Folate Analogues. 33. Synthesis of Folate and Antifolate Poly-γ-glutamates by [(9-Fluorenylmethoxy)oxy]carbonyl Chemistry and Biological Evaluation of Certain Methotrexate Polyglutamate Polylysine Conjugates as Inhibitors of the Growth of H35 Hepatoma Cells[1]

Ann Abraham,[†] M. G. Nair,*[,†] R. L. Kisliuk,[§] Y. Gaumont,[§] and J. Galivan*[,‡]

Drug Development Laboratory, University of South Alabama Cancer Center, and Department of Biochemistry, University of South Alabama, Mobile, Alabama 36688, Health Science Campus, Tufts University, Boston, Massachusetts 02111, and Wardsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201.
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Representative examples of folate and antifolate poly-γ-glutamyl metabolites were synthesized via the [(9-fluorenylmethoxy)oxy]carbonyl (Fmoc) chemistry using the KH polyamide resin. Polyglutamate yields were consistently better in all cases compared to the previous Merrifield method, and the crude products were obtained in greater than 85% purity. The symmetrical anhydride (7) derived from α-*tert*-butyl *N*-Fmoc-L-glutamate (6) was used for the initial coupling of the first glutamate residue to the KH resin and also for subsequent chain elongation. The α-*tert*-butyl protective groups were not labile under the conditions used for the cleavage of the finished peptide from the resin. A series of poly-γ-glutamyl metabolites of methotrexate (MTX) with a chain length ranging from two to five glutamyl residues were synthesized and coupled with poly(L-lysine) having an average molecular weight of 27 000 and 52 000. Each conjugate was tested for its ability to inhibit the growth of wild type (H35) and MTX transport resistant (H35R) strains of hepatoma cells in culture, the latter having a 100-fold reduced sensitivity to MTX. 4-Amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamylpoly(L-lysine) conjugate [MTX(G_2)-poly-L-Lys-52000] and MTX(G_4)-poly-L-Lys-52000 were among the most active (I_{50} = 8.0 and 10 nM against H35 cells) MTX–polylysines synthesized to date, and they were somewhat more inhibitory to the transport resistant cells. MTX(G_5)-poly-L-Lys-52000 was ~1000 times more effective than MTX(G_5)-poly-D-Lys-52000 in inhibiting the growth of H35R hepatoma cells in culture, indicating that internal cleavage of the γ-glutamate chain of the conjugate with subsequent release of MTX or shorter chain polyglutamates of MTX is unlikely to be an important determinant of MTX-polyglutamate polylysine cytotoxicity. The results indicate that MTX-polyglutamate poly(L-lysine) conjugates are taken up by the cells independently of MTX and probably via endocytosis.

The role of poly-γ-glutamyl metabolites of antifolates as determinants of antifolate cytotoxicity has been established.[2,3] Like folate coenyzmes, several antifolates including methotrexate (MTX) are metabolized to their poly-γ-glutamyl derivatives of varying chain length in tumor cells,[2-6] murine tissues,[7,8] and in humans.[8] Kisliuk[9,10] and co-workers found that polyglutamyl metabolites of MTX are potent inhibitors of thymidylate synthase (TS, EC 2.1.1.45) and dihydrofolate reductase (EC 1.5.1.3) derived from several species. The effects of various folate and MTX polyglutamates on human thymidylate synthase activity was investigated by Cheng et al.,[11] and they found that increasing the number of glutamyl residues increases their respective binding affinities toward the enzyme. The poly-γ-glutamyl metabolites of 10-deazaaminopterin and 10-ethyl-10-deaazaaminopterin were remarkably more inhibitory to human TS than the parent compounds.[12] The potent thymidylate synthase inhibitor N^{10}-propargyl-5,8-dideazafolate (PDDF, CB 3717) is also metabolized to the polyglutamyl derivatives,[13] and these compounds effectively inhibited TS derived from a number of sources.[14-16]

Homofolate polyglutamates and their reduced derivatives were found to be inhibitory toward glycinamide ribonucleotide formyl transferase,[17] which is a newly discovered target for the design of novel antitumor agents. Investigations by Jolivet and co-workers established that the conversion of MTX to poly-γ-glutamates in human MCF 7 and ZR 75 B cells was dose and time dependent.[5] The rate of disappearance of the metabolites from these cells decreased with increasing chain length. Preferential polyglutamylation of 4-amino antifolates in tumor versus normal proliferative tissues in tumor bearing animals has also been documented.[18] All currently available biochemical and pharmacological data indicate that polyglutamylation of antifolates enhances their cytotoxicity, and these metabolites interfere more effectively with

[†] University of South Alabama.
[§] Tufts University.
[‡] New York State Department of Health.

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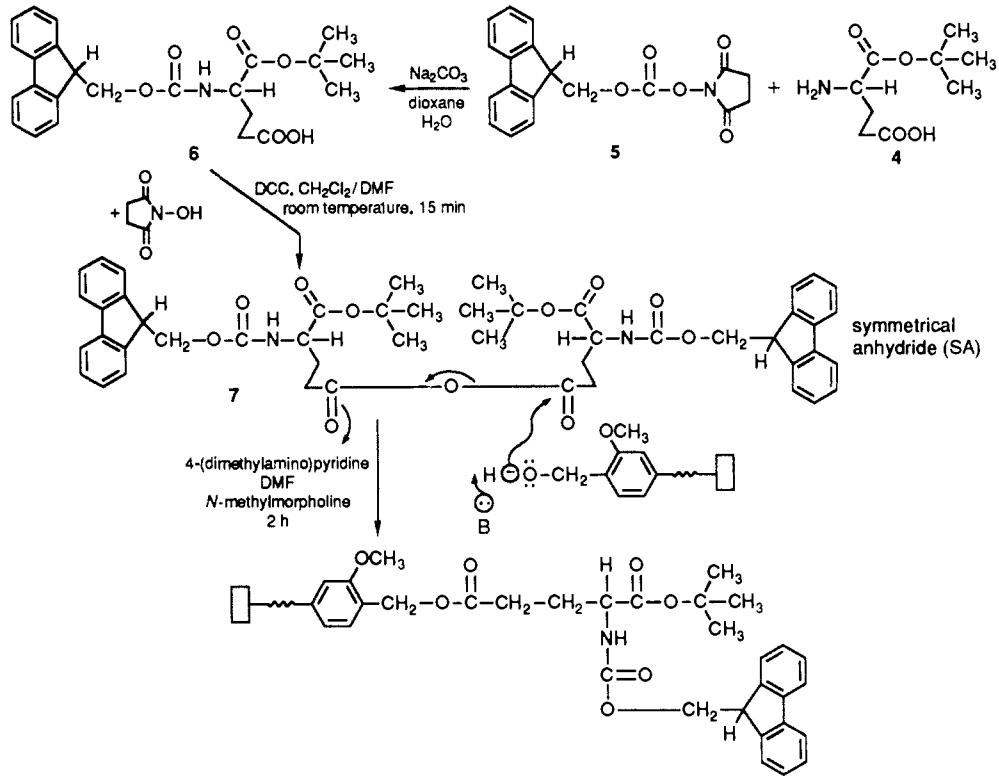
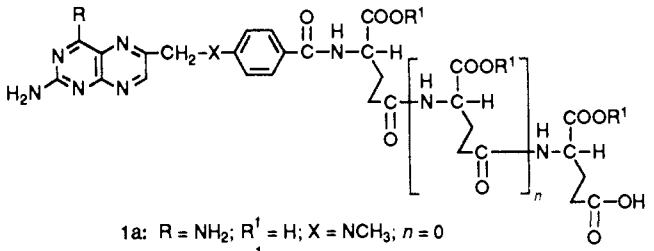
Scheme I. Synthesis of Fmoc-α-*tert*-butyl-L-glutamate and Coupling to Resin



tetrahydrofolate utilization at various stages of folate metabolism.[2,3] Antifolate polyglutamates of higher chain length are not transported efficiently to mammalian cell lines.[14,15] Therefore, currently available biological data on these compounds are derived mainly from in vitro enzyme inhibition studies. In order to assess the biochemical and pharmacological effects of these metabolites in vivo in relation to their cytotoxicity, it is desirable to develop methods for efficient delivery of these metabolites to target cells. We approached this difficult problem first by developing a general, convenient, and efficient method for the synthesis of antifolate polyglutamates, followed by an attempt to deliver these metabolites to target cells via conjugation with poly(L-lysine).[19] We selected MTX-polyglutamates (compounds **1a–d**) as examples of anti-



1a: R = NH_2; R^1 = H; X = NCH_3; n = 0
b: R = NH_2; R^1 = H; X = NCH_3; n = 1
c: R = NH_2; R^1 = H; X = NCH_3; n = 2
d: R = NH_2; R^1 = H; X = NCH_3; n = 3
e: R = NH_2; R^1 = $C(CH_3)_3$; X = NCH_3; n = 2
f: R = OH; R^1 = $C(CH_3)_3$; X = $NC(O)CF_3$; n = 1
g: R = OH; R^1 = H; X = NH; n = 1
h: R = OH; R^1 = $C(CH_3)_3$; X = $NC(O)CF_3$; n = 2
i: R = OH; R^1 = H; X = NH; n = 2
j: R = NH_2; R^1 = $C(CH_3)_3$; X = CH_2; n = 1
k: R = NH_2; R^1 = H; X = CH_2; n = 1

folate metabolites and poly(L-lysine) as a carrier because these compounds have been studied extensively in the past.[2,3,20,21] The existing synthetic methods for MTX-polyglutamates are laborious and yields are relatively low.[7] In order to prepare large amounts of polyglutamates of MTX that will be needed for future animal studies, it was essential to develop alternate synthetic methods.

In addition, antifolate polyglutamate intermediates of structural type **1e** will be needed during the second phase of this study for regiospecific conjugation of these metabolites to macromolecules. Taking these requirements into account, we report in this paper the development of a general and convenient synthesis of folate and antifolate polyglutamates (**1a–k**) by utilizing the [(9-fluorenylmethyl)oxy]carbonyl (Fmoc) chemistry, and the preliminary evaluation of various MTX poly-γ-glutamyl poly(L-lysine) conjugates as growth inhibitors of wild type H35 and transport-resistant H35R hepatoma cells in culture.

Chemistry

The synthetic strategy was based on our future requirements for a series of α-carboxyl-protected antifolate poly-γ-glutamates with a free γ-carboxyl group for their regiospecific conjugation to carrier molecules such as poly(L-lysine). Acid-labile *tert*-butyl ester was selected as a reversible protective group for the α-carboxyl groups of glutamic acid. Selective acid lability of the peptide resin linkage of KH polyamide resin in the presence of *tert*-butyl ester side chain protection has been previously established.[22] For reversible protection of the amino group of glutamic acid, the Fmoc functionality was selected due to its base lability.[23,24] The combination of Fmoc and

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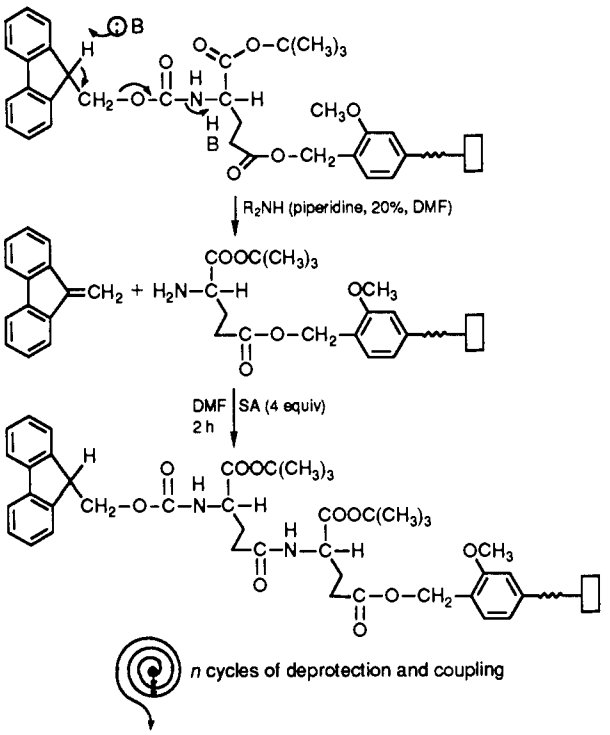
Scheme II

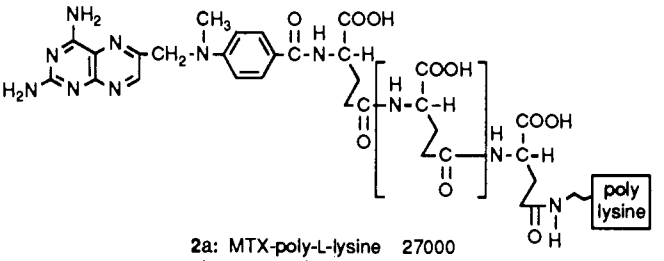


tert-butyl groups for the protection of the amino group and the α-carboxyl groups of glutamic acid, respectively, offered a viable alternative to the traditional *t*-Boc method[25,26] for the preparation of α-carboxyl-protected poly-γ-glutamates of folate and antifolates. In addition, the choice of side-chain protection by the *tert*-butyl group offered the advantage of preventing a potential γ ⇄ α bond migration[25,26] during base treatment of the γ-peptide derivatives.

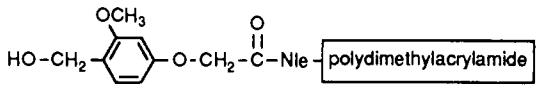
Commercially available α-*tert*-butyl L-glutamate (**4**) was reacted with *O*-Fmoc-succinimide (**5**) according to standard procedure (Scheme I) to obtain the central intermediate α-*tert*-butyl *N*-Fmoc-L-glutamate (**6**). Compound **6** was converted to the symmetrical anhydride **7** by the carbodiimide method.[24] An apparatus that was capable of recycling four different reagents through a column containing a stationary bed of resin was locally designed and was used for the synthesis (see the Experimental Section). The first glutamate moiety was coupled to the resin by recycling a solution of the symmetrical anhydride **7**, *N*-methylmorpholine, and a trace of 4-(dimethylamino)pyridine over a bed of KH resin through the apparatus until the UV absorption of the circulating reagents (2 h) remained unchanged as a function of time. Deprotection of the amino group was accomplished by treatment of the above resin with a solution of piperidine in DMF.[23,24] The deprotected, glutamate-bound resin ester was reacted with the symmetrical anhydride as described above for the introduction of the second glutamate moiety, and the cycles of deprotection and coupling were continued until the desired glutamate chain length (**8**) was reached. Activation of N^{10}-(trifluoroacetyl)pteroic acid and analogues of pteroic acid for coupling was carried out by converting them to their respective mixed anhydrides with isobutyl chloroformate as described previously from this laboratory.[7,15]

Solutions of these mixed anhydrides in DMF were allowed to recirculate over the deprotected resin to accomplish final coupling, and the resulting resin-bound α-carboxyl-protected poly-γ-glutamates of folate or antifolates (**9**) were cleaved from the resin with a 1% solution of TFA in CH_2Cl_2. Subsequent treatment of the cleaved product with a solution of HCl (<0.1 N) generated the desired polyglutamyl derivatives in overall yields ranging from 40 to 50%, which were judged to be 85–90% pure by HPLC analysis. Final purifications were accomplished by ion-exchange chromatography over DEAE-cellulose.[7,15] All polyglutamyl derivatives synthesized by this procedure were identical with authentic standards prepared previously in this laboratory by the *t*-Boc method. Independent evidence for the structure of all compounds was also obtained by FAB mass spectrometry.

Poly(L-lysines) with average molecular weights of 27 000 and 52 000 were selected as carriers of MTX-polyglutamates. The polyglutamates were linked to polylysines via peptide linkages between the carboxyl groups of MTX polyglutamate and the ε-amino groups of lysine residues, by the cardodiimide method.[19] The conjugates were purified by a combination of extensive dialysis and gel filtration. All conjugates were free of MTX-polyglutamates as judged by HPLC analyses. The exact structures of the conjugates were difficult to determine due to the possibility of the reaction of various carboxyl groups of the polyglutamate with the amino groups of poly(L-lysine). The conjugates thus obtained were quantitated for MTX content by UV absorption spectroscopy and for polylysine content on a weight basis. The numbers of MTX molecules bound to each poly(L-lysine) polymer as determined by this procedure are presented in Table III. All compounds were lyophilized to a fine powder and stored below 0 °C. No changes in their UV spectra were noted after a 5-month period.



2a: MTX-poly-L-lysine 27000
b: *n* = 0; poly-L-lysine 27000
c: *n* = 1; poly-L-lysine 27000
d: *n* = 2; poly-L-lysine 27000
e: *n* = 3; poly-L-lysine 27000
f: MTX-poly-L-lysine 52000
g: *n* = 0; poly-L-lysine 52000
h: *n* = 1; poly-L-lysine 52000
i: *n* = 2; poly-L-lysine 52000
j: *n* = 3; poly-L-lysine 52000
k: *n* = 3; poly-D-lysine 52000



KH resin

Biological Evaluation and Discussion

Although the Fmoc chemistry described here offers a convenient alternative for the synthesis of folate and antifolate polyglutamates, further refinements in the chemistry appear to be warranted. The crude products obtained by this method are much cleaner and devoid of appreciable amounts of shorter chain polypeptides even without the blockade of unreacted amino groups by acetylation in each step. The major difficulty appears to be the quantitative

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Table I

compound	molecular formula	MW calcd	[MH+]	λ_{max},[a] nm	% yield
4-amino-4-deoxy-N^{10}-methylpteroyltetraglutamate (1c)	$C_{35}H_{43}N_{11}O_{14}$	841	842	257, 303, 372	27.5
α-*tert*-butyl 4-amino-4-deoxy-N^{10}-methylpteroyltetraglutamate (1e)	$C_{51}H_{75}N_{11}O_{14}$	1065	1066		
α-*tert*-butyl N^{10}-(trifluoroacetyl)pteroyltriglutamate (1f)	$C_{43}H_{56}F_3N_9O_{13}$	963	964		
pteroyltriglutamate (1g)	$C_{29}H_{33}N_9O_{12}$	699	700	253.5, 282.7, 362.0	40
α-*tert*-butyl N^{10}-(trifluoroacetyl)pteroyltetraglutamate (1h)	$C_{52}H_{71}F_3N_{10}O_{16}$	1148	1149		
pteroyltetraglutamate (1i)	$C_{34}H_{40}N_{10}O_{15}$	828	829	254.2, 283.6, 362.2	46.9
α-*tert*-butyl 4-amino-4-deoxy-10-deazapteroyltriglutamate (1j)	$C_{42}H_{59}N_9O_{11}$	865	866		
4-amino-4-deoxy-10-deazapteroyltriglutamate (1k)	$C_{30}H_{35}N_9O_{11}$	697	698	254.3, 368.6	51

[a] All UV spectra were determined in 0.1 N NaOH.

cleavage of the finished product from the resin with 1% TFA in CH_2Cl_2. Mass spectral evidence indicated that prolonged treatment of the resin with this reagent also resulted in appreciable deprotection of the α-carboxyl groups. However, deprotection of the α-carboxyl groups of the finished peptide did not take place during the first 60 min, during which time only about 80% of the product was cleaved from the resin. It should be emphasized that the peptide synthesizer used in this procedure was locally designed and that no provision was made to rigorously exclude moisture or air from the apparatus. This defect in the design of the apparatus may have contributed to the observed moderate yields of the product. Contrary to the Merrifield method of folate polyglutamate synthesis using *t*-Boc-glutamic acid α-benzyl ester, a suspected base induced potential $\gamma \rightleftarrows \alpha$ bond migration[25,26] during the final deprotection and cleavage of the product from the resin is impossible via the current procedures.[26] The identity of the folate and antifolate polyglutamates synthesized by the current procedure in all respects including HPLC with those prepared by our earlier methods conclusively established that there was no $\gamma \rightarrow \alpha$ bond migration during polyglutamate synthesis by the Merrifield method in which *t*-Boc-glutamic acid α-benzyl ester was used as the glutamate intermediate.

Extracellular hydrolysis of MTX-polyglutamate polylysine conjugates to free MTX or shorter chain MTX-polyglutamates by conjugase or nonspecific endopeptidases was a primary concern at the outset of this study. In order to avoid this ambiguity, parallel studies were performed in MTX-sensitive (H35) and MTX-resistant (H35R) hepatoma cell lines. It has been established previously that the MTX-resistant strain (H35R) of hepatoma cell is defective in the transport of MTX. Therefore, if extracellular hydrolysis of MTX-polyglutamate polylysine conjugates takes place, these compounds will be incapable of exhibiting cytotoxicity to H35R cell lines. On the other hand, if both H35 and H35R cell lines are equally sensitive to the drugs, one might conclude that these conjugates are taken up by these cell lines by mechanisms other than mediated transport such as endocytosis. A number of MTX-polyglutamates with a chain length ranging from two to five glutamate residues were coupled by the carbodiimide method to poly(L-lysines) with average molecular weights of 27 000 and 52 000. Methotrexate was also coupled to these polylysines and these two conjugates were used as standards. The inhibitory activities of all polylysine conjugates (M_r 52 000) toward human dihydrofolate reductase were determined in vitro. The results are summarized in Table II. As expected, the polylysine conjugates of MTX and MTX-polyglutamates were only weak inhibitors of this enzyme. All 10 conjugates were evaluated for their growth inhibition with use of the MTX-sensitive H35 and MTX resistant transport defective H35R cell lines under identical conditions. These data are summarized in Table III. The high molecular weight conjugates

Table II. Inhibition of Dihydrofolate Reductase by Methotrexate (MTX) Poly(L-lysine) Conjugates

compound[a]	IC_{50}, μM: *Lactobacillus casei*[b]	IC_{50}, μM: human[c]
MTX	0.015	0.033
MTX-poly-L-Lys (2f)	1.3	5.0
MTX(G_2)-poly-L-Lys (2g)	0.39	0.64
MTX(G_3)-poly-L-Lys (2h)	1.2	3.0
MTX(G_4)-poly-L-Lys (2i)	0.4	1.2
MTX(G_5)-poly-L-Lys (2j)	0.6	2.2

[a] The number in parentheses denotes the total number of glutamate residues in the molecule. [b] Assayed spectrophotometrically in a solution containing Tris-HCl (pH 7.4, 50 mM), Na_2EDTA (1 mM), NADPH (0.1 mM), and dihydrofolate (0.06 mM) at 30 °C (ref 10). [c] Assayed spectrophotometrically in a solution containing potassium phosphate (pH 7.0, 50 mM), NADPH (0.14 mM), dihydrofolate (0.1 mM) at 24 °C. Enzyme kindly provided by Dr. J. H. Freisheim (ref 29).

Table III[a]

compound	I_{50}, nM: H35	I_{50}, nM: H35R	MTX/polymer
MTX-poly-L-Lys-27000 (2a)	32.0	15.5	4.2
MTX(G_2)-poly-L-Lys-27000 (2b)	18.0	12.0	3.5
MTX(G_3)-poly-L-Lys-27000 (2c)	32.0	16.0	4.5
MTX(G_4)-poly-L-Lys-27000 (2d)	14.0	9.0	2.3
MTX(G_5)-poly-L-Lys-27000 (2e)	23.0	19.5	2.5
MTX-poly-L-Lys-52000 (2f)	18.5	12.0	10
MTX(G_2)-poly-L-Lys-52000 (2g)	8.0	8.0	10
MTX(G_3)-poly-L-Lys-52000 (2h)	19.5	14.5	11.3
MTX(G_4)-poly-L-Lys-52000 (2i)	10.0	7.5	4.7
MTX(G_5)-poly-L-Lys-52000 (2j)	18.0	22.5	5.9
MTX(G_5)-poly-D-Lys-52000 (2k)	1300 ± 90	2180 ± 230	
methotrexate (3)	10.0	906.0	

[a] Cells were plated at 2×10^5 in 4 mL swims S77 in a 60-mm culture dish. Cell growth conducted for 72 h at which time the cells were counted spectrophotometrically by the method of Finlay.[30] The results are the average of duplicate plates. Quadruplicate assays of individual random points with H35 and H35R cells had an average standard deviation of ±10%.

(M_r 52 000) were generally more active than conjugates with low molecular weights (M_r 27 000), indicating that the former conjugates are taken up more effectively by both cell lines. MTX(G_2)- and MTX(G_4)-Lys-52000 conjugates were among the most active (I_{50} = 8.0 and 10 nM) MTX–polylysines synthesized to date.[20] It is possible that release of intact polyglutamates from the conjugates would lead to greater cytotoxicity due to enhanced retention of these compounds[4,5] and possibly inhibition of other folate-dependent enzymes.[9,12] The observed results do not support that possibility since the even-chain polyglutamate conjugates appear to be more active than adjacent odd chain derivatives. These results were consistent with H35 and H35R cells, although the latter were generally somewhat more sensitive to inhibition by the conjugates.

The differential effect between monoglutamate and polyglutamate derivatives of MTX is most clearly observed

Scheme III

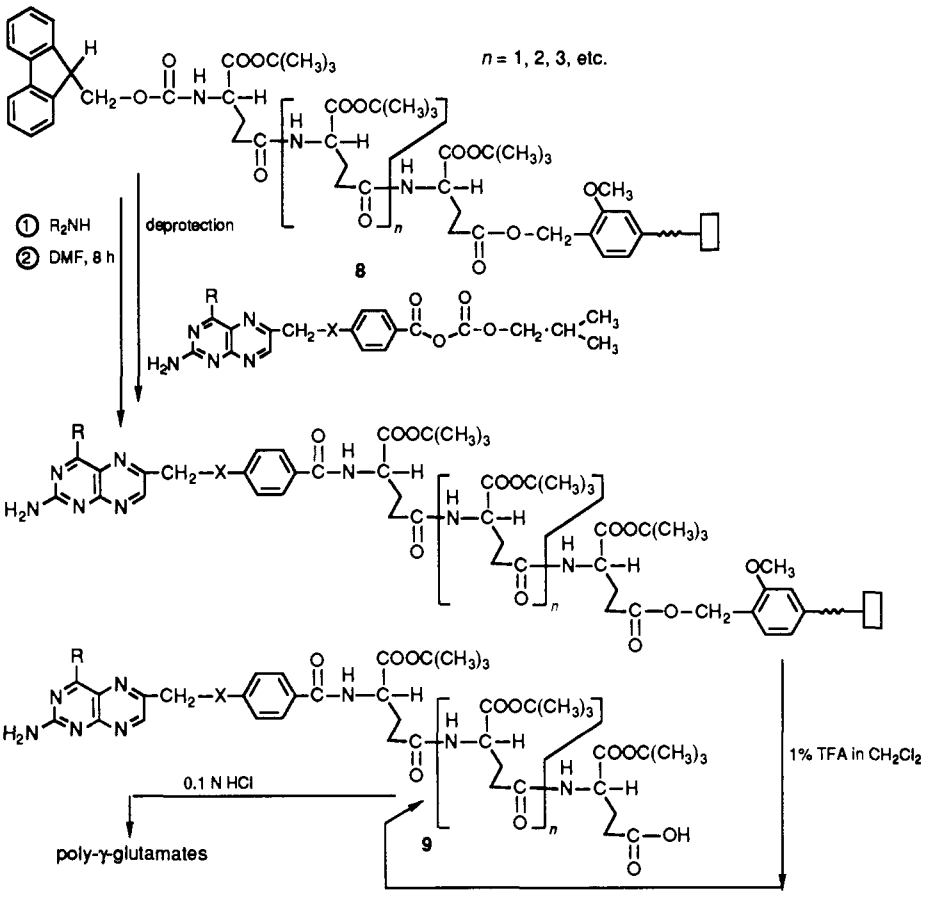

Table IV. Effect of a 6-h Pulse (h 24–30) during a 72-h Growth Cycle of H35 Cells

compd	glutamate residues	I_{50}, nM
2f	1	197
2g	2	92
2h	3	232
2i	4	132
2j	5	1550
MTX		75

when short term pulse exposure conditions are utilized.[28] Thus, when cells are exposed to forms of MTX that cannot be glutamylated (e.g., 4-fluoromethotrexate) throughout the growth cycle, it is only 10-fold less effective than MTX, whereas this difference is increased to 2300-fold following a 2-h pulse. To further determine if the longer polyglutamate chain length is a determinant of growth inhibition by the conjugates, the H35 cells were exposed to compounds **2f–j**. The results (Table IV), which are consistent with the data in Table III, show that the even chain length polyglutamate conjugates have the greatest activity. In addition, the longer chain length polyglutamates are less active. This is the opposite of what one would expect if similar amounts of intact MTX-polyglutamates were being released into the cell at constant rates from each conjugate. Thus, these results suggest that the glutamate chain length is an important parameter in determining qualitative and/or quantitative aspects of cellular internalization and cleavage to active MTX derivatives. The growth inhibitory effect of MTX(G_n) polylysine conjugates was completely reversed by (6*RS*)-5-formyltetrahydrofolate, establishing that these compounds are acting by an antifolate mechanism.

The possibility of an internal cleavage of the polyglutamate chain connecting methotrexate to polylysine was next investigated. 4-Amino-4-deoxy-N^{10}-methylpteroylpentaglutamate [MTX(G_5)] was coupled to poly(D-lysine) by the carbodiimide method to obtain MTX(G_5)-poly-D-Lys (M_r 52 000). The growth-inhibitory activity of this compound against both strains of H35 hepatoma cells was evaluated as above. It has been shown that poly(D-lysine) enters this cell line as rapidly as poly(L-lysine).[21] If internal cleavage of the polyglutamate chain took place with both MTX(G_5) polylysine conjugates, then the cytotoxicity of the poly(L-lysine) and the poly(D-lysine) conjugates might have been approximately equal. However, a striking difference in the toxicity between these conjugates was observed. MTX(G_5)-poly-L-Lys was 1000 times more active than the corresponding poly(D-lysine) conjugate. This result indicated that internal cleavage of the polyglutamate chain by lysosomal endopeptidase is not an important determinant of the cytotoxicity of these conjugates. The alternate explanation of sequential cleavage of the polylysine backbone followed by the release of MTX-polyglutamates that are further degraded to MTX metabolites of shorter glutamate chain length appears to be more consistent with the observed data. Since regiospecific conjugation of polyglutamates to polylysines was not accomplished in this work, the heterogeneity of the structures of the products resulting from the lysosomal degradation of the conjugates might explain the lack of correlation

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between the observed biological activity and the length of the polyglutamate chain. Our results, clearly established that MTX-polyglutamates can be delivered to H35 hepatoma cells that are resistant to MTX by virtue of a transport defect, and presuambly to a number of other tumor cells via conjugation with poly(L-lysine), and these conjugates can exhibit antifolate activity equal to or superior to that of methotrexate. We have also shown that antifolate polyglutamate poly(L-lysines) can survive extracellular hydrolysis and be taken up by cells via endocytosis. Comparative toxicity results with poly(L-lysine) and poly(D-lysine) conjugates indicated that internal cleavage of the poly-γ-glutamate chain intracellularly is not an important determinant of methotrexate polyglutamate poly(L-lysine) cytotoxicity. The synthesis of a series of α-*tert*-butyl antifolate poly-γ-glutamates with a free terminal γ-carboxyl group has now been accomplished. Methods are being developed to couple these derivatives with poly(L-lysine) followed by deprotection of the α-carboxyl groups. Evaluation of the biological activity of such regiospecific conjugates should provide valuable insight regarding the role of polyglutamylation of antifolates as a determinant of antifolate cytotoxicity.

Experimental Section

All solvents were analytical grade and were kept on a bed of 3A molecular sieve for 24 h prior to use. Isobutyl chloroformate and *N*-methylmorpholine were redistilled, and the former was stabilized with calcium carbonate. Ultraviolet spectra were run on a Bausch and Lomb Model 2000 spectrophotometer interfaced with a Commodore SP9000 superpet computer. All HPLC analysis were done with a Waters Model 600 A solvent delivery system equipped with a Model 481 variable-wavelength detector and Model 740 data module. N^{10}-(Trifluoroacetyl)pteroic acid was purchased from Aldrich Chemical Co., Inc. Anion-exchange chromatography was done on DEAE-cellulose in the chloride form. Mass spectra were obtained by fast atom bombardment on an VG-70-250 SEQ magnetic sector instrument. Poly(L-lysine) and poly(D-lysine) were obtained from Sigma Chemical Co. Methotrexate was supplied by the National Cancer Institute through the courtesy of Dr. J. A. R. Mead.

α-*tert*-Butyl *N*-Fmoc-L-glutamate (6). To a stirring solution of 0.305 g (1.5 mmol) of α-*tert*-butyl L-glutamate (**4**) in 20 mL of 10% Na_2CO_3 was added 0.506 g (1.5 mmol) of *O*-Fmoc-succinimide (**5**) dissolved in 10 mL of dioxane. After 20 h at 24 °C, the reaction mixture was diluted with 90 mL of cold water, the pH adjusted to 8.0 with 1 M Na_2CO_3, and the mixture extracted twice with 100-mL portions of ether. The ether layer was discarded. About 100 mL of EtOAc was added to the aqueous layer, and with stirring the pH was lowered to 2.0 with 1 N HCl. The EtOAc layer was separated, washed twice with 20-mL portions of distilled water, dried with anhydrous Na_2SO_4, and evaporated. The crude product (**6**) was further purified by column chromatography over silica gel by elution with 3% MeOH in CH_2Cl_2: yield 85%; mp 108 °C; MS [MH^+] 426.

General Procedure for the Synthesis of Antifolate Polyglutamates. (a) Preparation of α-*tert*-Butyl *N*-Fmoc-L-glutamic Anhydride (7). To a stirring solution of 340 mg (0.8 mmol) of **6** in 8 mL of CH_2Cl_2 was added 82.5 mg (0.4 mmol) of dicyclohexylcarbodiimide in 1 mL of CH_2Cl_2. After 15 min, the precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated under reduced pressure. The resulting residue was dissolved in 7 mL of DMF and was used immediately for the coupling reaction.

(b) Addition of the First Glutamate Residue to the Resin. An Omnifit model all-glass column with a fritted disk and Teflon endings through which up to four solvents could be circulated was used as the reaction chamber. The KH resin was packed in this chamber with the aid of DMF. The inlet and outlet of the column were connected by Teflon tubings to Teflon valves in such a way that four different solvents or reagents could be recirculated through the column from their respective reservoirs with the aid of a Model RP-G20 lab pump (Fluid Metering, Inc.). Each solvent or reagent was introduced to the reaction chamber by vacuum, created by the manual operation of a syringe that was connected to the chamber by Teflon tubing. After circulation of the reagent or solvent, for the desired period, it could be diverted to waste by opening of one of the valves. There was no provision to rigorously exclude air or moisture in the design of the apparatus. This apparatus was found to be useful for the preparation of small amounts of the desired polyglutamates.

Pepsyn KH resin with a capacity of 0.09 mequiv/g for esterification was obtained from Milligen Biosearch Division. For a typical synthesis, 1 g of the resin was used. The resin was suspended in the reaction chamber and washed three times with 20-mL portions of DMF, and to the washed resin was added the solution of anhydride **7** followed by the addition of 4.88 mg (0.04 mmol) of 4-(dimethylamino)pyridine in 1 mL of DMF and 0.045 mL (0.4 mmol) of *N*-methylmorpholine in 2 mL of DMF. The reagent mixture was recycled over the resin bed for 2 h and filtered. The resin was washed four times with 20-mL portions of DMF.

(c) Deprotection, Coupling, and Isolation of the Products. A solution of 20% piperidine in DMF (20 mL) was allowed to recirculate through the resin bed for 15 min for deprotection of the amino group. After filtration, the resin was washed five times with 20-mL portions of DMF and treated with the symmetrical anhydride **7** as described previously. Addition of 4-(dimethylamino)pyridine was omitted in this and all subsequent coupling steps. The process of deprotection and coupling was continued as described until the desired glutamate chain length was reached. After final deprotection, the resin-bound polyglutamate derivative was reacted with the activated pteroic acid analogue to effect the final coupling. The pteroic acid analogues were activated as the mixed anhydride with isobutyl chloroformate. For the synthesis of pteroylpolyglutamates, pteroic acid was used as the N^{10}-trifluoroacetyl derivative. However, for MTX-polyglutamates and 10-deazaaminopterin polyglutamates unprotected 4-amino-4-deoxy-N^{10}-methylpteroic acid and 4-amino-4-deoxy-10-deazapteroic acid were used for activation.

In a typical procedure, a solution of 0.15 mmol of the pteroic acid analogue in 10–15 mL of DMF was made in a stoppered cylinder by heating to ~80 °C. In the case of 4-amino-4-deoxy-N^{10}-methylpteroic acid, 2 mL of DMSO was added to DMF. The measuring cylinder containing the solution was placed in an ice bath, and after 10 min 0.021 mL (0.1875 mmol) of *N*-methylmorpholine was added. After 15 min at 0 °C, 0.016 mL (0.125 mmol) of freshly distilled isobutyl chloroformate was added to the cylinder and the reaction mixture was kept at this temperature for an additional 30 min. The cylinder was then removed from the ice bath and allowed to warm to 25 °C during the next 25 min. The mixed-anhydride solution was then introduced to the reaction chamber, recirculated for 17 h, and filtered. The resin was washed successively with three 20-mL portions of DMF and six 20-mL portions of CH_2Cl_2. The resin was transferred to a vessel, shaken with 25 mL of a solution of 1% TFA in CH_2Cl_2 for 1 h, filtered to a round-bottomed flask containing 10 mL of benzene, and immediately evaporated under reduced pressure at 25 °C. After evacuation under vacuum, a sample was withdrawn for mass spectral analysis. The residue was suspended in 25 mL of 0.1 N HCl, and 25 mL of THF was added dropwise with stirring until a clear solution was obtained. After 18 h at 25 °C, the pH of the solution was adjusted to 7.5, evaporated off THF, diluted to 50 mL with distilled water, and applied on a DEAE-cellulose (chloride form) column (5 × 20 cm). The column was washed with water and eluted with 0.1 N HCl. The column effluents were monitored by UV absorption at 255 nm, and the single UV-absorbing band that was eluted from the column was pooled and lyophilized to obtain the polyglutamate sample. Quantitation was carried out spectrophotometrically by using the known appropriate ε value for each compound.[28]

For the synthesis of pteroylpolyglutamates, N^{10}-(trifluoroacetyl)pteroic acid was used for final coupling. However, after cleavage of the product from the resin and removal of the *tert*-butyl groups, the product was treated with 0.1 N NaOH (3-fold molar excess) to deprotect N^{10}. The solution was then adjusted to pH 7.5 and applied to a DEAE column as described above. The molecular weights of selected α-carboxyl-protected polyglutamates and final products as determined by FAB mass spectrometry, their percentage yields, and UV spectral data are given in Table I.

General Method for the Preparation of MTX(G_n) Polylysine Conjugates. In a typical procedure 137 mg of poly(L-lysine) (M_r 52 000) was dissolved in 5 mL of 0.01 M potassium phosphate buffer containing 0.15 M NaCl at pH 7.2. A solution of 28 μmol/mL of MTX or the appropriate MTX(G_n) was prepared in the same manner as above, and 1 mL was added to the polylysine solution. Then 0.5 mL of a solution (200 mg/mL) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was added, and the reaction mixture was mixed and allowed to stand at 25 °C in the dark for 18 h. The reaction mixture was then applied on a column (1 × 25 cm) of Sephadex G 25, previously equilibrated with the buffer used for making the reagents. The column was eluted with 0.01 M potassium phosphate buffer, and the first yellow band eluted from the column was collected and dialyzed with a bag made of spectra/por (Fisher) membrane with a MW cutoff of 3500 for 4 days against 4 L of distilled water. The water was changed every 24 h. The dialyzed material was diluted with distilled water to a known volume, and methotrexate or MTX-polyglutamate content was estimated by UV spectroscopy with a standard curve. All conjugates exhibited the typical UV absorption of MTX or its corresponding polyglutamates, adducing independent evidence for the structural ingegrity of the 4-amino-4-deoxy-N^{10}-methylpteroylglutamate part of the molecule. The MTX(G_n) solutions thus prepared were lyophilized to a fine solid, and the weights were determined to estimate the number of MTX or its polyglutamates covalently bound to the polylysine molecule.

Aqueous solutions of all conjugates were analyzed by HPLC for the presence of free MTX or MTX-polyglutamates.[27] A mixture of polylysine and MTX or its polyglutamates on HPLC analyses monitored at 299 nm gave single-absorption peaks corresponding to MTX or its polyglutamates that were quantitatively eluted from the column.

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Registry No. **1a**, 41600-13-9; **1b**, 41600-14-0; **1c**, 73610-81-8; **1d**, 80801-54-3; **1e**, 124126-66-5; **1f**, 63948-16-3; **1g**, 89-38-3; **1h**, 124153-32-8; **1i**, 29701-38-0; **1j**, 124126-67-6; **1k**, 105099-94-3; **3**, 59-05-2; **4**, 45120-30-7; **5**, 82911-69-1; **6**, 84793-07-7; **7**, 124126-68-7; 4-amino-4-deoxy-N^{10}-methylpteroic acid, 19741-14-1; 4-amino-4-deoxy-10-deazapteroic acid, 33047-42-6; L-lysine homopolymer, 25104-18-1; poly(L-lysine), SRU, 38000-06-5; D-lysine homopolymer, 26853-89-4; poly(D-lysine), SRU, 26913-90-6; N^{10}-TFA-pteroic acid, 37793-53-6; dihydrofolate reductase, 9002-03-3.

Synthesis and Antiviral and Cytotoxic Activity of Iodohydrin and Iodomethoxy Derivatives of 5-Vinyl-2′-deoxyuridines, 2′-Fluoro-2′-deoxyuridine, and Uridine

Rakesh Kumar,[†] Lihua Xu,[†] Edward E. Knaus,[†] Leonard I. Wiebe,*[,†] Dorothy R. Tovell,[‡] D. Lorne Tyrrell,[‡] and Theresa M. Allen[§]

Faculty of Pharmacy and Pharmaceutical Sciences, Department of Infectious Diseases and Medical Microbiology, and Department of Pharmacology, University of Alberta, Edmonton, Canada T6G 2N8. *Received April 7, 1989*

A series of new 5-(1-hydroxy-2-iodoethyl)-2′-deoxyuridine and uridine compounds (**11**, **16**) was synthesized by the regiospecific addition of HOI to the vinyl substituent of 5-vinyl-2′-deoxyuridine (**10a**), 5-vinyl-2′-fluoro-2′-deoxyuridine (**10b**), 5-vinyluridine (**10c**), and (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (**4b**). Treatment of the iodohydrins **11a–c** with methanolic sulfuric acid afforded the corresponding 5-(1-methoxy-2-iodoethyl) derivatives (**12a–c**). In contrast, reaction of 5-(1-hydroxy-2-iodoethyl)-2′-deoxyuridine (**11a**) with sodium carbonate in methanol afforded a mixture of 5-(1-hydroxy-2-methoxyethyl)-2′-deoxyuridine (**13**) and 2,3-dihydro-3-hydroxy-5-(2′-deoxy-β-D-ribofuranosyl)-furano[2,3-*d*]pyrimidin-6(5*H*)-one (**14**). The most active compound, 5-(1-methoxy-2-iodoethyl)-2′-deoxyuridine (**12a**, ID_{50} = 0.1 μg/mL), which exhibited antiviral activity (HSV-1) 100-fold higher than that of the 5-(1-hydroxy-2-iodoethyl) analogue (**11a**), was less active than IVDU or acyclovir (ID_{50} = 0.01–0.1 μg/mL range). The C-5 substituent in the 2′-deoxyuridine series was a determinant of cytotoxic activity, as determined in the in vitro L1210 screen, where the relative activity order was $CH(OH)CHI_2$ (**16**) > $CH(OMe)CH_2I$ (**12a**) > $CH(OH)CH_2I$ (**11a**) ≃ $CH(OH)CH_2OMe$ (**13**). The 2′-substituent was also a determinant of cytotoxic activity in the 5-(1-hydroxy-2-iodoethyl) (**11a–c**) and 5-(1-methoxy-2-iodoethyl) series of compounds, where the relative activity profile was 2′-deoxyuridine > 2′-fluoro-2′-deoxyuridine > uridine (**11a** > **11b** ≥ **11c**; **12a** > **12b** > **12c**). The most active cytotoxic agent (**16**), possessing a 5-(1-hydroxy-2,2-diiodoethyl) substituent (ED_{50} = 0.77 μg/mL), exhibited an activity approaching that of melphalan (ED_{50} = 0.15 μg/mL). All compounds tested, except for **13** and **14**, exhibited high affinity (K_i = 0.035–0.22 mM range relative to deoxyuridine, K_i = 0.125) for the murine NBMPR-sensitive erythrocyte nucleoside transport system, suggesting that these iodohydrins are good permeants of cell membranes.

In an earlier study we reported the syntheses of the bromohydrin (**1a**) and chlorohydrin (**1b**) derivatives of 5-vinyl-2′-deoxyuridine (VDU, **2**).[1] 5-(1-Hydroxy-2-chloroethyl)-2′-deoxyuridine (**1b**) and 5-(1-methoxy-2-bromoethyl)-2′-deoxyuridine (**3a**) were moderately active (ID_{50} = 0.1–1.0 μg/mL, relative to acyclovir, ID_{50} = 0.01 μg/mL) against herpes simplex virus type 1 (HSV-1), but demonstrated only weak cytotoxicity against murine L1210 cells in vitro. This weak activity is a strong contrast to the potent in vitro anti-HSV-1 activity exhibited by BVDU (**4a**), IVDU (**4b**), and 5-(2-chloroethyl)-2′-deoxyuridine (CEDU, **5**).[2,3] BVDU and VDU are rapidly catabolized to the corresponding pyrimidine bases by the action of pyrimidine phosphorylase enzymes,[4,5] whereas CEDU is

[†] Faculty of Pharmacy and Pharmaceutical Sciences.
[‡] Department of Infectious Diseases and Medical Microbiology.
[§] Department of Pharmacology.

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