheumatology 38:997–1002
12. Tattersall MHN, Slowiaczek P, deFazio A (1982) Regional variation in human extracellular purine levels. J Lab Clin Med 102:5159–5164
13. Gordon JL (1986) Extracellular ATP: effects, sources and fate. Biochem J 233:309–319
14. Saugstad OD (1975) Hypoxanthine as a measurement of hypoxia. Paediatr Res 9:158–161
15. Zimm S, Johnson GE, Poplack DG (1986) Modulation of thiopurine cytotoxicity in the HL-60 cell line by physiological concentrations of hypoxanthine. Cancer Res 46:6286–6289
16. Fairchild CR, Maybaum J, Straw JA (1988) Enhanced cytotoxicity with methotrexate in conjunction with hypoxathine in L1210 cells in culture. Cancer Chemother Pharmacol 22:26–32
17. Kwok JBJ, Tattersall MHN (1992) DNA fragmentation, dATP pool elevation and potentiation of antifolate cytotoxicity in L1210 cells by hypoxathine. Br J Cancer 65:503–508
18. Taswell C (1981) Limiting dilution assays for the determination of immunocompetent cell frequencies. 1. Data analysis. J Immunol 126:1614–1619
19. Mann GJ, Fox RM (1986) Deoxyadenosine triphosphate as a mediator of deoxyguanosine toxicity in cultured T lymphoblasts. J Clin Invest 78:1261–1269
20. Sherman PA, Fyfe JA (1989) Enzymatic assay for deoxyribonucleotides as template primers. Anal Biochem 180:222–226
21. Hull-Ryde EA, Cummings RG, Lowe JE (1983) Improved method for high energy nucleotide analysis of canine cardiac muscle using reversed-phase high- performance liquid chromatography. J Chromatogr 275:411–417
22. McGuire JJ (2003) Anticancer antifolates: current status and future directions. Curr Pharm Des 9(31):2593–2613
23. Takemura Y, Kobayashi H, Miyachi H (1997) Cellular and molecular mechanisms of resistance to antifolate drugs: new analogues and approaches to overcome the resistance. Int J Hematol 66(4):447–459
24. Assaraf YG (2007) Molecular basis of antifolate resistance. Cancer Metastasis Rev 26(1):153–181
25. Natsumeda Y, Ikegami T, Olah E, Weber G (1989) Significance of purine salvage in circumventing the action of antimetabolites in rat hepatoma cells. Cancer Res 49:88–92
26. Smith SG, Lehman NL, Moran RG (1993) Cytoxicity of antifolate inhibitors of thymidylate and purine synthesis to WiDr colonic carcinoma cells. Cancer Res 53:5697–5706
27. Toohey JI (1978) Methylthioadenosine nucleoside phosphorylase deficiency in methylthio-dependent cancer cells. Biochem Biophys Res Commun 83:27–35
28. Batova A, Diccianni MB, Omura-Minamisawa M, Yu J, Carrera CJ, Bridgeman LJ, Kung FH, Pullen J, Amylon MD, Yu AL (1999) Use of alanosine as a methylthioadenosine phophorylase selective therapy for T-cell acute lymphoblastic leukemia in vitro. Cancer Res 59:1492–1497
29. Baggott JE, Vaughn WH, Hudson BB (1986) Inhibition of 5′-aminoimidazole-4 arboxamide ribotide transformylase, adenosine deaminase and 5′-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5′-aminoimidazole-4-carboxamide riboside and ribotide. Biochem J 236:193–200
30. Cronstein BN (1997) The mechanism of action of methotrexate. Rheum Dis Clin North Am 23:739–755
31. Hirai S, Hayashi Y, Koizumi T, Nakanishi N, Fukui T, Ichikawa A (1993) Fibroblast growth factor-dependent metabolism of hypoxanthine via the salvage pathway for purine synthesis in porcine aortic endothelial cells. Biochem Pharmacol 45:1695–1701
32. Li WW, Lin JT, Tong WP, Trippett TM, Brennan MF, Bertino JR (1992) Mechanisms of natural resistance to antifolates in human soft tissue sarcomas. Cancer Res 52:1434–1438
33. Watkins D, Cooper BA (1983) Acritical intracellular concentration of fully reduced non-methylate folate polyglutamates prevents macrocytosis and diminished growth rate of human cell line K562 in culture. Biochem J 214:465–470
34. Born GVR, Kratzer MAA (1984) Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. J Physio (London) 354:419–429
35. Chong L, Tattersall MNH (1995) 5, 10-dideazatetrahydrofolic acid reduces toxicity and deoxyadenosine triphosphate pool expansion in cultured L1210 cells treated with inhibitors of thymidylate synthase. Biochem Pharmacol 49:819–827