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IN VITRO METABOLISM OF 5,8-DIDEAZAFOLATES AND 5,8-DIDEAZAISOFOLATES BY MAMMALIAN FOLYLPOLY-γ-GLUTAMATE SYNTHETASE

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Abstract—The *in vitro* metabolism of a variety of 5,8-dideazafolate and 5,8-dideazaisofolate analogues by pig liver folylpolyglutamate synthetase and the specificity of the enzyme for some polyglutamate derivatives of these analogues have been investigated. All 4-oxo-quinazoline analogues were metabolized to long chain polyglutamate derivatives, primarily the pentaglutamate, whereas 4-amino-quinazolines were metabolized to a lesser extent, with the accumulation of di- and triglutamate derivatives. This pattern of metabolism was consistent with the large drop in V_{max}/K_m and V_{max} values for folylpolyglutamate synthetase observed with diglutamate derivatives of 4-aminofolate analogues. The extent of metabolism of the various analogues did not correlate with the relative substrate effectiveness of their parent monoglutamate derivatives. The 5-chloro and 5-methyl substitutions of quinazolines enhanced the addition of glutamate residues to 4-amino derivatives but markedly impaired the metabolism of 4-oxo derivatives.

The enzyme folylpolyglutamate synthetase [FPGS§; tetrahydrofolate:L-glutamate γ-ligase (ADP-forming), EC 6.3.2.17] catalyzes the conversion of folates and antifolate analogues possessing a terminal L-glutamate moiety to poly-γ-glutamyl derivatives [1,2]. Intracellular metabolism of folates to polyglutamates is required for their cellular retention and for the formation of more effective intracellular substrates for the enzymes of one carbon metabolism [1,2]. The metabolism of some antifolates to polyglutamate forms is believed to play an important role in their cytotoxic efficacy due to the increased retention of drug within the cell and also to the increased affinity of polyglutamate forms of some antifolates for target enzymes [1–8].

A number of studies have explored the structure–activity relationships of various folates and folate analogues for mammalian FPGS. Most of these studies have utilized monoglutamate forms of these compounds [9–14]. However, limited studies with polyglutamate derivatives have suggested that the specificity for diglutamate derivatives may be quite different from that displayed by monoglutamates [2,10,15,16]. Cellular retention of folates and antifolates requires their metabolism to triglutamate or longer chain length derivatives [17,18] and, consequently, specificities noted for monoglutamate derivatives may not necessarily indicate whether a particular compound will be metabolized to a form that is effectively retained by cells.

In the current study, the *in vitro* metabolism of a variety of 5,8-dideaza analogues of folate, isofolate, aminopterin, and isoaminopterin by purified pig liver FPGS was assessed to evaluate how structural modifications of these compounds affect their ability to be metabolized to long chain polyglutamyl derivatives.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]Glutamic acid (10 mCi/mmol) was obtained from Amersham. Folic acid and aminopterin were obtained from the Sigma Chemical Co. Methotrexate (MTX) was obtained from the Aldrich Chemical Co. Folic acid polyglutamates ($PteGlu_{2,3}$) and (6*S*)-$H_4PteGlu_{1-3}$ were synthesized, purified and characterized as described previously [19]. MTX polyglutamates were obtained from American Radiolabeled Chemicals. The methods for preparing the 5,8-dideaza-folate and aminopterin analogues have been described previously [14,20–24].

Folylpolyglutamate synthetase. Pig liver FPGS was purified to homogeneity as described previously [2]. The specific activity of the purified enzyme with (6*S*)-$H_4PteGlu$ as the substrate was 123 units/mg protein at saturating substrate concentrations. One unit equals 1 μmol $H_2PteGlu_2$ formed/hr.

Folylpolyglutamate synthetase assay. Assay mixtures for the measurement of kinetic parameters contained 100 mM Tris/50 mM glycine buffer, pH 9.75 (at 22°), analogue (various concentrations), L-[^{14}C]glutamate (2 mM; 2.5 mCi/mmol), ATP (1 mM), $MgCl_2$ (10 mM), KCl (20 mM), β-mercaptoethanol (100 mM), bovine serum albumin (50 μg), and enzyme in a total volume of 0.5 mL. The enzyme concentration was adjusted to ensure that less than 5% of the substrate was converted to product at the lowest substrate concentrations tested.

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§ Abbreviations: FPGS, folylpolyglutamate synthetase; PteGlu, pteroylmonoglutamic acid, folic acid; $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylpoly-γ-glutamate, n indicating the number of glutamate moieties; and MTX, methotrexate.

The reaction tubes were capped and incubated at 37° for 1 hr. The pH of the assay mixture was 9.0 at 37°. The reaction was stopped by the addition of ice-cold 30 mM β-mercaptoethanol (1.5 mL) containing 10 mM unlabeled glutamate, and the mixture was applied to a 2 × 0.7 cm DEAE cellulose (Whatman DE52) column equilibrated with 10 mM Tris buffer, pH 7.5, containing NaCl (80 mM) and β-mercaptoethanol (30 mM). The column was washed with the equilibration buffer (3 × 5 mL) to remove labeled glutamate and the folate or analogue product was eluted with 0.1 N HCl (3 mL).

Metabolism of folates and analogues. Assay-mixtures for the *in vitro* metabolism of folates and analogues (1 or 10 μM) were as described above except that all mixtures contained 20 mUnits of FPGS (6 nM) and 1 mM [^{14}C]glutamate (10 mCi/mmol) and the reaction was allowed to proceed for 20 hr. After washing the DE52 columns with 10 mM Tris buffer, pH 7.5/80 mM NaCl/30 mM β-mercaptoethanol to remove labeled glutamate, the columns were washed with 25 mM HCl (1 mL) and the products eluted with 25 mM HCl (3 mL).

Labeled folate products were cleaved to *p*-aminobenzoylpolyglutamates and separated, according to glutamate chain length, by HPLC on a strong anionic exchanger (Whatman SAX), as described previously [25]. Labeled analogue products were analyzed without prior cleavage. A 250 mM concentration of diammonium phosphate (0.1 mL) was added to an aliquot (1 mL) of the 25 mM HCl eluent, the mixture was filtered and 0.5 mL was applied directly to the SAX HPLC column. The column was eluted as described previously for aminobenzoylpolyglutamate derivatives [25].

RESULTS

Substrate activity of polyglutamate derivatives. Kinetic constants for the polyglutamate analogues tested are shown in Table 1 and are compared with values obtained with their respective monoglutamate derivatives. V_{max}/K_m, the pseudo first order rate constant, can be used to compare the relative substrate effectiveness of the different analogs at concentration ranges considerably below their K_m values. For the FPGS reaction, which is ordered Ter Ter, V_{max}/K_m is also a reflection of the on rate of the substrate for the enzyme [2,10].

The V_{max}/K_m values of the mono- and diglutamate of 10-methyl-5,8-dideazafolate were similar although V_{max} was decreased for the diglutamate analogue. The 5,8-dideazaisofolate series also demonstrated a progressive drop in V_{max} as its glutamate chain length was increased from one to three residues and a progressive drop in V_{max}/K_m, which was more pronounced with the triglutamate derivative. These trends with the 4-oxo analogues were similar to those seen with unsubstituted folate derivatives, which generally demonstrate reduced V_{max} values with increased glutamate chain length but similar V_{max}/K_m values for mono- and diglutamates.

Extension of the glutamate chain of dideaza-isoaminopterin caused a very large drop in V_{max} and V_{max}/K_m and the diglutamate was a very poor substrate. Similar effects were noted with 4-amino-folates such as MTX, the diglutamate of which was a very poor substrate and the triglutamate was almost inactive.

Metabolism of 5,8-dideazafolate analogues. Products of extended *in vitro* metabolism studies with a variety of 5,8-dideazafolates (1 μM) are shown in Table 2, which also shows the V_{max}/K_m values for the corresponding monoglutamate analogues. Polyglutamate standards were not available for most of the compounds tested. The identity of products eluted from the HPLC columns was determined by carrying out short-term incubation studies with higher concentrations of analogues. Di- and triglutamates accumulated under these conditions. In cases where standards were available, co-elution of labeled metabolites with unlabeled standards was observed. In addition, the elution positions of the polyglutamate forms of the different analogues could be predicted by the elution behaviour of their respective monoglutamate derivatives.

It has been previously shown that compounds that accumulate in extended incubation studies with FPGS are extremely poor substrates for the enzyme [26]. Under the described conditions, H_4PteGlu, the most effective folate substrate for the enzyme [10], was metabolized primarily to the hexaglutamate, the predominant folate derivative found *in vivo* in pig liver [19]. Most of the dideazafolate analogues were also converted to fairly long chain length polyglutamates, predominantly the pentaglutamate derivative, but the extent of metabolism did not correlate with their substrate efficacies as monoglutamates (Table 2). For example, in the dideazafolate series, **1**–**7**, the 10-propargyl derivative, **4**, was metabolized primarily to the pentaglutamate despite being a very poor monoglutamate substrate for FPGS and substitution of the 10-nitrogen of dideazafolate, **1**, by sulfur, **5**, markedly impaired substrate activity but resulted in a compound that was metabolized more extensively than any other analogue, with the formation of significant amounts of hexaglutamate. However, the 5-chloro substitution, **7**, which increases the substrate effectiveness by 25-fold and generates the most effective 4-oxo substrate known for this enzyme [9,14], markedly impaired the ability of the compound to be metabolized to long chain derivatives.

5,8-Dideazaisofolate, **8**, was not metabolized as extensively as its normal bridge homologue, **1**. In the isofolate series (**8**–**12**, Table 2), the 9-methyl substitution, **9**, had little effect on its metabolism, while substitution at the 5 position with a methyl group, **11**, or chlorine, **12**, markedly impaired metabolism despite these compounds being more effective monoglutamate substrates. However, the 5,9-dimethyl derivative, **10**, despite being a very poor substrate for FPGS, was metabolized more extensively than for other isofolate analogue tested.

Metabolism of 5,8-dideazaaminopterin analogues. Products of extended *in vitro* metabolism studies with a variety of 5,8-dideazaaminopterin analogues (1 μM) are shown in Table 3. MTX was metabolized primarily to di- and triglutamates, consistent with the poor substrate activities of these compounds (Table 1). Aminopterin is a better substrate for

Table 1. Kinetic constants of folate and 5,8-dideazapteroylpolyglutamate analogues for folylpolyglutamate synthetase

	K_m (μM)	V_{max} (μmol/hr/mg)	V_{max}/K_m (rel)*
(6*S*)-H_4PteGlu†‡	7.7	123	100
(6*S*)-H_4PteGlu$_2$	3.4	55	102
(6*S*)-H_4PteGlu$_3$	1.1	11	62
PteGlu†	93	75	5.1
PteGlu$_2$	62	74	7.4
PteGlu$_3$	119	41	2.2
10-Methyl-5,8-dideazafolate	49	96	12
10-Methyl-5,8-dideazafolate (Glu$_2$)	16	39	15
5,8-Dideazaisofolate	21	92	28
5,8-Dideazaisofolate (Glu$_2$)	16	30	12
5,8-Dideazaisofolate (Glu$_3$)	111	18	1.0
Methotrexate	142	63	2.8
Methotrexate (Glu$_2$)	118	13	0.8
Methotrexate (Glu$_3$)			<0.004
5,8-Dideazaisoaminopterin	44	101	14
5,8-Dideazaisoaminopterin (Glu$_2$)	112	7.0	0.4

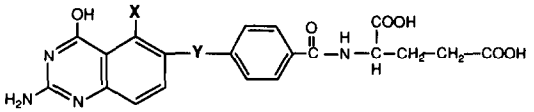
Assay mixtures, containing the indicated analogues, were as described in Materials and Methods.

* Relative to that obtained with (6*S*)-H_4PteGlu (equals 100).

† Values for folates are from Ref. 10.

‡ Glu$_n$ refers to the total number of glutamate moieties in the molecule.

Table 2. Metabolism of 5,8-dideaza analogues of folic acid and isofolic acid by folylpolyglutamate synthetase



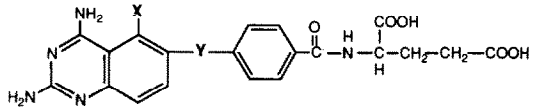
	X	Y	Distribution of products (%)* di	tri	tetra	penta	hexa	hepta	V_{max}/K_m†
1	H	CH_2NH	3	12	7	69	10	0	68
2	H	$CH_2N(CHO)$	0	22	62	16	0	0	19
3	H	$CH_2N(CH_3)$	3	8	21	65	4	0	12
4	H	$CH_2N(CH_2C{\equiv}CH)$	4	11	21	59	5	0	3.2
5	H	CH_2S	0	0	15	51	34	0	17
6	H	CH_2O	0	6	32	62	0	0	8.7
7	Cl	CH_2NH	0	3	71	26	0	0	1750
8	H	$NHCH_2$	7	7	40	44	3	0	28
9	H	$N(CH_3)CH_2$	10	10	29	48	3	0	39
10	CH_3	$N(CH_3)CH_2$	5	8	17	62	8	0	3.9
11	CH_3	$NHCH_2$	12	47	41	1	0	0	65
12	Cl	$NHCH_2$	29	25	41	5	0	0	102
		(6*S*)-H_4PteGlu	0	9	12	28	41	10	100
		PteGlu	12	36	40	13	0	0	5.1

Assay conditions, and the separation of products by HPLC, are described in Materials and Methods. Assay mixtures containing the indicated analogue (1 μM), L-[^{14}C]glutamate (1 mM), ATP (5 mM) and enzyme (20 mUnits) were incubated for 20 hr.

* Adjusted to account for increasing specific activity of product with increasing glutamate chain length.

† Relative to that obtained with (6*S*)-H_4PteGlu (equals 100). Values were reported earlier in Refs. 9 and 14.

Table 3. Metabolism of 5,8-dideaza analogues of aminopterin and isoaminopterin by folylpolyglutamate synthetase



	X	Y	Distribution of products (%)* di	tri	tetra	penta	hexa	hepta	V_{max}/K_m†
13	H	CH_2NH	84	16	0	0	0	0	86
14	H	$CH_2N(CHO)$	85	15	0	0	0	0	122
15	H	$CH_2N(CH_3)$	69	31	0	0	0	0	13
16	CH_3	CH_2NH	7	83	10	0	0	0	19
17	Cl	CH_2NH	14	49	31	6	0	0	2210
18	H	$NHCH_2$	88	13	0	0	0	0	14
19	H	$N(CHO)CH_2$	85	15	0	0	0	0	44
20	H	$N(CH_3)CH_2$	84	16	0	0	0	0	3.3
21	Cl	$NHCH_2$	15	60	23	1	0	0	92
		Aminopterin	71	30	0	0	0	0	32
		Methotrexate	33	64	3	0	0	0	2.8

Assay mixtures, containing the indicated analogues (1 μM), were as described in the legend to Table 2.

* Adjusted to account for increasing specific activity of product with increasing glutamate chain length.

† Relative to that obtained with (6*S*)-H_4PteGlu (equals 100). Values were reported earlier in Refs. 9 and 14.

FPGS than MTX but was metabolized to a lesser extent (Table 3), suggesting that its diglutamate derivative is a very poor substrate. The limited extent of metabolism of these 4-amino-folates was also mirrored in the metabolism of the 5,8-dideazaaminopterin and isoaminopterin analogues which, for the most part, were only converted to di- and triglutamates.

5,8-Dideazaaminopterin, **13**, was a more effective substrate for FPGS than aminopterin but was metabolized to a lesser extent. The 10-methyl substitution, **15**, decreased substrate activity but increased the extent of metabolism of the compound, as was observed with MTX, while the 10-formyl substitution, **14**, had little effect. Substitution with a methyl group, **16**, or chlorine, **17**, at the 5 position had variable effects on substrate activity but markedly improved the extent of metabolism of both compounds. 5-Chloro-dideazaaminopterin, **17**, is the best substrate known for FPGS [14] and was the most extensively metabolized of the 4-amino derivatives tested in these *in vitro* studies.

The isoaminopterin series, **18**–**21**, behaved similarly to their normal bridge homologues with the 5-chloro substitution, **21**, markedly increasing the extent of metabolism, although the 9-methyl substitution, **20**, had no effect.

Metabolites obtained after extended incubation using 10 μM analogues were similar to those shown in Tables 2 and 3 with a slightly shorter distribution of glutamate chain lengths with some of the analogues.

DISCUSSION

Previous studies have shown that the 4-amino substitution of pteroylmonoglutamate improves its substrate activity for pig liver FPGS, whereas this substitution impairs activity with the diglutamate [2]. The limited studies reported here with diglutamate quinazoline analogues show a similar effect. V_{max}/K_m values for the diglutamates of the two 4-oxoquinazolines tested were similar or slightly reduced compared to the values for their respective monoglutamate derivatives, whereas the 4-amino-quinazoline diglutamate tested had a greatly reduced V_{max}/K_m value compared to its monoglutamate form. Similar results have been reported for polyglutamates of 5,8-dideazaisofolate, **8**, using partially purified FPGS from HCT-8, a human colon carcinoma cell line. The triglutamate of **8** retains good substrate activity although the V_{max} is reduced [7]. Catalytic rates were lower with all the diglutamate derivatives but the drop was especially pronounced for the 4-amino derivative. A progressive decrease in catalytic rate with extension of the glutamate chain has been observed with pteroylpolyglutamates and has been suggested to indicate a decreased ability to position the terminal glutamate of the molecule in the active site as the glutamate chain is extended [2,10]. The K_m for glutamate was lowered for all the diglutamate analogues tested (data not shown), a phenomenon previously shown for pteroyldiglutamates [10], indicating that the reduction in V_{max} was not due to an increased requirement for glutamate.

The results obtained from the *in vitro* metabolism studies, which used a FPGS concentration that was in the physiological range, were consistent with the substrate specificities noted above. All 4-oxoquinazoline analogues, including those that are poor substrates, were metabolized to long glutamate chain length products, while 4-amino derivatives were metabolized primarily to di- and triglutamate products, which is similar to the pattern seen with 4-oxo- and 4-amino-folates [26]. The major exceptions were 5-chloro-dideazaaminopterin and 5-chloro-dideazaisoaminopterin, which were metabolized more extensively than other 4-amino-quinazolines. The increased substrate activity of these compounds for FPGS, which also appears to hold for polyglutamate forms of these analogues, may explain why they are more potent cytotoxic agents than other dideazaaminopterins and dideazaisoaminopterins for a variety of tumor cell lines, despite no apparent advantage in their ability to be transported into cells or to inhibit dihydrofolate reductase [21,27]. The 5-methyl substitution of dideazaaminopterin also improves its metabolism, despite a drop in substrate activity for FPGS, and this substitution has also been shown to improve the cytotoxicity of the compound [21,27].

The 5-chloro substitution of 4-oxo analogues also lowers the K_m and increases the substrate effectiveness of analogues for FPGS [9,14], but it dramatically impairs the ability of the compounds to be metabolized to longer chain length polyglutamate derivatives indicating that this substitution has a detrimental effect on the substrate activity of 4-oxo-analogue polyglutamate derivatives. Similarly, the 5-methyl substitution, **11**, had an adverse effect on the metabolism of 4-oxo compounds. These substitutions have been shown to impair the cytotoxic efficacy of 4-oxoquinazolines [21].

In vitro metabolism experiments of the type described in this study may suggest which metabolites of the various analogues, once transported into the cell, would accumulate *in vivo* due to the extremely poor substrate activity of particular polyglutamate metabolites for FPGS [26]. However, this model does not necessarily mimic *in vivo* conditions as the potential efflux of short chain length intermediates is ignored. The retention and concentration of these compounds within cells require their metabolism at least to the triglutamate derivative [2,17] which is dependent on the substrate effectiveness of the mono- and diglutamate forms of these drugs. With folates or 4-oxo-analogues that are effective mono- and diglutamyl substrates for FPGS, similar metabolites were found in these studies to those found in cells [19, 28]. With some substrates, such as MTX, short glutamate chain lengths are often found in cells [2,28], although in some cells longer chain length derivatives not predicted by *in vitro* metabolism studies are found. This phenomenon has been mimicked recently in model cells expressing a wide range of human FPGS activities. MTX accumulation and metabolism to polyglutamates by these cells were highly dependent on FPGS activity whereas folate accumulation and metabolism were unresponsive to a wide range of FPGS activities [17]. The poor substrate activities of diglutamates of 4-amino compounds for FPGS suggest that metabolism of the di- to the triglutamate is a major rate-limiting step in their accumulation by the cell. Dihydrofolate reductase is the major target enzyme of 2,4-diaminofolate analogues. While polyglutamylation of 4-amino compounds does not increase their affinity for this enzyme, it allows the accumulation of free drug in the cell. In the cells described above [17], MTX was more cytotoxic to cells expressing higher levels of FPGS (unpublished data).

The sensitivity of cells to antifolate agents that require metabolism to polyglutamates for effective inhibition of target enzymes and/or accumulation by the cell will be dependent on the substrate activity of mono- and diglutamates of the analogues for FPGS and, at least for poor FPGS substrates, the level of FPGS activity. Modifications of the molecule that improve the substrate activity of the diglutamate derivative should enhance drug accumulation and, consequently, cytotoxicity.

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