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ACTIVATION AND CYTOTOXICITY OF 2-α-AMINOACYL PRODRUGS OF METHOTREXATE

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Abstract—In an effort to improve the selectivity of the anticancer drug methotrexate (MTX), a series of potential prodrugs in which the 2-amino group was acylated with various α-amino acids (as well as L-pyroglutamic acid) was synthesized. Such derivatives are anticipated to be hydrolysed to MTX by appropriate aminopeptidases localized (over-expressed naturally or targeted as anti-tumor antibody conjugates) in the vicinity of the tumor. The L-leucyl, L-valyl, L-isoleucyl, D-alanyl and L-pyroglutamyl derivatives were assessed as to their suitability as prodrugs. Except for the L-pyroglutamyl compound, all derivatives decomposed slowly when incubated in phosphate buffer, pH 7.3; the formation of MTX was minimal. No major differences were observed when serum was included in the incubation medium, except for the L-leucyl compound, which was hydrolysed to MTX. The L-leucyl, L-valyl and L-isoleucyl derivatives were hydrolysed readily to MTX by aminopeptidase M (EC 3.4.11.2), while the L-pyroglutamyl and D-alanyl compounds were activated by pyroglutamate aminopeptidase (EC 3.4.19.3) (from *Bacillus amyloliquefaciens*) and D-aminopeptidase (from *Ochrobactrum anthropi*), respectively. When tested for inhibition of the target enzyme dihydrofolate reductase (DHFR; EC 1.5.1.3), 2-L-valyl-MTX showed inhibition two orders of magnitude poorer than that given by MTX, in agreement with the expectation that acylation of the 2-amino group reduces binding to DHFR. After treatment of this derivative with aminopeptidase M, the extent of inhibition correlated with the amount of MTX formed. MTX derivatives alone or in combination with the complementary peptidase were tested for cytotoxicity on murine L1210 cells in culture. The above-listed derivatives were considerably less cytotoxic than MTX, except for the L-leucyl derivative which showed considerable cytotoxicity. When the appropriate exogenous peptidase was included, the cytotoxicity of the activated prodrugs approached that of MTX. These results indicate that 2-L-leucyl-MTX is unsuitable as a prodrug since it is activated prematurely by serum enzymes. Although the L-valyl and L-isoleucyl derivatives do not hydrolyse to MTX in serum and are readily activated, they are not ideal prodrugs since they decompose under physiological conditions; the properties of the decomposition product will have a bearing on the ultimate suitability of these compounds. 2-L-Pyroglutamyl-MTX is the best candidate prodrug, showing stability and ready activation by the appropriate aminopeptidase.

Key words: methotrexate; prodrugs; aminopeptidase; cytotoxicity; antibody-directed enzyme prodrug therapy; dihydrofolate reductase

Most drugs currently used in cancer treatment lack selectivity for tumor tissues, and toxic effects in normal tissues are commonly seen. One approach to minimize toxicity towards normal cells is to develop inactive prodrugs that selectively generate the active drug at the tumor site [1]. Such activation may be effected by endogenous enzymes that are over-expressed in tumors or by exogenous enzymes, conjugated to tumor-associated antibodies, which localize on the tumor surface [2, 3]. The latter strategy, known as ADEPT§ [3], has the advantage that a large number of active drug molecules can be generated from a limited amount of enzyme-antibody conjugate, and a clinical trial reported recently showed some promising results [3].

MTX is a commonly used drug in cancer chemotherapy, but has major toxic effects on normal bone marrow, mucosal and lymphoid cells and is also an immunosuppressant [4]. It acts by strongly inhibiting DHFR (EC 1.5.1.3), causing depletion of tetrahydrofolate and, hence, of thymidylate and other metabolites on which DNA synthesis vitally depends.

Prodrugs of MTX previously studied include the ester and amide analogues, derivatized at the α- and/or the γ-carboxylate groups of the glutamate residue. Derivatives of the α-carboxylate group, in particular, are expected to be relatively non-cytotoxic *in vitro*, since a free α-carboxylate group contributes significantly to the binding of MTX to DHFR [5]. Among these studies, diesters [6, 7] and bisamides

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§ Abbreviations: ADEPT, antibody-directed enzyme prodrug therapy; MTX, methotrexate, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid; DHFR, dihydrofolate reductase; TFA, trifluoroacetic acid; Leu-, L-leucyl; Val-, L-valyl; Ile-, L-isoleucyl; D-Ala, D-alanyl; and Pyr-, L-pyroglutamyl.

[8, 9] of MTX were investigated for *in vivo* cytotoxic activity. The observed results suggest that MTX is released slowly *in vivo* by the action of endogenous enzymes. In our laboratory, a series of α-monoamides of MTX was prepared [10], and *in vivo* studies suggested that these derivatives also were acting as slow-release agents [11]. In an effort to obtain selective activation of MTX prodrugs at tumor sites, MTX-α-peptides were prepared by Kuefner *et al.* [12] with the expectation that these derivatives would be cleaved by endogenous peptidases that are over-expressed by tumors or by antibody-carboxypeptidase conjugates targeted to the tumor. The best candidate prodrug identified was MTX-α-alanine, which was stable in serum, was readily cleaved by carboxypeptidase A, and was considerably less cytotoxic than MTX *in vitro* [12]. In a recent extension of this study, this prodrug showed immunologically specific cell growth inhibition when combined with an antibody-carboxypeptidase-A conjugate *in vitro* [13].

Another group of MTX prodrugs potentially suitable for tumor targeting comprises the 2-α-aminoacyl derivatives of MTX, viz. those with α-aminoacyl substituents at the 2-NH_2 of the pteridine ring. These compounds are expected to have greatly reduced inhibitory effects on DHFR since a free amino group at the 2-position of the pteridine ring is essential for tight binding [5]. Furthermore, such prodrugs may be cleaved by exopeptidases with the appropriate substrate specificity. Exopeptidases appropriate to the present work include aminoacyl and pyroglutamyl peptide hydrolases, which cleave N-terminal α-amino acids and pyroglutamic acid, respectively, from peptides [14, 15]. In this paper, these enzymes are, for convenience, collectively described as aminopeptidases.

Elevated levels of aminopeptidase activity have been detected in association with the plasma membrane of several human tumor cell lines and mammalian metastatic tumor cells. The enzyme identified is aminopeptidase N (EC 3.4.11.2) [16–20] (also known as aminopeptidase M or alanine aminopeptidase), which prefers L-alanyl- and L-leucyl-β-naphthylamides as substrates. Hence, suitable prodrugs in these situations are 2-L-alanyl-MTX and 2-L-leucyl-MTX, which are structurally similar to the above substrates.

Alternatively, exogenous aminopeptidase activity may be made to localize at the tumor via antibody targeting. In this case, the enzymes may be chosen from a range of bacterial, plant or mammalian aminopeptidases that are capable of hydrolysing peptides containing α-amino or imino acids at the N-terminus. Examples of these enzymes are porcine kidney aminopeptidase M (microsomal) (EC 3.4.11.2), which hydrolyses β-naphthylamides of L-α-amino acids [21], pyroglutamate aminopeptidase (pyroglutamyl peptide hydrolase, pyrrolidonyl peptidase or pyrrolidonecarboxy peptidase) (EC 3.4.19.3) from various bacteria and plants [15, 22, 23], and bacterial D-aminopeptidase [24, 25].

A requirement of any prodrug approach to cancer chemotherapy is that the prodrug should not be activated prematurely before reaching the tumor. Of considerable importance is the stability of prodrugs in the body. Human plasma, liver and kidney are known to contain aminopeptidase activity [26–28]. Hence, the specificities of both exogenous and endogenous enzymes are taken into account in the design of prodrugs.

Recently, we synthesized a number of 2-α-aminoacyl derivatives of MTX, as well as 2-L-pyroglutamyl-MTX* [29]. In this paper, we report our examination of the suitability of these compounds as prodrugs. These studies cover stability towards buffer and serum, hydrolysis by complementary aminopeptidases, DHFR inhibition and cytotoxicity. Brief reports of some of these results have been presented at conferences [30, 31].

MATERIALS AND METHODS

Materials. *t*-Butoxycarbonyl-protected 2-α-aminoacyl derivatives of MTX di-*t*-butyl ester were prepared as described elsewhere* [29] and were deprotected, shortly before use, by treatment with TFA* [29]. Upon removal of TFA, the residues were reconstituted in 2 M phosphate buffer, pH 7.3, containing 10% dimethyl sulfoxide. The resultant 2-α-aminoacyl derivatives of MTX were assayed by HPLC (see below) for contamination by MTX, and concentrations were determined by comparison of peak areas with those of standard MTX solutions. The percentage contamination by MTX for the L-leucyl (Leu), L-valyl (Val), L-isoleucyl (Ile), D-alanyl (D-Ala), D-valyl, D-leucyl, D-norleucyl and L-pyroglutamyl (Pyr) derivatives (unless stated otherwise) were: 7.0, 0.6, 4.6, 8.4, 6.8, 2.5, 2.0 and 2.6%, respectively. MTX was from David Bull Laboratories. Human serum was obtained from healthy volunteers and was stored frozen prior to use. All other reagents were from the Sigma Chemical Co.

Enzymes. Aminopeptidase M (microsomal) from porcine kidney was purchased from Boehringer Mannheim. D-Aminopeptidase was isolated from *Ochrobactrum anthropi* [24]. Purified recombinant pyroglutamate aminopeptidase from *Bacillus amyloliquefaciens* [32] was a gift from Dr. T. Yoshimoto of Nagasaki University, Japan. Pure DHFR from *Lactobacillus casei* with a specific activity of 9.4 U/mg was provided by Dr. J. Feeney of the National Institute of Medical Research, London.

Stability and enzyme cleavage studies. Solutions of MTX derivatives were diluted in 1 M phosphate buffer, pH 7.3. Human serum and/or the appropriate aminopeptidase were added, and the mixtures were incubated at 37°. At the appropriate times, 25-μL samples were withdrawn and analysed by HPLC. The HPLC system consisted of a Spectra Physics Isochrom LC pump, a Rheodyne injector fitted with a 20-μL sampling loop, a Spectra Physics 100 variable wavelength detector set at 300 nm and a Spectra Physics Data Jet recorder/integrator, and a Merck 5 μm Lichrospher 100 RP-18 column was used. The mobile phase, which was pumped at the flow rate of 0.8 mL/min, was a mixture of methanol and 0.1 M phosphate buffer, pH 7.3; solvent ratios ranged from

* Boadle DK, Cheung HTA, Dong Z, Smal MA and Tran TQ, manuscript in preparation.

Table 1. Stability of MTX prodrug derivatives: composition after incubation in buffer and in serum

Prodrug	Initial MTX content (%)	Composition in buffer* (%)			Composition in serum† (%)		
		MTX	Prodrug	Decomp.‡	MTX	Prodrug	Decomp.‡
2-Leu-MTX	8.5	18.8	38.1	43.1	28.4	29.6	42.0
2-Val-MTX	0.7	2.1	47.6	48.2	2.5	49.7	47.8
2-Ile-MTX	0.5	2.9	46.4	50.7	6.1	44.7	49.1
2-D-Ala-MTX	8.4	20.2	59.1	18.5	20.4	57.5	19.4
2-Pyr-MTX	2.6	7.9§	92.1§	0	8.0‖	92.0‖	0

* Unless otherwise stated, compositions are after a 90-min incubation at 37° in 1 M phosphate buffer, pH 7.3, as analysed by HPLC (single determinations).
† Incubation as for in buffer, but in medium containing 20% serum (v/v).
‡ Decomposition product.
§ Mean (±0.7) of two determinations.
‖ Mean (±0.2) of four determinations.

20/80 (v/v) for 2-D-Ala- and 2-Pyr-MTX to 32/68 (v/v) for 2-Leu- and 2-Ile-MTX. Under these conditions, the MTX derivatives eluted at 4–8 min, and the decomposition products eluted several minutes later. For quantitation purposes, the extinction coefficient at 300 nm (the *p*-aminobenzoyl chromophore) of the decomposition products was assumed to be the same as that of MTX and its 2-aminoacyl derivatives.

Inhibition of DHFR. Inhibition of the DHFR-catalysed NADPH-dependent reduction of dihydrofolate by MTX and its derivatives was measured by a modification of the method of Mathews *et al.* [33]. Briefly, to 1.9 mL of an aqueous solution containing DHFR (60 mU/mL), inhibitor, 0.08 mM NADPH, 0.15 M potassium chloride and 0.05 M Tris, pH 7.4, was added 100 μL of 1.0 mM dihydrofolate in 0.1 M mercaptoethanol. The change in absorbance at 340 nm was monitored, and the initial velocity was calculated. Inhibition results are expressed as a percentage of the average control (uninhibited) rate, which was determined in duplicate.

In vitro *cytotoxicity.* Murine L1210 cells were grown in suspension culture at 37° in RPMI 1640 medium supplemented with 10% non-dialysed fetal bovine serum, 2 mM L-glutamine and 32 μg/mL gentamycin in a humidified 10% CO_2, 5% O_2 atmosphere. Cell doubling times were approximately 12 hr. Twenty-four hours before drug addition, the cells were suspended in fresh medium at a concentration of 5×10^4 cells/mL and plated out into 24-well plates. MTX or its derivatives and/or enzyme were administered as a single dose and remained in culture for the duration of the experiment (performed in duplicate). Cells counts were made at 24 and 48 hr using trypan blue exclusion to assess cell viability. Results are presented as a percentage of control (no additions) cell counts.

RESULTS

When the aminoacyl derivatives of MTX were incubated at 37° in buffer alone, gradual decomposition with time to an unknown product was observed; the results after a 90-min incubation are shown in Table 1. No decomposition product was observed in the case of 2-Pyr-MTX. For each derivative, the retention time of the decomposition product was considerably greater than that of the parent compound or of MTX, and varied according to the aminoacyl substituent. MTX did not decompose under these conditions. For all derivatives, small amounts of MTX were formed by hydrolysis (generally less than 10%/hr). The L-leucyl and D-alanyl derivatives had the highest hydrolysis rates of the compounds tested.

The stability of MTX derivatives upon incubation in a mixture of phosphate buffer, pH 7.3, and 20% serum was followed, and the results after 90 min are shown in Table 1. Stability studies were not conducted in whole serum or plasma due to difficulties with subsequent HPLC analysis; however, recent studies have indicated that peptide degradation rates are linearly proportional to the serum concentration [34]. Serum did not alter the stabilities of L-pyroglutamyl or the D-alanyl derivative. Very small increases in the hydrolysis rates were noted for the L-isoleucyl and L-valyl compounds. However, the addition of 20% serum doubled the hydrolysis rate for 2-Leu-MTX, which was not unexpected since serum has substantial leucine aminopeptidase activity [26].

All compounds were tested for hydrolysis by aminopeptidase M (0.08 U/mL) in the presence of human serum. Among these, the D-aminoacyl and L-pyroglutamyl derivatives were not substrates for this enzyme. 2-Leu-MTX was an excellent substrate for aminopeptidase M under these conditions, with over 80% conversion to MTX in 1 hr (Table 2). The amount of decomposition was greatly diminished due to depletion of the substrate by hydrolysis. Under similar conditions, 2-Ile-MTX and 2-Val-MTX were also substrates, the rate of enzymic hydrolysis being of a magnitude similar to the decomposition rate (Table 2). A comparison of the chemical versus enzymic hydrolysis/decomposition for 2-Val-MTX is illustrated in Fig. 1. Towards the end of the enzyme-catalysed reaction, formation of MTX ceased and the amount of the decomposition

Table 2. Enzymic hydrolysis of MTX prodrug derivatives

Prodrug	Initial concentration (μM)	Enzyme	Enzyme activity (U/mL)	Composition* (μM)		
				MTX	Prodrug	Decomposition product
2-Leu-MTX†	12	Aminopeptidase M	0.08	9.8	0.6	1.6
2-Val-MTX	48	Aminopeptidase M	0.08	23	8.9	16
2-Ile-MTX	97	Aminopeptidase M	0.08	43	13	40
2-D-Ala-MTX	20	D-Aminopeptidase	0.32	13	4.2	2.6
2-Pyr-MTX†	10	Pyroglutamate aminopeptidase	0.02	7.8 ± 0.4	2.3 ± 0.2	0

* Unless otherwise stated, compositions are after a 90-min incubation at 37° with enzyme in 1 M phosphate buffer, pH 7.3, containing 20% serum (v/v), as analysed by HPLC (single determinations).
† After a 60-min incubation; mean ± range of two determinations, where indicated.

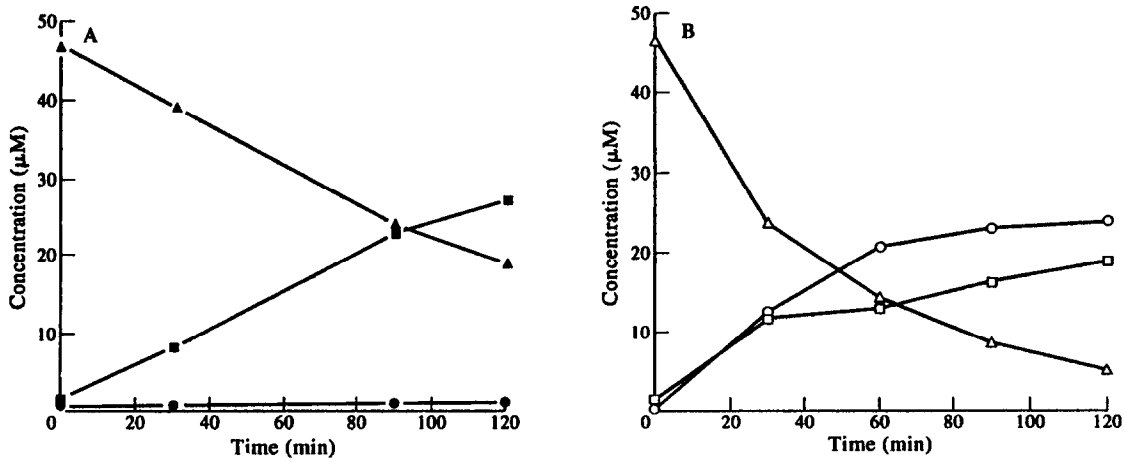

Fig. 1. (A) (closed symbols): conversion of 2-Val-MTX (▲) to MTX (●) and decomposition product (■) in 1 M phosphate buffer, pH 7.3, containing 20% serum (v/v). (B) (open symbols): corresponding conversion in the presence of aminopeptidase M (0.08 U/mL). Each point represents a single determination.

product did not decrease. These observations suggest that the decomposition product is not a substrate for aminopeptidase M. When serum was omitted from the enzymic reactions, no significant differences were observed in the rate of hydrolyses. Full kinetic analyses were not performed due to limited quantities of substrates being available.

D-Aminopeptidase at 0.32 U/mL was found to release MTX from 2-D-Ala-MTX (20 μM) (Table 2), but did not hydrolyse the corresponding D-leucyl, D-valyl and D-norleucyl derivatives of MTX under similar conditions. The rate of hydrolysis of 2-D-Ala-MTX increased with increasing enzyme concentration; initial rates of enzymic hydrolysis at pH 7.3 of 20 μM substrate were 0.045, 0.114 and 0.313 μM/min at enzyme concentrations of 0.14, 0.32 and 1.4 U/mL, respectively (no serum added).

Pyroglutamate aminopeptidase from *B. amyloliquefaciens* was found to hydrolyse the 2-L-pyroglutamyl derivative of MTX (Table 2). Similar to the above results, the hydrolysis rate was proportional to the enzyme concentration.

2-Val-MTX was examined for inhibition of *L. casei* DHFR, and its action was compared with that of MTX (Fig. 2). The IC_{50} for 2-Val-MTX was 1.7 μM and for MTX, 0.047 μM. Allowing for contamination by 0.6% MTX in the sample of 2-Val-MTX, the estimated IC_{50} of 2-Val-MTX was approximately 4 μM. Upon hydrolysis of 2-Val-MTX (10 μM) by aminopeptidase M (0.2 U/mL) for 40 min at 25°, the IC_{50} decreased to 0.12 μM. HPLC analysis indicated that there was 37% conversion to MTX, which correlates well with the observed change in DHFR inhibition. Aminopeptidase M itself was found to have only a minimal effect on DHFR activity (<20% inhibition at 0.08 U/mL). Similar effects on DHFR activity were observed when 2-Pyr-MTX was hydrolysed by pyroglutamate aminopeptidase (not shown). In a further experiment, a sample of 2-Val-MTX at 2 μM was allowed to decompose at 37° for 1 hr. The resulting sample inhibited DHFR to a similar extent as a fresh sample of 2-Val-MTX.

The effects of MTX derivatives alone and combined with the appropriate aminopeptidase on

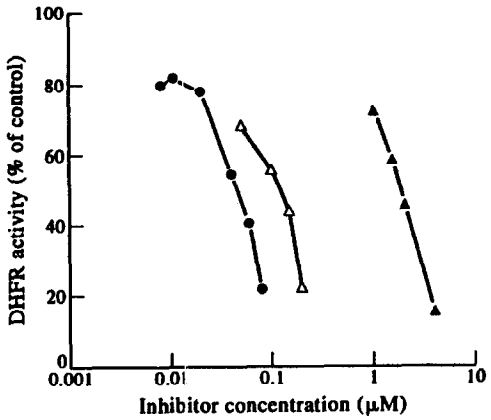

Fig. 2. Inhibition of *L. casei* DHFR activity by MTX (●), 2-Val-MTX (▲) and 2-Val-MTX (containing 37% MTX) activated by aminopeptidase (△). Inhibition results (single determinations) are expressed as a percentage of the control (uninhibited) rate of 16.1 ± 0.6 nmol tetrahydrofolate reduced/min (mean ± range of two determinations).

the growth of L1210 leukemia cells after 48 hr are shown in Table 3. The progress of inhibition of cell growth by 2-D-Ala-MTX at 0.02 μM with and without D-aminopeptidase is shown in Fig. 3. Although similar trends were apparent after 24 hr, generally the effects were not as marked as at 48 hr. In all cases, the derivatives were less cytotoxic than the parent drug MTX, but not devoid of cytotoxicity. The major part of this residual cytotoxic activity may be attributed to contamination by MTX. For example, 2-Pyr-MTX (at 0.1 μM), which was contaminated with 7.0% MTX, reduced growth to 14.0 ± 1.3% of control after 48 hr (Table 3), whereas in another experiment using 2-Pyr-MTX containing 2.6% MTX, cell growth under similar conditions was 49.9 ± 3.7% of control. 2-Val-MTX was the least cytotoxic, being at least 10-fold less active than MTX. However, 2-Leu-MTX displayed approximately half the activity of MTX, presumably because it was hydrolysed substantially by serum contained in the culture medium (see Table 1). Upon inclusion of the complementary aminopeptidase, the cytotoxic activity of all MTX derivatives increased. In particular, 2-Pyr-MTX and 2-D-Ala-MTX became as cytotoxic as MTX when activated by pyroglutamate aminopeptidase and D-aminopeptidase, respectively. Aminopeptidase M and D-aminopeptidase alone did not inhibit cell growth; pyroglutamate aminopeptidase at 0.025 U/mL had a small growth inhibitory effect of 13.1 ± 5.9% after 48 hr.

DISCUSSION

The 2-α-aminoacyl derivatives of MTX were examined for their suitability as prodrugs in either the ADEPT strategy [2, 3] or for use in conjunction with enzymes that are over-expressed by tumors, in particular aminopeptidase M [16–20]. Use of prodrugs in such therapies requires that the prodrugs are inactive, do not prematurely release free active drug, and are readily activated by the appropriate enzyme.

First, results of the studies on the inhibition of DHFR indicated that the IC_{50} of 2-Val-MTX is two orders of magnitude higher than that of MTX. Thus, the conversion of the 2-amino group on the pteridine ring into an amide derivative greatly reduces the enzyme inhibition. This is to be expected since in this conversion the strong interaction between the N1/2-NH_2 region of MTX and a critical Asp or Glu on DHFR [5] has been removed. After 2-Val-MTX was incubated with its complementary aminopeptidase, the increase in enzyme inhibition corresponded to the quantity of MTX released.

Second, the chemical stability of the candidate prodrugs was examined. All the tested prodrugs, with the exception of the pyroglutamate derivative, underwent slow decomposition under physiological

Table 3. Cytotoxicity to L1210 cells of MTX prodrug derivatives with or without activation by aminopeptidases*

Prodrug/Drug	Enzyme	Concentration (U/mL)	Viable cells (% control) at drug concentrations: 1.0 μM	0.1 μM	0.02 μM	0.01 μM
MTX			2.6 ± 0.8	4.9 ± 0.5	13.8 ± 2.0	16.8 ± 0.9
2-Leu-MTX			6.7 ± 0.8	10.0 ± 0.4	39.8 ± 2.2	
2-Leu-MTX	Aminopeptidase M	0.005	2.4 ± 0.6	8.1 ± 0.2	12.1 ± 1.7	
2-Val-MTX			9.0 ± 0.5	49.4 ± 17.6	116†	
2-Val-MTX	Aminopeptidase M	0.005	4.5 ± 0.2	12.7 ± 1.2	110 ± 3	
2-Ile-MTX			9.5 ± 0.8	36.4 ± 3.9	78.3 ± 8.2	
2-Ile-MTX	Aminopeptidase M	0.005	5.5 ± 0.3	7.9 ± 0.3	39.9 ± 1.5	
2-D-Ala-MTX			4.7 ± 0.1	9.8 ± 1.0	61.0 ± 2.6	
2-D-Ala-MTX	D-Aminopeptidase	0.14		6.9 ± 0.5	20.1 ± 1.3	
2-Pyr-MTX‡				14.0 ± 1.3	83.9 ± 4.3	76.3 ± 8.2
2-Pyr-MTX‡	Pyroglutamate aminopeptidase	0.025		6.0 ± 0.8	18.7 ± 2.5	76.1 ± 3.8

* After 48 hr. Values are means (±range) of duplicate determinations, unless otherwise stated.
† Single determination.
‡ Initial MTX content 7.0% by HPLC.

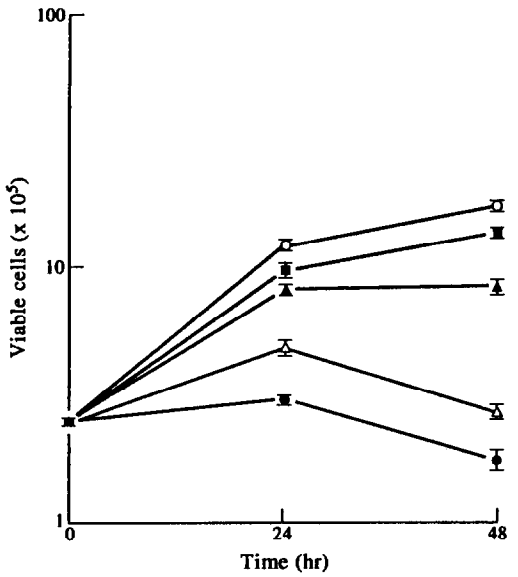

Fig. 3. Cytotoxic effects on L1210 cells in culture caused by 0.02 μM MTX (●), 0.02 μM 2-D-Ala-MTX (▲), 0.14 U/mL D-aminopeptidase (□) and 0.02 μM 2-D-Ala-MTX plus 0.14 U/mL D-aminopeptidase (△); control (■). Each point represents the mean (±range) of two determinations.

conditions. These compounds have half-lives in the order of 1–2 hr, and thus some of them may survive long enough to allow adequate delivery to the tumor. A major consideration is whether or not the decomposition product is cytotoxic. A preliminary experiment on 2-Val-MTX examining the effect of decomposition on inhibition of DHFR activity showed no major changes in inhibition. The chemical structures of the decomposition products are unknown, but indications, based on HPLC retention times, are that the α-aminoacyl group is retained. Experiments are currently underway to isolate and characterize this compound; biological activity will also be assessed.

With the exception of 2-Leu-MTX, α-aminoacyl derivatives did not release free MTX in the presence of serum, and hence they may be suitable prodrugs. The extent of release of MTX from the 2-L-α-aminoacyl derivatives is unknown in an intracellular environment, where there is high aminopeptidase activity [26]. This would be dependent on the extent of internalization of these derivatives. Being folate derivatives with α- and γ-carboxylate groups, they may be internalized via the reduced folate carrier [35]; however, our cytotoxicity experiments indicate that these derivatives (except for 2-Leu-MTX) are not as cytotoxic as MTX, suggesting that in L1210 cells in culture, little internalization followed by intracellular hydrolysis occurs. 2-D-Ala-MTX is not hydrolysed in serum and is not expected to be cleaved in an intracellular environment, since mammalian aminopeptidases are specific for peptides containing natural L-amino acids [14]. The resistance of 2-Pyr-MTX to hydrolysis by serum is consistent with the results obtained by Szewczuk and Kwiatkowska [23], who found no pyroglutamate aminopeptidase activity in human serum. Additionally, the more recent studies by Mantle and coworkers [36–38] have shown little pyroglutamate aminopeptidase activity (less than 5% of total aminopeptidase activity) in the soluble extracts (cytosolic) of human skeletal muscle, kidney and cerebral cortex. These data suggest that 2-Pyr-MTX may not be rapidly hydrolysed in the cytosolic environment, even if internalized.

Finally, hydrolysis of MTX derivatives by complementary aminopeptidases was studied. 2-Val-, 2-Leu- and 2-Ile-MTX were cleaved rapidly by porcine kidney aminopeptidase M, while *B. amyloliquefaciens* pyroglutamate aminopeptidase activated 2-Pyr-MTX. Results of enzymic cleavage studies were supported by cytotoxicity data. D-Aminopeptidase, a recently discovered enzyme [24], hydrolysed 2-D-Ala-MTX, but did not cleave the corresponding D-valyl, D-leucyl and D-norleucyl derivatives. Earlier substrate specificity studies on this enzyme indicated that D-norleucine amide was a substrate, albeit a poor one [24]. However, substitution of the small amide group by a bulky MTX structure has resulted in no observable hydrolysis. No other investigations were carried out on the latter three derivatives as they were judged to be totally unsuitable as prodrugs.

The α-aminoacyl derivatives of MTX studied may be graded as to their suitability as prodrugs, based on all of the above results. 2-Leu-MTX is unsuitable as a prodrug, primarily since it is activated rapidly by serum aminopeptidases. 2-Val-MTX and 2-Ile-MTX may be potential prodrugs since they are not activated by serum enzymes. Decomposition may be a problem, especially if the decomposition product turns out to be cytotoxic; this is currently being investigated. The other concern is that decomposition would reduce the amount of prodrug reaching the tumor site; this may be compensated for by increasing the dose. Since these two derivatives are good substrates for aminopeptidase M (which is the same as aminopeptidase N), they may be candidates for prodrug therapy utilizing aminopeptidase N activity, which is over-expressed on some tumor surfaces [16–20]. Alternatively, they may be used in the ADEPT approach, where exogenous aminopeptidase M is targeted to the tumor surface by conjugation with an antibody specific for the appropriate tumor-surface antigen. 2-D-Ala-MTX is of a similar potential as the above L-α-aminoacyl derivatives. This prodrug would be useful in therapy based on the ADEPT strategy, wherein an exogenous D-aminopeptidase is to be localized via antibody targeting. Recent cloning of this enzyme [39] should make large quantities available for conjugation to the appropriate antibodies, and this prodrug will have the advantage of being unlikely to undergo premature cleavage by endogenous aminopeptidases, which have specificity for L-aminoacyl groups.

2-Pyr-MTX is the best candidate prodrug, being superior to the previous compounds in that it does not decompose. The use of this prodrug in therapy would be in the ADEPT approach in conjunction with exogenous pyroglutamate aminopeptidase targeted to the tumor. The source of this enzyme

may be bacterial, as it has been cloned recently from three sources [32, 40, 41].

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