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Regiospecific γ-Conjugation of Methotrexate to Poly(L-lysine)

Chemical and Biological Studies

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SUMMARY

Regiospecific syntheses of γ- and α-conjugates of methotrexate and poly(L-lysine) are described. The α- and γ-*t*-butyl esters, respectively, of methotrexate were coupled to poly(L-lysine) with diphenylphosphoryl azide in *N*,*N*-dimethylformamide, the ester-protecting group was cleaved with 15% hydrogen bromide in acetic acid, and small molecules were removed by dialysis. Poly(L-lysine) of M_r = 1,500–8,000 and 8,000–30,000 was used to prepare six different conjugates, which were characterized by ultraviolet absorbance measurement and quantitative amino acid analysis. The degree of substitution varied from one methotrexate per 4.7 lysines to one methotrexate per 10.2 lysines. Dihydrofolate reductase inhibition in a cell-free assay was observed with α- and γ-conjugates, but the latter had the greater affinity (only 3-fold less than that of methotrexate itself). The binding of the conjugates exhibited a slight pH dependence, with affinity being greater at pH 7.2 than at pH 8.5 for both α- and γ-conjugates. Toxicity to cultured rat hepatoma cells (H35) was also greater for the γ-conjugates, and showed some dependence on the chain-length and degree of substitution of the poly(L-lysine) carrier. Cells resistant to methotrexate by virtue of a transport defect ($H35R_{0.3}$ line) retained their sensitivity to the γ-conjugate, but less so to the α-conjugate. There was also some retention of sensitivity in a more highly resistant cell line ($H35R_{10}$) with impaired methotrexate transport and a concomitant increase in dihydrofolate reductase activity. γ-Conjugation was likewise more favorable in cytotoxicity assays against L1210 murine leukemia cells, and there was partial retention of activity against highly methotrexate-resistant lines (L1210/R71 and L1210/R81) with a transport defect and/or an elevation of dihydrofolate reductase content. In antitumor assays against intraperitoneal L1210 leukemia in mice, a γ-conjugate with M_r = 8,000–30,000 and one methotrexate per 5.5 lysines produced a 35–75% increase in lifespan when administered intraperitoneally at single doses equivalent to 10–20 mg/kg of methotrexate. A similar increase in lifespan with methotrexate alone on the single-dose regimen required 50–150 mg/kg. An α-conjugate of similar M_r and degree of substitution was inactive at nontoxic doses, as were other γ-conjugates of lower M_r and/or degree of substitution. Thus, with a favorable combination of carrier size and methotrexate/lysine ratio, a γ-conjugate of methotrexate to poly(L-lysine) is capable of markedly reducing the amount of methotrexate needed to elicit a therapeutic response in mice with L1210 leukemia.

INTRODUCTION

Covalent attachment of the anticancer drug MTX[1] to poly(L-lysine) was first described by Ryser and Shen (1, 2) as a novel approach to altering the mode of cellular uptake and overcoming MTX resistance based on impaired transport. Among the prominent features of the action of MTX-poly(L-lysine) are that (i) the intact conjugate binds less well to dihydrofolate reductase than does MTX itself (3); (ii) uptake of the conjugate probably occurs via pinocytosis (1, 2); (iii) uptake, especially in cells with defective MTX transport, is markedly superior to that of MTX (2, 4, 5), is unaffected by inhibitors of

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[1] The abbreviations used are: MTX, methotrexate, *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-glutamic acid; IC_{50}, 50% inhibitory concentration; ILS, increase in lifespan.

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MTX transport (6), but is stimulated by complexation with the polyanion heparin (7); (iv) cytotoxicity is dependent on lysosomal degradation, as indicated by loss of activity on trypsinization (2, 8), addition of lysosomal inhibitors (4, 9), or replacement of poly(L-lysine) by poly(D-lysine) (3, 9); and (v) conjugates appear to have some differential tumor versus nontumor cell selectivity *in vitro* (4) and *in vivo* (10, 11). Lysosomal degradation of MTX-poly(L-lysine) yields at least some free MTX, since MTX polyglutamates are recovered from cells upon incubation with MTX-poly(L-lysine) (1–4, 5, 9). However, there is evidence that the major lysosomal products are small MTX-linked oligomers (1–4, 5, 9), and it has been speculated that these may contribute also to the toxic effects of MTX-poly(L-lysine) (12).

An important aspect of the chemistry of all MTX-poly(L-lysine) conjugates studied to date (1–11) is that they are probably mixtures of α- and γ-linked species. This is readily understood when one considers that the method used for their preparation (2, 5) involves direct carbodiimide-mediated coupling of MTX, with both carboxyl groups free, to poly(L-lysine). Under these conditions it is very likely that reaction occurs at the γ- and/or α-positions (13). Small MTX-containing lysine oligomers generated in cells by lysosomal degradation of the poly(L-lysine) carrier would thus be expected to be mixtures of γ- and α-substituted species, and it is known that a free α-carboxyl group is necessary in MTX derivatives for tight binding to the target enzyme dihydrofolate reductase (14–16). Accordingly, to the extent that α-linked MTX-oligolysines might arise from a mixture of MTX-poly(L-lysine) and MTX-α-poly(L-lysine), a less than optimal effect on tumor cell growth would be predicted. Further complications inherent in direct coupling reactions between MTX and poly(L-lysine) via the carbodiimide route include racemization (13) and the likely formation of cross-linked MTX-poly(L-lysine) species that would elute with the desired products in the exclusion volume of gel filtration columns (2, 5). Cross-linked MTX-poly(L-lysine) might resist cleavage by lysosomal enzymes, or else might be cleaved to inactive cross-linked oligolysine fragments. This would again compromise biological activity, and would make it difficult to properly assess the potential of poly(L-lysine) conjugation as a novel approach to antifolate therapy. In this paper, we report the first regiochemically unambiguous synthesis of pure MTX-γ-poly(L-lysine) and MTX-α-poly(L-lysine) and present data on their biological activity *in vitro* and *in vivo*. We have observed that MTX-γ-poly(L-lysine) is more active than MTX-α-poly(L-lysine) both as an inhibitor of dihydrofolate reductase in a cell-free assay and as an inhibitor of tumor cell growth in culture. The γ-conjugate is also the more active one *in vivo* against L1210 leukemia in mice, and when single doses of MTX versus MTX-γ-poly(L-lysine) are compared there is a 5- to 10-fold reduction in the amount of antifol needed for a therapeutic effect. However, the chainlength and degree of substitution of the polymer can markedly influence therapeutic efficacy, and have to be very precisely balanced to maximize antitumor activity while minimizing host toxicity.

MATERIALS AND METHODS

MTX was obtained from the National Cancer Institute, Bethesda, MD, as the sterile, freeze-dried sodium salt for clinical use. Poly(L-lysine) hydrobromide, M_r = 1,500–8,000 and 8,000–30,000, was from Chemical Dynamics, South Plainfield, NJ, and diphenylphosphoryl azide was from Aldrich Chemical Company, Milwaukee, WI. Spectrapor dialysis tubing, M_r = 1,000 and 3,500, was from Fisher Scientific, Boston, MA. Swims medium S77, horse serum, and fetal bovine serum were from Grand Island Biological Company, Grand Island, NY. RPMI 1640 medium and fetal bovine serum were from Kansas City Biologicals, Kansas City, MO.

Synthesis of MTX-Poly(L-lysine) Conjugates from α- or γ-t-Butyl Esters of MTX

Preparation of MTX-PLL8. Poly(L-lysine) hydrobromide, M_r = 8,000–30,000 (209 mg, 1.0 mmol of lysine) was added to dry *N,N*-dimethylformimide (20 ml) containing triethylamine (141 mg, 1.4 mmol) and the mixture was subjected to prolonged sonication and magnetic stirring until all the polymer dissolved, leaving triethylammonium bromide as a fine suspension. The mixture was cooled to 0° and MTX α-*t*-butyl ester (17) (107 mg, 0.2 mmol) was added, followed by diphenylphosphoryl azide (55 mg, 0.2 mmol) in a small volume of *N,N*-dimethylformamide. After 3–4 hr of stirring at 0° and 24 hr at room temperature, the solvent was evaporated and the residue was triturated with ether, filtered, and air-dried. The product was suspended directly in glacial acetic acid (10 ml), 30% hydrogen bromide in acetic acid (10 ml) was added, and the mixture was stirred at room temperature for 1 hr and then poured into a large volume of ether. The ether was decanted, the residue was washed twice more with ether, and the remaining solid was taken up in water (30 ml). The pH was adjusted to 7 with concentrated ammonia, and the slightly cloudy solution was dialyzed for 3 days against 4 liters of 0.05 M sodium acetate buffer, pH 5.0, using M_r = 3,500 Spectrapor tubing. Buffer changes were made daily, and the final dialysate was clear yellow.

Preparation of MTX-PLL6. The same procedure as described for MTX-PLL8 was followed except that MTX γ-*t*-butyl ester (17) was used.

Preparation of MTX-PLL1. The method described above was used, except that the amounts of reagents were: triethylamine, 120 mg (1.2 mmol); MTX α-*t*-butyl ester, 54 mg (0.1 mmol); diphenylphosphoryl azide, 28 mg (0.1 mmol).

Preparation of MTX-PLL2. The same procedure as in the synthesis of MTX-PLL1 was followed, except that dialysis was performed with M_r = 1,000 Spectrapor tubing.

Determination of the MTX Concentration in Dialyzed Conjugates

The ultraviolet absorbance of each batch of exhaustively dialyzed product was measured at 260 nm, and the effective MTX concentration was calculated by assuming ϵ = 25,600. Absence of MTX in the buffer outside the dialysis bag after the third daily change (see above) was established by ultraviolet spectroscopy and thin layer chromatography. Each dialyzed solution was diluted to give a final "MTX concentration" in the 1–4 mM (0.45–1.8 mg/ml) range, and was divided into small aliquots for storage at −20°. The Lys/Glu ratio in each batch was determined by amino acid analysis. The Lys/Glu values and "MTX concentrations" for the various MTX-poly(L-lysine) conjugates studied are given in Table 1. No free MTX could be detected by ultraviolet spectroscopy or thin layer chromatography after 3-day dialysis of mixtures of poly(L-lysine) and free MTX combined in ratios of 5:1 or 10:1.

Cells

Monolayers of H35 rat hepatoma cells and of the MTX-resistant sublines $H35R_{0.3}$ and $H35R_{10}$ were grown at 37° under a 5% CO_2 atmosphere in 60-mm plastic dishes containing Swims S77 medium supplemented with 20% horse serum, 5% fetal bovine serum and 4 mM L-glutamine (18).

TABLE 1

Characteristics of MTX-poly(L-lysine) conjugates

Compound	Poly(L-lysine)	Lys-Glu linkage	Lys-Glu ratio	
			Theory	Found
	M_r			
MTX-PLL1	8,000–30,000	γ-ε	10.0	9.7
MTX-PLL2	1,500–8,000	γ-ε	10.0	10.2
MTX-PLL4	8,000–30,000	γ-ε	5.0	5.5
MTX-PLL6	8,000–30,000	α-ε	5.0	6.8
MTX-PLL7	1,500–8,000	γ-ε	5.0	6.5
MTX-PLL8	8,000–30,000	γ-ε	5.0	4.7

Suspension cultures of L1210 cells were grown as previously described (19), in RPMI 1640 medium containing 5% fetal bovine serum and antibiotics. The MTX-resistant L1210/R81 cells with a 35-fold elevation of dihydrofolate reductase and highly defective MTX transport were maintained in medium containing 10 μM MTX. The MTX-resistant L1210/R71 cell line with an 88-fold elevation of dihydrofolate reductase was initially provided by Dr. R. C. Jackson (Warner-Lambert, Ann Arbor, MI) and was maintained in medium containing 3 μM MTX. The L1210/R71 and L1210/R81 cells were maintained in the absence of MTX for at least three transfer generations before harvesting in order to eliminate MTX bound to dihydrofolate reductase. Both cell lines have population doubling times of 12–14 hr.

Cell Growth Inhibition

In the cytotoxicity assays on H35, $H35R_{0.3}$, and $H35R_{10}$ cells, an initial inoculum of 2×10^5 cells per plate was used and the number of cells was determined after 96 hr (18). The cells were detached by treatment with 0.05% trypsin and were counted with a Model Z_{B1} Coulter counter. In the assays on L1210, L1210/R71, and L1210/R81 cells, an initial inoculum of 5×10^4 cells/ml was used in 24-well cluster dishes and the cells were counted after 48 hr. The IC_{50}, defined as the drug concentration inhibiting cell growth by 50% of the untreated control value, was obtained in each assay from the appropriate growth curve. All assays were performed in duplicate tubes and had a standard deviation of ±10%.

Dihydrofolate Reductase Inhibition

Dihydrofolate reductase from L1210 cells was purified by affinity chromatography as previously described (20), and enzyme activity was assayed spectrophotometrically. Initial rates were derived from the change in absorbance continuously recorded with a Cary Model 219 spectrophotometer. The assay mixture, in a total volume of 1 ml, consisted of 0.01 M Tris-chloride buffer (pH 7.2), 75 μM NADPH, and 50 μM dihydrofolate. The standard unit of enzyme activity was determined from the change in absorbance at 340 nm using $\Delta\epsilon = 12{,}300$ at 22° (21). Inhibition studies were carried out by preincubating the enzyme, NADPH, and inhibitor in the assay buffer for 2 min at 22°, and residual enzyme activity was determined after the addition of dihydrofolate. Remaining activity was expressed as a percentage relative to the activity of the enzyme without inhibitor added. Assays were also performed in 0.01 M Tris-chloride buffer (pH 8.5). Triplicate tubes were used at each inhibitor concentration, and results were averaged.

In Vivo Antitumor Activity

The effect of MTX-poly(L-lysine) conjugates on the growth of L1210 leukemia in mice was measured according to standard methods (22). The tumor inoculum was 10^5 cells per mouse, given intraperitoneally. Animals were weighed on days 1 and 7 after tumor implantation, and the 7-day weight change was calculated as a percentage. Groups of five mice were used at each dose, and the 7-day weight change was averaged (SD ± 10%). Antitumor activity was expressed as the per cent ILS based on median survivals of treated versus untreated animals. A control experiment using MTX and poly(L-lysine) as a noncovalently linked mixture was also performed.

RESULTS AND DISCUSSION

In order to address the question of whether MTX-poly(L-lysine) conjugates can directly inhibit dihydrofolate reductase, we compared the affinity of MTX and of two of the conjugates, MTX-PLL6 and MTX-PLL8, for purified enzyme from L1210 cells. As shown in Table 2, the γ-linked conjugate MTX-PLL8 had an IC_{50} 2.4-fold lower than that of the α-linked conjugate MTX-PLL6. Since the two conjugates were both prepared from M_r = 8,000–30,000 poly(L-lysine) (see Table 1), this difference cannot be related simply to the chainlength of the carrier. Thus, the general principle that γ-substitution has a greater effect than α-substitution on the binding of MTX derivatives to dihydrofolate reductase (14–16) appears to be valid even when the substituent is a polymer. It is of interest to note that at least some of the MTX-poly(L-lysine) molecules used in these experiments, i.e., those at the upper end of the M_r range, must be comparable in size to the target enzyme itself. It can be calculated for MTX-PLL8, in which the M_r of the carrier is 8,000–30,000 and the experimentally determined Lys/Glu ratio is about 5.0, that the conjugate contains 60–240 lysine residues and 12–48 MTX residues per polymer molecule. Assuming the M_r distribution in the 8,000–30,000 range to be uniform and therefore using 20,000 as a reasonable mean, the "average" MTX-PLL8 molecule would have to contain roughly 30 MTX residues. We had thought it very unlikely on steric grounds that more than two or three of these MTX residues could become enzyme-bound, and thus were surprised to find only a 3.8-fold difference in IC_{50} between MTX-PLL8 and MTX itself. It is possible that the polypeptide chain makes an additional contribution to dihydrofolate reductase binding over and above that of the MTX moiety. Another possibility that is difficult to completely rule out is that some free MTX might be present. We consider this unlikely because control experiments were performed in which it was shown that, when mixtures of poly(L-lysine) and nonconjugated MTX were dialyzed exhaustively in the same way as the conjugates, there was no detectable free MTX. In a recent paper (12), we reported that MTX derivatives with 1 to 3 lysines linked to the γ-position

TABLE 2

Inhibition of dihydrofolate reductase by MTX-poly(L-lysine) conjugates

The concentration (IC_{50}) of polymer-bound MTX required to inhibit enzyme activity by 50% was determined spectrophotometrically in 0.05 M Tris-chloride buffer, pH 7.2 or 8.5, as described in Materials and Methods. The M_r and found Lys/Glu ratio for each conjugate are given in Table 1. The numbers in parentheses at pH 7.2 are normalized relative to MTX (=1.0).

Compound	pH 7.2		pH 8.5	
	IC_{50}	$[IC_{50}]/[E]$	IC_{50}	$[IC_{50}]/[E]$
	μM		μM	
MTX-PLL6	0.45 (0.11)	5.0	0.24	12
MTX-PLL8	0.19 (0.26)	2.1	0.16	7.7
MTX	0.050 (1.0)	0.56	ND[a]	ND

[a] ND, not determined.

bind to L1210 dihydrofolate reductase with an IC_{50} of 0.09–0.14 μM, i.e., with as much as one-half the affinity of MTX. The data given here suggest that intact MTX-poly(L-lysine) could inhibit dihydrofolate reductase nearly as well as do the small MTX-oligolysine derivatives potentially formed in cells via lysosomal degradation.

The effect of pH on the inhibition of dihydrofolate reductase by MTX-PLL6 and MTX-PLL8 was also examined, with the aim of assessing how an alteration in net charge might influence binding of these α- and γ-linked conjugates to the enzyme. The $[IC_{50}]/[E]$ ratio, where $[E]$ is the enzyme concentration as determined from the residual activity in the absence of inhibitor, was used. This expression compensates for the fact that the enzyme has less reducing activity for dihydrofolate at pH 8.5 than at pH 7.5. As shown in Table 2, the $[IC_{50}]/[E]$ ratio increases for both MTX-PLL6 and MTX-PLL8 at the higher pH. However, the increase is 2.5-fold for the α-conjugate MTX-PLL6 but 4-fold for the γ-conjugate MTX-PLL8. Thus, it appears that, at least for this pair of conjugates with a comparable M_r and degree of substitution, the one with free α-carboxyl groups shows greater pH dependence in its binding to dihydrofolate reductase. Since the α-carboxyl in MTX is about 20 times more acidic than is the γ-carboxyl (23), our results may be explained on the basis that the net positive charge in the γ-conjugate MTX-PLL8, representing the balance of positive charge on polylysine ε-amino groups and negative charge on MTX α-carboxyls, is affected to a greater degree on lowering the pH from 8.5 to 7.5 than is the net charge in the α-conjugate MTX-PLL6.

The cytotoxicity of α- and γ-linked conjugates differing in size and degree of substitution was evaluated against H35 rat hepatoma cells and two MTX-resistant sublines, $H35R_{0.3}$ and $H35R_{10}$. The $H35R_{0.3}$ cells were shown in previous work (5) to be resistant by virtue of a defect in MTX active transport. Moreover, MTX-poly(L-lysine) prepared by carbodiimide coupling from poly(L-lysine) and unprotected MTX was found to be approximately as toxic to the $H35R_{0.3}$ cells as it was to the parental cells (5). The $H35R_{10}$ cells that were used in the present study are more highly resistant than the $H35R_{0.3}$ cells (Table 3), and are similar to the previously described $H35R_6$ cells, whose resistance is based on a combination of impaired transport and decreased dihydrofolate reductase content (5).

When α- and γ-conjugates of equal M_r and comparable degree of substitution were compared (MTX-PLL4 and MTX-PLL8 versus MTX-PLL6) against the parental H35 cells (Table 3), the γ-conjugate proved to be 5- to 10-fold more toxic, in qualitative agreement with the dihydrofolate reductase inhibition data (Table 2) and the common finding that γ-substituted MTX derivatives are more cytotoxic than their α-isomers (14–16). When γ-conjugates of differing M_r were compared (MTX-PLL4 and MTX-PLL8 versus MTX-PLL7), there was again a 10- to 20-fold difference in IC_{50}, indicating greater activity with the longer chain carrier. When γ-conjugates with the same M_r but different degree of substitution were compared, a 20-fold difference in favor of 10:1 substitution was seen with the shorter chain carrier (MTX-PLL2 versus MTX-PLL7). On the other hand, in the conjugates containing the longer chain carrier (MTX-PLL1 versus MTX-PLL4 and MTX-PLL8), the difference between 5:1 and 10:1 substitution was less than 2-fold, though the lower ratio still favored activity. The most active conjugate among the six tested was MTX-PLL1 (M_r = 8,000–30,000; Lys/Glu = 9.7), which had the same IC_{50} as MTX itself. The structure-activity correlations that emerge from these observations are as follows: (i) γ-linkage of MTX to poly(L-lysine) is preferred over α-linkage; (ii) increasing the degree of substitution (the "MTX load") decreases the activity of conjugates of comparable size, especially with a shorter chain carrier; (iii) when carrying a low MTX load, carriers of different size are equally effective, but with a high MTX load, the shorter chain carrier is less effective. In general, the difference in IC_{50} between α- and γ-conjugates was less than differences based on the M_r or the MTX load. The correlation of activity with degree of substitution may reflect the fact that the total positive charge per carrier molecule decreases as more lysine residues are MTX-linked. Indeed, each MTX molecule not only removes one positive charge from the carrier, but also adds a negative charge at physiologic pH in the form of a glutamate carboxyl. Our findings are consistent with the accepted view that positive charges are important to ensure adequate cellular uptake of a polymer.

In the assays against $H35R_{0.3}$ cells (Table 3), α- and γ-conjugates of equal M_r and Lys/Glu ratio (MTX-PLL4 and MTX-PLL8 versus MTX-PLL6) again showed a 10-fold difference in favor of γ-conjugation. When 5:1 γ-conjugates of different M_r were compared (MTX-PLL4 and MTX-PLL8 versus MTX-PLL7), there was greater toxicity with the longer chain conjugates, while in 10:1 γ-conjugates (MTX-PLL1 versus MTX-PLL2) there

TABLE 3

Effect of MTX-poly(L-lysine) conjugates on the growth of MTX-sensitive and MTX-resistant rat hepatoma cells in culture

The parental H35 cells and the resistant $H35R_{0.3}$ subline have been described previously (5); the $H35R_{10}$ cells are very similar to the $H35R_6$ cells described in the same paper. Resistance to MTX in the $H35R_{0.3}$ cells is due to defective transport, whereas in $H35R_{10}$ cells there is both a transport defect and an increase in dihydrofolate reductase activity. Numbers in parentheses for the resistant cells are normalized relative to the parent cells.

Compound	Cells	IC_{50}		
		H35	$H35R_{0.3}$	$H35R_{10}$
			μM	
MTX-PLL1		0.011	0.21	
MTX-PLL2		0.010	0.25	
MTX-PLL4		0.014 (1.0)	0.012	
			0.038	
			0.055	
		Mean	0.035 (2.5)	
MTX-PLL6		0.11	0.35	
		0.14		
MTX-PLL7		0.20	4.4	
MTX-PLL8		0.019 (1.0)	0.032 (1.7)	3.0 (160)
MTX		0.012 (1.0)	1.6 (130)	50 (420)

was little dependence on carrier size. Thus, in $H35R_{0.3}$ cells as in H35 cells, carrier size appears to be more important at the higher drug load. However, when the effect of drug load was evaluated in MTX-resistant $H35R_{0.3}$ cells, the results were less consistent than in H35 cells. When γ-conjugates of equal M_r but different Lys/Glu ratio were compared (MTX-PLL2 versus MTX-PLL7), there was 20-fold greater toxicity in favor of the lower degree of substitution at $M_r = 1{,}500$–$8{,}000$, whereas with the larger carrier (MTX-PLL4 and MTX-PLL8 versus MTX-PLL1) the opposite effect was observed, i.e., there was 8-fold greater potency at the higher drug load. The contrast between the latter results and those in H35 cells indicates that, in looking for structure-activity correlates among MTX-poly(L-lysine) conjugates, it is important to consider not only the nature of the conjugate but also the nature of the target cell.

A further interesting feature of MTX-PLL8 emerged when its activity was compared in $H35R_{0.3}$ and $H35R_{10}$ cells (Table 3). While there was virtually no cross-resistance between MTX and MTX-PLL8 in $H35R_{0.3}$ cells, whose resistance is based only on a transport defect, substantial cross-resistance developed in the $H35R_{10}$ cells, in which increased DHFR is present in addition to the transport defect. Thus, in this rat hepatoma system at least, MTX-PLL8 was able to overcome MTX resistance only up to a point. Since the other conjugates were not tested against the $H35R_{10}$ cells, we are unable to state whether the cross-resistance seen between MTX and MTX-PLL8 in the more highly MTX-resistant cells is general.

The effects of MTX-PLL6 and MTX-PLL8 on L1210 murine leukemia cells and of MTX-PLL6 on two MTX-resistant sublines, L1210/R71 and L1210/R81, in suspension culture were examined (Table 4) in order to confirm that the biological activity of the conjugates was not confined to hepatoma cells. The L1210/R81 cells have been described previously (19) and were 110,000-fold resistant to MTX as a result of a 35-fold increase in dihydrofolate reductase activity combined with virtually total absence of MTX active transport. The L1210/R71 cells (24) were 9,500-fold resistant and showed an 88-fold elevation in dihydrofolate reductase activity, i.e., 2.5 times as much enzyme as the L1210/R81 cells. As indicated in Table 4, MTX-PLL8 was 17-fold less active than MTX against the parental L1210 cells, and MTX-PLL6 was 38-fold less active. Thus, the γ-linked conjugate was again the more toxic one, though its potency was less impressive in this system, relative to MTX, than against hepatoma cells. Perhaps this is due to the shorter drug exposure time (48 versus 96 hr) in the L1210 assay, which allows less time for the conjugate to be taken up. Alternatively, it is possible that lysosomal activity is greater in hepatoma cells than in lymphoid cells. Other workers have noted that MTX-poly(L-lysine) conjugates are not equally toxic to all cell types (4). Of greater interest than the results against parental L1210 cells, however, were those against the L1210/R71 and L1210/R81 sublines, against which even MTX-PLL6, the less active of the two conjugates against the parental line, showed activity equal to, or greater than, that of MTX. In the more striking instance (L1210/R81 cells), there was approximately a 30-fold difference in favor of the conjugate. These results confirm that MTX-poly(L-lysine) conjugates can at least partially overcome MTX resistance even when the latter stems from a combination of impaired transport and dihydrofolate reductase overproduction. It may be noted, however, that this need not necessarily apply in all instances, as illustrated by the fact that $H35R_{10}$ cells, which are 4,000-fold resistant to MTX, display considerably more cross-resistance than do the L1210/R71 cells, which are 9,500-fold resistant.

In vivo antitumor testing of the MTX-poly(L-lysine) conjugates was carried out in L1210 leukemic mice and the results are shown in Table 5. Unlike the experiments of Chu and Howell (11), in which MTX-poly(L-lysine) with $M_r = 40{,}000$–$60{,}000$ was used and treatment was given intraperitoneally or intrapleurally to mice with intrapleural tumor, our assays involved the intraperitoneal route for both tumor implantation and drug treatment. As in the earlier study (11), a single dose of conjugate was administered, on the day following inoculation with 10^5 L1210 cells. Several structure-activity features emerge from the results: (i) the shorter chain γ-conjugates MTX-PLL2 and MTX-PLL7, regardless of the degree of substitution (Lys/Glu ratio), are inactive (<25% ILS) at nontoxic doses; (ii) the longer chain γ-conjugate MTX-PLL1 with a low degree of substitution (Lys/Glu of about 10:1) is likewise inactive; (iii) the longer chain γ-conjugates MTX-PLL4 and MTX-PLL8 with a higher degree of substitution (Lys/Glu of about 5:1) show moderate activity (25–75% ILS) at MTX-equivalent doses of 10–20 mg/kg; (iv) MTX alone, given as a single dose, was about as active as MTX-PLL4 and MTX-PLL8 but required a dose several times higher (e.g., 150 versus 16.4 mg/kg for a 75% ILS) to achieve a comparable therapeutic effect; (v) the α-conjugate MTX-PLL6 was inactive at a nontoxic dose; (vi) a noncovalently linked combination of MTX and $M_r = 8{,}000$–$30{,}000$ poly(L-lysine) equivalent to MTX-PLL4 and MTX-PLL8 was inactive; and (vii) toxicity was evident with nearly all the conjugates and the toxic dose varied with both the M_r and the degree of substitution. In the

TABLE 4

Effect of MTX-poly(L-lysine) conjugates on the growth of MTX-sensitive and MTX-resistant L1210 murine leukemia cells in culture

The culture conditions for L1210 and L1210/R81 cells have been described previously (19). The L1210/R81 cells are MTX-resistant by virtue of a combination of a 37-fold increase in dihydrofolate reductase activity and a nearly total absence of MTX active transport. The L1210/R71 subline, which is less resistant, has 88-fold increased dihydrofolate reductase activity but normal MTX transport. Numbers given in parentheses for each cell line are normalized relative to MTX (=1.0).

Compound	Cells	IC_{50}		
		L1210	L1210/R71	L1210/R81
			μM	
MTX-PLL6		0.075 (38)	16.6 (0.87)	7.5 (0.034)
MTX-PLL8		0.034 (17)	ND[a]	ND
MTX		0.002 (1.0)	19.0 (1.0)	220 (1.0)

[a] ND, not determined.

TABLE 5

Antitumor activity of MTX-poly(L-lysine) conjugates against L1210 leukemia in mice

Groups of five male B6D2F$_1$J mice were inoculated intraperitoneally with 10^5 L1210 cells on day 0, and drugs were injected intraperitoneally on day 1 or on days 1, 5, and 8 in sterile water. The MTX-equivalent concentration in each injected sample was determined spectrophotometrically (see Materials and Methods). In the experiment in which MTX and poly(L-lysine) were given as a noncovalently linked mixture, the MTX was dissolved in sterile water at the desired final concentration and poly(L-lysine), M_r-8,000–30,000, was added in an amount corresponding to a calculated Lys/Glu ratio of 5.0. Untreated controls had a mean survival of 8, 9, or 10 days depending on the experiment.

Compound	Schedule	MTX-equivalent dose	7-day weight change	Median survival	%ILS
		mg/kg	*%*	*days*	
MTX-PLL1	Day 1	7.5	+10	10/8	+25
		12.5	Toxic (3/5)		
MTX-PLL2	Day 1	13.1	+11	10/8	+25
			Toxic (3/5)		
MTX-PLL4	Day 1				
	Expt. 1	10.0	+7	11/8	+38
		16.2	+11	12/8	+50
	Expt. 2	10.9	+12	11/8	+38
		16.4	−8	14/8	+75
		22.4	Toxic (3/5)		
	Days 1, 5, 8	5.7	+7	12/9	+33
		11.4	+7	14/9	+56
		17.1	Toxic (5/5)		
MTX-PLL6	Day 1	17.6	+3	11/10	+10
		26.8	Toxic (4/5)		
MTX-PLL8	Day 1	10.4	+12	11/8	+38
		20.8	+2	12/9	+33
MTX + poly(L-lysine)	Day 1	20.4	+3	10/9	+11
MTX alone	Day 1	50	+18	11/8	+38
		100	+10	12/8	+50
		150	+8	14/8	+75
		200	0	14/8	+75

short chain as well as long chain conjugates, toxicity proved to be greater when the MTX load was low than when it was high. This suggests that the number of free protonated amino groups on the carrier contributes to toxicity just as it does to antitumor activity. In this regard it is of interest to note that poly(L-lysine) alone is known for its toxic effects (20), which are thought to be due to its polycationic nature and detergent-like properties. The reported toxicity of poly(L-lysine) is one of the reasons that prompted this study of high load conjugates in which the amount of poly(L-lysine) per molecule of MTX was deliberately chosen to be less than that used in other investigations.

In one experiment (Table 5), we also compared single dose administration versus a multiple dose regimen. When MTX-PLL4 was given on days 1, 5, and 8 following tumor implantation, there was a 56% ILS at an MTX-equivalent dose of 11.4 mg/kg, as compared with single-dose treatment, which produced a 50% ILS at 16.2 mg/kg. On both schedules, however, the dose-response curve was very steep and host toxicity was dose-limiting.

Overall, our results are consistent with those of Chu and Howell (11), who reported that mixed γ/α-conjugates of MTX and M_r = 3,000 or 40,000–60,000 poly(L-lysine) produced a 25–40% ILS in L1210 leukemic mice when both tumor and drug were given intrapleurally. The conjugates were inactive when the tumor was intrapleural and treatment was intraperitoneal, suggesting that these macromolecules have to be confined to the tumor compartment in order to be effective. This could offer an advantage for intracavitary chemotherapy. An MTX-equivalent dose of 1.4 mg/kg of the M_r = 40,000–60,000 conjugate led to about the same 30–35% ILS as a single dose of 95 mg/kg of MTX, while a 6.0 mg/kg dose of the M_r = 3,000 conjugate gave a 25% ILS. The conjugates used by Chu and Howell (11) thus appear to be more potent than ours, but this may be due to the difference in site of tumor implantation (intrapleural versus intraperitoneal) in the two studies. Another and probably more significant difference is in the MTX content of the conjugates. Chu and Howell stated that their M_r = 40,000–60,000 conjugate contained 83–103 mg of MTX per 1000 mg of poly(L-lysine). Assuming average values of 50,000 and 93 mg/1000 mg for the M_r and MTX content, respectively, it can be calculated that the Lys/Glu ratio in this conjugate is about 40:1, which would leave considerably more positive groups than are present in our conjugates and might well explain why the latter are less potent on a molar basis, and also less toxic than those of Chu and Howell.

In summary, this paper describes a method of regiospecific synthesis of α- and γ-conjugates of MTX to poly(L-lysine). The γ-conjugates appear to be more toxic than the α-conjugates *in vitro*, but activity seems to depend to a greater degree on the MTX load and carrier chainlength than on the position of MTX substitution. The intact conjugates are themselves moderately potent inhibitors of dihydrofolate reductase, suggesting that

their cell-killing effect need not necessarily require preliminary lysosomal breakdown to smaller MTX-oligolysine fragments or to free MTX. Low cross-resistance between MTX and MTX-poly(L-lysine) in MTX-resistant cells with a defect in MTX active transport is confirmed, and is shown to be more pronounced for γ- than α-conjugates. Lack of complete cross-resistance is also observed in cells that are MTX-resistant by virtue of a combination of impaired transport and increased dihydrofolate reductase activity. These results offer continued evidence of the potential therapeutic utility of MTX-homopolypeptide conjugates, while underscoring the need for novel approaches to dissociating antitumor activity from host toxicity, perhaps by making changes in the nature of the polypeptide carrier. Work is ongoing in our laboratory to address this problem.

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