Drugs of the Future 2003, 28(10): 967-974

CCC: 0377-8282/2003

Review Article

Folylpolyglutamate synthetase as a target for therapeutic intervention

John J. McGuire[1*] and James K. Coward[2]

[1]Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA and
[2]Departments of Medicinal Chemistry and Chemistry, University of Michigan, Ann Arbor, MI USA

CONTENTS

Abstract

The antifolate methotrexate (MTX), which targets the folate-dependent enzyme dihydrofolate reductase (DHFR), has been used effectively in cancer chemotherapy for over 50 years. Extensive efforts have been mounted to develop DHFR inhibitors that are more selective and/or have a wider tumor range than MTX and to develop inhibitors of other folate-dependent enzymes, principally thymidylate synthase and the enzymes of *de novo* purine synthesis. To date, these efforts have met with limited success. Another potential pathway for exploitation is the synthesis of poly(γ-glutamyl) forms of reduced folates. Folylpolyglutamates are essential for folate-mediated one-carbon metabolism to function and hence are essential for cell survival. Thus, folylpolyglutamate synthetase (FPGS), which is responsible for synthesis of these metabolites, is suggested as a target for drug development. This article reviews the rationale for synthesis of FPGS inhibitors and the progress to date in developing potent, cell-permeable inhibitors of this critical enzyme.

Introduction

Methotrexate (MTX), a potent inhibitor of the folate-dependent enzyme dihydrofolate reductase (DHFR), is the only antifolate approved for wide clinical use. MTX has a number of desirable properties as a clinical agent. It has well-behaved pharmacokinetics, does not exhibit cumulative toxicity, has well-understood and manageable acute toxicities, and expected or unexpected acute toxicity can be reversed by the administration of leucovorin (a reduced folate) "rescue" (1). Although MTX is useful in a number of cancers (*e.g.*, childhood acute lymphocytic leukemia, head and neck squamous cell cancer) its clinical use is limited by the occurrence of natural resistance (limited tumor spectrum) and acquired resistance by a number of mechanisms (1). MTX has now been a part of the cancer treatment armamentarium for over 50 years. Despite extensive efforts during that time period to improve on its pharmacologic and biochemical properties, no other DHFR inhibitor has been proven superior to MTX in randomized clinical trials (2). Because of this shortcoming, other folate-dependent enzymes, especially thymidylate synthase and glycinamide ribonucleotide and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferases (enzymes in *de novo* purine synthesis) have received considerable attention in the past two decades as drug targets because of their role in providing dTTP, dATP and dGTP for DNA synthesis. Several agents derived in these efforts are still in clinical trials, but so far only raltitrexed (Tomudex™), an inhibitor of thymidylate synthase, has achieved any success (2). Ralitrexed is now approved in Europe, but not the U.S., for the treatment of colon cancer.

A potential target in folate metabolism that has not yet been exploited clinically is folylpolyglutamate synthetase (FPGS), the enzyme that is responsible for synthesis of the ubiquitous and essential poly(γ-glutamyl) folates, which are the functional forms of reduced folates in cells. This review will discuss the occurrence and function of folylpolyglutamates, their synthesis by FPGS and the progress that has been made to date in developing inhibitors of this enzyme.

Natural occurrence and function of folylpoly(γ-glutamates)

Folates are a family of essential human vitamins based on the structure of folic acid (PteGlu) that, when

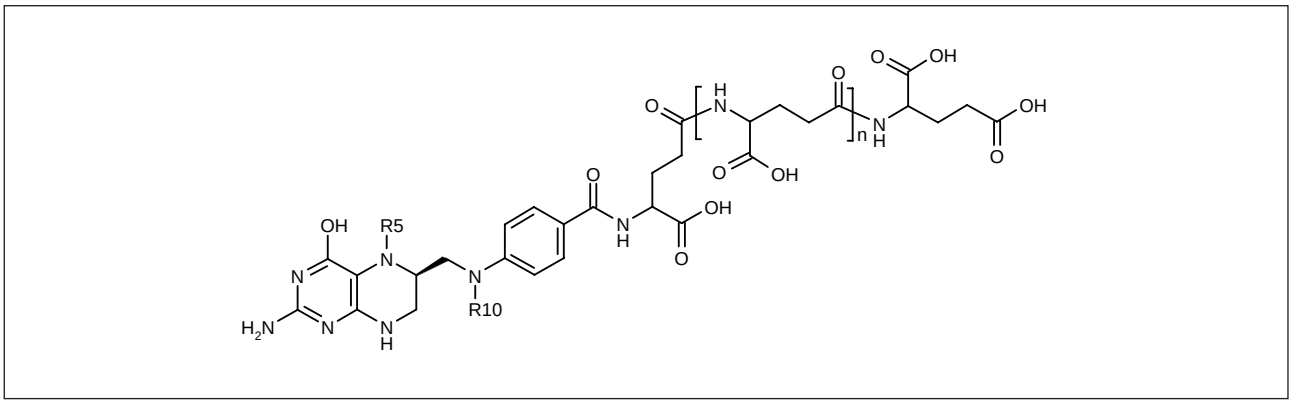


Fig. 1. General structure of 5,6,7,8-tetrahydrofolylpoly(γ-glutamates) and their one-carbon substituted forms. Only folates at the 5,6,7,8-tetrahydro reduction level are physiologically active. 5,6,7,8-Tetrahydrofolic acid contains one intrinsic glutamic acid; intracellularly, additional glutamates are added in γ-linkage. One-carbon substituents at the oxidation level of methanol, formaldehyde or formate can be present at N^5, N^{10} or bridging N^5 and N^{10}.

fully reduced (5,6,7,8-tetrahydro), serve as cofactors to transfer one-carbon moieties at the oxidation level of methanol, formaldehyde and formate (3). These one-carbon units are used in catabolic and anabolic reactions, including those in the synthesis of serine, glycine, methionine, purines and thymidylate (3). Across the phylogenetic spectrum, intracellular folates consist almost exclusively as poly(γ-glutamyl) metabolites (Fig. 1) (4). A distribution of polyglutamate lengths generally occurs that is cell lineage-specific; within a given cell lineage, the length distribution is generally identical for individual folate species containing different one-carbon substituents. In human cells, the usual range of lengths is 5-8 total glutamates with a total folate pool estimated to be 1-10 μM. Monoglutamyl folates (which are transport forms of the vitamin) must be present transiently in the intracellular space, but intracellular levels of monoglutamate are low or undetectable under physiological conditions. Since polyglutamylation is not coupled directly to transport (5), the fact that monoglutamates are not observed suggests that transport, rather than the level of synthesis itself (below), is rate-limiting to folylpolyglutamate synthesis.

Folylpolyglutamates perform a number of functions in folate/one-carbon metabolism (4), but two functions predominate. The first one is retention of folates at levels far in excess of the extracellular concentration (human plasma is ≈ 10 nM in folates (6)). Two mechanisms contribute to polyglutamate retention. First, both known folate transport systems, the reduced folate carrier (RFC) and the membrane-bound folate binding proteins (FBPs), transport monoglutamyl folates, but polyglutamyl folates are poorly transported (if at all), especially if more than two glutamates are present. Since folylpolyglutamates also cannot use these systems for efflux, and other known folate monoglutamate efflux systems (*e.g.*, MRP1 and MRP3 (7)) also do not efflux polyglutamates, mediated efflux does not occur. Second, because of the high negative charge on folylpolyglutamates, they cannot passively diffuse through cell membranes.

Serving as the kinetically preferred substrates (higher V_{max}/K_m) for most folate-dependent reactions is the second function of folypolyglutamates in folate/one-carbon metabolism. Studies with purified folate-dependent enzymes suggest that folylpolyglutamates are usually more catalytically efficient (up to 10^4-fold increased V_{max}/K_m) than folylmonoglutamates (4). The K_m is most often substantially lower, but in some cases V_{max} is increased. Catalytic efficiency may also be enhanced by preferential channeling of folylpolyglutamate between active sites in multifunctional folate-dependent enzymes.

Although retention is clearly essential at low physiological extracellular folate concentrations, increased catalytic efficiency is apparently also essential since a Chinese hamster ovary (CHO) cell line that transports folates normally, but cannot synthesize folylpolyglutamates, is unable to survive even in the presence of supraphysiological levels of reduced folates (8). Thus, at least one folate-dependent reaction is insufficiently active with a monoglutamate substrate to supply required levels of a critical metabolite.

Folylpolyglutamate synthetase (FPGS)

FPGS is the enzyme that synthesizes folate and antifolate poly(γ-glutamate) metabolites (reaction 1) of all lengths. Although there is only one human FPGS gene,

$$H_4PteGlu_n + \text{L-Glu} + \text{Mg-ATP} \rightarrow H_4PteGlu_n\text{–}\gamma\text{–Glu} + \text{Mg-ADP} + P_i \quad (1)$$

at least two classes of mRNA transcripts are synthesized, one of which contains a putative mitochondrial leader sequence. FPGS expression at the activity level (9) or activity and protein levels (10) has been observed in CHO and human leukemia cell cytosol and mitochondria, respectively, confirming that two isoforms are expressed. FPGS is capable of synthesizing *in vitro* all the folylpoly-

glutamate lengths observed in each subcellular compartment. For ligation of the first γ-glutamate, the enzyme mechanism is ordered ter-ter with substrates binding in the order Mg-ATP, folate, glutamate and products being released in the order Mg-ADP, folate-γ-glutamate, P_i (11).

Rationale for FPGS as a drug target

A critical role for folylpolyglutamates is demonstrated by the observations that all organisms synthesize them and that metabolically active intracellular reduced folate pools consist entirely of folylpolyglutamates (6). More importantly, the synthesis of folylpolyglutamates is essential for cell survival since the absence of folylpolyglutamate synthesis in CHO cells caused by mutational inactivation of FPGS leads to failure to accumulate folate pools and induces auxotrophy for glycine, a purine, thymidine and methionine (8, 12). If these metabolites are not supplied continuously, the auxotrophy is lethal. Thus, FPGS-specific inhibition should induce the FPGS-minus phenotype and be lethal through induction of folate deficiency and the consequent deficiencies in critical metabolites; cell death may occur through apoptosis (13, 14).

Other studies also support targeting FPGS (2). Early preclinical and clinical studies showed that folate deficiency induced nutritionally could decrease tumor growth rate without adversely affecting the host. Since one function of folylpolyglutamates is to retain folates intracellularly, drug-induced FPGS inhibition might similarly lead to selective folate deficiency in tumors. Clinically, it is very difficult to achieve and maintain folate deficiency by diet, thus a drug-based approach should be superior. Inhibition of FPGS expression in cell culture has been achieved by means of antisense constructs (15); this led to decreased dTMP synthesis and decreased cell growth, providing further support for FPGS as a target.

FPGS inhibition might be useful in overcoming one clinically identified (16) mechanism of acquired (and perhaps natural) antifolate resistance, decreased FPGS activity (17). Even if FPGS-deficient tumors have normal or near normal folate pools (because of the high catalytic efficiency of FPGS with natural folate substrates (18, 19)), their low FPGS levels should mean that less inhibitor will be required to totally eliminate FPGS activity and to induce lethal folate deficiency (above). Thus, FPGS-deficient antifolate-resistant tumors should be collaterally sensitive to FPGS-specific inhibitors (18).

Importantly for cancer chemotherapy, FPGS inhibition may be tumor selective through at least two mechanisms (2). First, as noted above, dietary folate deficiency selectively decreases tumor growth without adversely affecting the host. Secondly, tumor and normal cells (20) and even different normal tissues (21) may express different FPGS isozymes; thus, an FPGS inhibitor more specific for tumor FPGS might be developed.

Some unique issues may complicate the development of FPGS inhibitors. For example, all cell lines examined to date apparently express at least 10-fold higher levels of FPGS than are required to maintain the folylpolyglutamate pools essential for normal growth (22, 23). This means that potent FPGS inhibition will probably be required, since FPGS activity will need to be essentially fully suppressed. This may appear to be an unreachable goal. However, there is ample precedent for achieving therapeutically effective inhibition of enzyme targets displaying a similar requirement for a high degree of inhibition; examples even occur within folate metabolism. For example, from the studies of Goldman *et al.* (24), it is known that inhibition of DHFR by MTX must be > 98% complete before any effect on DNA synthesis occurs; to inhibit DNA synthesis, and thus inhibit growth, with this tight-binding inhibitor requires an even greater level of inhibition. Since this level of inhibition is manifestly achieved in the clinic by patients who respond to MTX, it indicates that the requirement for a high degree of inhibition is in itself not a disqualification for an enzyme as a chemotherapeutic target.

It is also worth emphasizing that sustained FPGS inhibition will be required to induce functional folate deficiency (meaning a decrease in the absolute level of the folylpolyglutamate pool and/or conversion to the “inactive” and potentially effluxable monoglutamates). During sustained FPGS blockade, a decrease in the absolute level of the folylpolyglutamate pool would occur as the preformed folylpolyglutamate pool is diluted out by cell division and/or as tetrahydrofolylpolyglutamates are inactivated by nonenzymatic C9-N10 cleavage (25); the latter process is believed to be slow. Conversion of folylpolyglutamates to monoglutamates could occur through the action of γ-glutamyl hydrolase (26). Functional folate deficiency would occur once the folate pool was reduced below a critical threshold level (27). This critical threshold in CCRF-CEM human leukemia cells in culture appears to be about 10% of the normal folate pool and should require 4 or more days of sustained FPGS inhibition to occur (27).

Another potential complication to the development of effective FPGS inhibitors has been noted recently. Human FPGS occurs as cytosolic (cFPGS) and mitochondrial (mFPGS) isoforms (above). This raises the question as to whether both FPGS isoforms must be inhibited to induce cell death. Inhibition of mFPGS could be difficult because mitochondria are impermeable to many (28), but not all (29), classes of antifolates. Data from FPGS-minus cells transfected to express both isoforms, only cFPGS, or only mFPGS (30) suggest that full auxotrophy is observed only when neither isoform is expressed, while cFPGS expression alone leads to glycine auxotrophy, and with mFPGS expression alone (as would occur with a potent cFPGS inhibitor) no auxotrophy is observed. Although the level of expression of each isoform in these studies may be a confounding factor, these data suggest that an ideal FPGS inhibitor must target both isoforms. The lack of auxotrophy when no cFPGS is expressed apparently occurs because folylpolyglutamates synthesized by mFPGS in the mitochondria are “released” into the cytosol. However, the levels of

folylpolyglutamates in the cytosol are significantly lower than normal when only mFPGS is expressed. If the requirement for end-products of folate-dependent pathways occurring exclusively in the cytosol (especially purines and thymidylate) is higher than can be sustained by folylpolyglutamates released into the cytosol from mitochondria, inhibition of cell growth by a cFPGS-specific inhibitor might still occur; this may be tumor-type dependent. A corollary practical issue is that methods used to study FPGS inhibitors must take these characteristics into account. For example, standard cell culture growth inhibition assays that use drug exposure periods of 48-120 h may underestimate or miss completely an anti-FPGS effect because of its delayed mechanism of action.

Aside from the quadruple auxotrophy cited above (8, 12), there are few data about the biological effects FPGS inhibition may have and how those effects could be distinguished from those of inhibitors of other folate-dependent enzymes. We have shown (J.J. McGuire, unpublished observation) that the delayed growth inhibition of cells depleted of folylpolyglutamates (by growth in folate-depleted medium) can be differentiated readily from the rapid growth inhibition induced by inhibitors of DHFR (MTX, trimetrexate), TMPS (raltitrexed, AG-331) and purine biosynthesis (DDATHF). The result with DDATHF is important because cytotoxicity of DDATHF may be delayed (31), but growth inhibition is not (J.J. McGuire, unpublished observation). However, demonstration that growth inhibition (even if delayed) truly results from FPGS blockade is not trivial. Multiple cellular and biochemical tests are required, all of which must give data consistent with FPGS inhibition.

Folate-based FPGS inhibitors

Potential folate-based FPGS inhibitors have been synthesized and characterized by several groups (32-34). Design of FPGS inhibitors has focused on folate-based structures, since analogs of glutamate or ATP, the other cosubstrates (reaction 1), would probably be too extensively metabolized or bound to other enzymes or protein receptors to be specific. In extensive structure-activity studies of folate-based structures conducted by several groups, structural changes in the pteroyl (heterocyclic) moiety critical for imparting FPGS inhibition have not yet been identified (35). To date, parallel changes in substrate and inhibitor activity occur, *i.e.*, poor FPGS substrates are poor FPGS inhibitors and good FPGS substrates cause apparent inhibition only by virtue of being good alternate substrates. This finding has led to use of changes in the pteroyl moiety to increase inhibitory potency and/or to control target specificity and to focus on changes in the integral glutamate moiety to impart FPGS inhibition (2). The use of different pterin heterocycles can lead to two classes of FPGS inhibitors: FPGS-specific and "dual-site" inhibitors that act against both FPGS and a second folate-dependent enzyme. The first class is of interest in answering the question of whether FPGS-specific inhibitors can be therapeutically effective. The second class of inhibitors may show enhanced therapeutic efficacy as a result of their two sites of action; they may have a unique self-potentiating effect (34) by causing a general decrease in cellular reduced folylpolyglutamate pools and thus decrease competition at the second site. In addition, the incidence of acquired resistance to dual-site FPGS inhibitors may be lower than that to inhibitors of either target alone. A large number of entities have been investigated as substitutes for the integral glutamate moiety (35). Almost all substitutes lead to loss of FPGS substrate activity, as is desired, but only a few impart significant FPGS inhibitory activity.

The first lead FPGS inhibitor was discovered by Shane and coworkers who found that substitution of ornithine (Orn) for glutamate in PteGlu (*i.e.*, PteOrn; Fig. 2) led to loss of substrate activity, but allowed potent FPGS inhibition (33). Orn is the optimal 2,ω-diaminoalkanoic acid for imparting inhibition (35). Based on this information, a number of Orn-containing analogs were synthesized and tested, including 5,8-dideazapteroyl-Orn (Fig. 2) by Piper and colleagues, which was the first specific, very potent folate analog inhibitor of isolated FPGS (36). However, the McGuire lab showed that this substitution leads to very poor cellular uptake because of the positive charge on the δ-amine of Orn at physiological

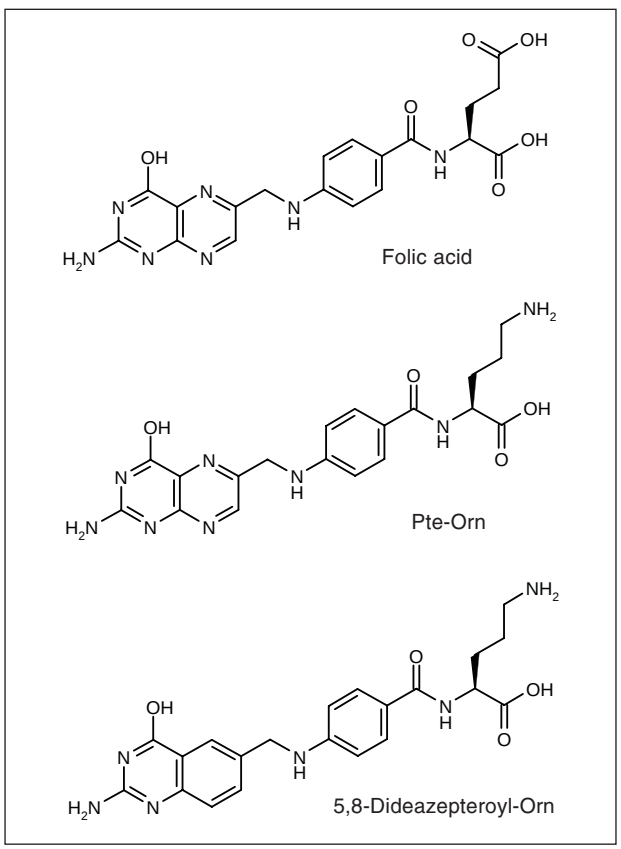
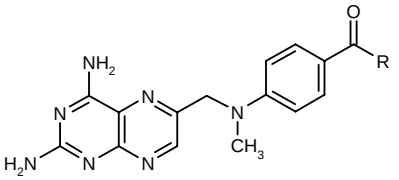
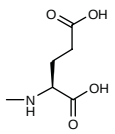
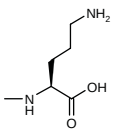
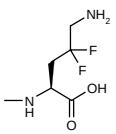
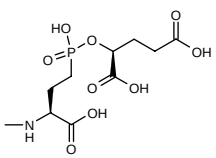
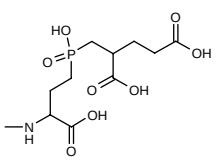


Fig. 2. Structures of some FPGS inhibitors containing ornithine substituted for glutamate.

Table I: Inhibitory potency against human FPGS of selected inhibitors based on the methotrexate (MTX) heterocycle.



R–

Name	Structure	IC_{50}, μM
Glu (MTX)		49 (K_m)
Orn		3.2
4,4-F_2Orn		>150
Glu-γ-{P}-O-glutarate		0.12
Glu-γ-{P}-CH_2-glutarate		0.008

pH (32, 36). A number of variations on Orn have been synthesized to attempt to increase uptake of this class of FPGS inhibitor. For example, reversible blocking of the δ-amine has been attempted (37; McGuire and Piper, unpublished data), but clear inhibition of FPGS in intact cells has not been demonstrated. In addition, 4,4-F_2Orn (δ-pKa, 6.9) was substituted for Orn (δ-pKa, 10.8) to decrease the δ-amine positive charge at physiological pH; transport was increased as hypothesized, but FPGS inhibitory activity was decreased dramatically (Table I) (38). The latter finding may implicate the protonated δ-amine of Orn as necessary for FPGS inhibition. Thus, changes to date have not succeeded in producing a potent Orn-containing FPGS inhibitor with uptake adequate to warrant further development. In this ornithine-containing class, potent FPGS inhibition and good transport may be mutually exclusive (38).

A second approach is to design FPGS inhibitors based on its enzyme mechanism. Studies from Coward and coworkers provided unambiguous proof that additions of at least the first two γ-glutamate residues by FPGS proceed through transient formation of an acyl phosphate at the γ-carboxyl of the growing polyglutamate chain (39). Concerted attack by the amino group of the incoming glutamate leads to transient formation of a tetrahedral intermediate at the γ-carbon (Fig. 3). Chemically stable (keto-phosphonate) analogs of the acyl phosphate were an initial synthetic target (40), but were bypassed when it was realized that the tetrahedral intermediate could be mimicked by stable phosphorous-containing structures (phosphonamidates, phosphonates and phosphinates) (41). Synthetic effort in this area has focused on the phosphonate and phosphinate mimics (Fig. 3), since acid-containing phosphonamidates tend to be unstable (42).

To establish the proof-of-principal that such structures can yield potent FPGS inhibitors, two phosphorous-containing analogs of the tetrahedral intermediate formed during the FPGS-catalyzed reaction were synthesized, namely, MTX-phosphonate and MTX-phosphinate

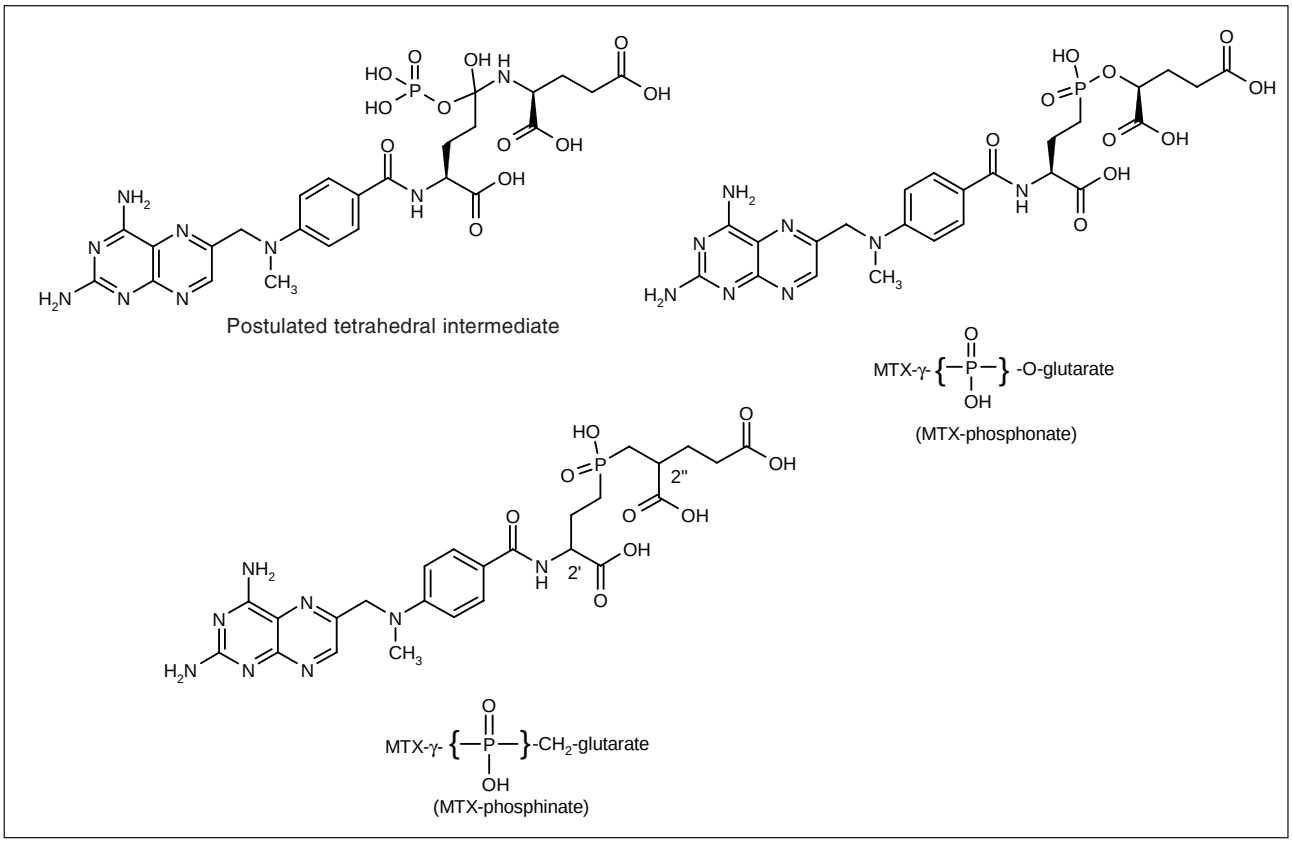


Fig. 3. Structures of the tetrahedral intermediate of the FPGS reaction and of MTX-phosphonate and MTX-phosphinate. Note that there are two chiral centers (2' and 2'') in MTX-phosphinate and the synthetic method yielded all 4 diastereomers.

(Fig. 3). The rationale for choosing the MTX heterocycle has been discussed in detail (43). The first analog to be synthesized and characterized was MTX-phosphonate (Fig. 3; 4-amino-10-methyl-pteroyl-L-Glu-γ-[ψ{P(O)(OH)-O}]glutarate) (44). (Use of ψ as a symbol for peptide surrogates is as described by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (45)). MTX-phosphonate is not an FPGS substrate, but is a very potent, competitive inhibitor of purified human FPGS with a K_i of 46 nM. The second phosphorous-containing analog is the -CH_2- variant of the previous compound, MTX-phosphinate (Fig. 3; 4-amino-10-methyl-pteroyl-L-Glu-γ-[ψ{P(O)(OH)-CH_2}]glutarate) that was synthesized initially as a mixture of four diastereomers (46). MTX-phosphinate is likewise not an FPGS substrate, but is 15-fold more potent (K_i = 3 nM) than MTX-phosphonate as a competitive inhibitor of purified human FPGS (43). Based on the known stereospecificity of human FPGS (4), it is likely that only the 2'*S*, 2''*S* MTX-phosphinate diastereomer is highly potent; thus, the actual K_i of the inhibitory species may be as low as 0.75 nM. A stereospecific synthesis of the 2'*S*, 2''*S* diastereomer is being elaborated to explore this possibility. Neither MTX-phosphonate nor MTX-phosphinate display time-dependent inhibition and preincubation of FPGS with the inhibitors does not increase the extent of inhibition. Thus, it appears that neither is a slow, tight-binding inhibitor that undergoes slow conversion to a tighter binding form (*e.g.*, via ATP-dependent phosphorylation). The substructure of MTX-phosphonate lacking the pterin (*i.e.*, the phosphonate moiety linked only to N-CH_3-*p*-aminobenzoate) is > 50-fold less inhibitory, underlining the significant contribution of the pterin heterocycle to inhibitor binding energy. It should be noted that neither MTX-phosphonate nor MTX-phosphinate is transported into intact CCRF-CEM human leukemia cells, presumably because of their negative charge and dipeptide structures (44). However, since the principle of inhibition of human FPGS by phosphorous-containing folate analogs is now established, the phosphinate pharmacophore is currently being incorporated into other folate analogs, to allow more potent and/or specific FPGS inhibition, and into phosphapeptide prodrugs, to increase uptake, in order to develop viable drug candidates.

Other classes of FPGS inhibitors

Some inhibitors that are structurally unrelated to any of the substrates of FPGS (reaction 1) have been characterized. These inhibitors include bromosulfophthalein

(47) and the polyanion suramin (27). Suramin is of particular interest because it is a very potent inhibitor of human FPGS and it also inhibits the enzyme that degrades the folylpolyglutamate chain, γ-glutamyl hydrolase (48). Although suramin is widely viewed as a growth factor inhibitor (49), its mechanism of action is not clearly defined and may include multiple important targets. Of note is the fact that suramin has a delayed effect on cell growth of similar duration (days) to that expected for induction of folate deficiency by FPGS inhibitors. Although of interest as inhibitors of purified FPGS and perhaps as lead compounds for further development, these compounds are not currently being pursued, because of their probable lack of specificity in cells.

Conclusions

Development of FPGS inhibitors remains an area of interest despite the fact that none of those described to date has advanced beyond early preclinical studies. This interest is based on the following: (a) the end-products of the enzyme-catalyzed reaction are essential for cell viability; (b) there is a strong rationale for suggesting that a therapeutically selective effect can be achieved by a specific FPGS inhibitor; (c) current clinical antifolates (MTX and raltitrexed) are well-tolerated agents with predictable and reversible acute toxicities; and (d) current antifolates do not exhibit cumulative toxicity and do not promote the occurrence of second cancers. With the next generation of mechanism-based FPGS inhibitors now under development, we should soon finally be able to test whether this interest has been justified.

Acknowledgements

This review is dedicated to Mr. William H. Haile on the occasion of his official retirement in appreciation of his valued service as a member of the staff of Roswell Park Cancer Institute and of his many contributions to the study of folate and antifolate metabolism and of FPGS. The authors wish to thank all of the members of their laboratories who participated in studies relevant to the development of FPGS inhibitors. Preparation of this review was supported by CA34500 (JJM), Cancer Center Support Grant CA16056 (RPCI) and CA28097 (JKC).

References

1. Bertino, J.R. *Ode to MTX.* J Clin Oncol 1993, 11: 5-14.

2. McGuire, J.J. *Anticancer antifolates: Current status and future directions.* Curr Pharm Des 2003, 9: 2593-613.

3. Shane, B. *Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism.* Vitam Horm 1989, 45: 263-335.

4. McGuire, J.J., Coward, J.K. *Pteroylpolyglutamates: Biosynthesis, degradation, and function.* In: Folates and Pterins Chemistry and Biochemistry of Folates. R.L. Blakley, S.J. Benkovic (Eds.). Wiley: New York 1984, 135-90.

5. Spinella, M.J., Brigle, K.E., Freemantle, S.J., Sierra, E.E., Goldman, I.D. *Comparison of methotrexate polyglutamylation in L1210 leukemia cells when influx is mediated by the reduced folate carrier or the folate receptor - Lack of evidence for influx route-specific effects.* Biochem Pharmacol 1996, 52: 703-12.

6. Cossins, E.A. *Folates in biological materials.* In: Folates and Pterins Chemistry and Biochemistry of Folates. R.L. Blakley, S.J. Benkovic (Eds.). Wiley: New York 1984, 1-59.

7. Zeng, H., Chen, Z.S., Belinsky, M.G., Rea, P.A., Kruh, G.D. *Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: Effect of polyglutamylation on MTX transport.* Cancer Res 2001, 61: 7225-32.

8. McBurney, M.W., Whitmore, G.F. *Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells.* Cell 1974, 2: 173-82.

9. Lin, B.F., Huang, R.F., Shane, B. *Regulation of folate and one-carbon metabolism in mammalian cells. III. Role of mitochondrial folylpoly-γ-glutamate synthetase.* J Biol Chem 1993, 268: 21674-9.

10. McGuire, J.J., Balinska, M., Russell, C.A. *Human cytosolic and mitochondrial folylpolyglutamate synthetase are electrophoretically distinct: Expression in antifolate-sensitive and -resistant human cell lines.* J Biol Chem 2000, 275: 13012-6.

11. Shane, B. *Pteroylpoly(γ-glutamate) synthesis by Corynebacterium species. Studies on the mechanism of folylpoly(γ-glutamate) synthetase.* J Biol Chem 1980, 255: 5663-7.

12. Taylor, R.T., Hanna, M.L. *Folate-dependent enzymes in cultured Chinese hamster ovary cells: Induction of 5-methyltetrahydrofolate homocysteine cobalamin methyltransferase by folate and methionine.* Arch Biochem Biophys 1975, 171: 507-20.

13. James, S.J., Basnakian, A.G., Miller, B.J. *In vitro folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells.* Cancer Res 1994, 54: 5075-80.

14. Koury, M.J., Horne, D.W. *Apoptosis mediates and thymidine prevents erythroblast destruction in folate deficiency anemia.* Proc Natl Acad Sci USA 1994, 91: 4067-71.

15. Liu, Y.N., Raghunathan, K., Hill, C. et al. *Effects of antisense-based folylpoly-γ-glutamate synthetase down-regulation on reduced folates and cellular proliferation in CCRF-CEM cells.* Biochem Pharmacol 1998, 55: 2031-7.

16. Rodenhuis, S., McGuire, J.J., Narayanan, R., Bertino, J.R. *Development of an assay system for the detection and classification of methotrexate resistance in fresh human leukemic cells.* Cancer Res 1986, 46: 6513-9.

17. McGuire, J.J. *Antifolate polyglutamylation in preclinical and clinical antifolate resistance.* In: Antifolate Drugs: Basic Research and Clinical Practice. A.L. Jackman (Ed.). Humana Press: Totowa, NJ 1999, 339-63.

18. McGuire, J.J., Heitzman, K.J., Haile, W.H., Russell, C.A., McCloskey, D.E., Piper, J.R. *Cross-resistance studies of folylpolyglutamate synthetase-deficient, methotrexate-resistant CCRF-CEM human leukemia sublines.* Leukemia 1993, 7: 1996-2003.

19. Lowe, S.W., Ruley, S.W., Jacks, T., Housman, D.E. *p53-dependent apoptosis modulates the cytotoxicity of anticancer agents.* Cell 1993, 74: 957-67.

20. Rumberger, B.G., Barrueco, J.R., Sirotnak, F.M. *Differing specificities for 4-aminofolate analogues of folylpolyglutamyl synthetase from tumors and proliferative intestinal epithelium of the mouse with significance for selective antitumor action.* Cancer Res 1990, 50: 4639-43.

21. Turner, F.B., Andreassi, J.L., Ferguson, J. et al. *Tissue-specific expression of functional isoforms of mouse folypoly-γ-glutamate synthetase: A basis for targeting folate antimetabolites.* Cancer Res 1999, 59: 6074-9.

22. McCloskey, D.E., McGuire, J.J., Russell, C.A. et al. *Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines.* J Biol Chem 1991, 266: 6181-7.

23. Shane, B., Lowe, K., Osborne, C., Kim, J., Lin, B. *Role of folylpolyglutamate synthetase in the regulation of folate and antifolate metabolism.* In: Chemistry and Biology of Pteridines 1989. H.-C. Curtius, S. Ghisla, N. Blau (Eds.). Walter de Gruyter: Berlin 1989, 891-5.

24. White, J.C., Goldman, I.D. *Mechanism of action of methotrexate. IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro.* Mol Pharmacol 1976, 12: 711-9.

25. Scott, J.M. *Catabolism of folates.* In: Folates and Pterins Chemistry and Biochemistry of Folates. R.L. Blakley, S.J. Benkovic (Eds.). Wiley: New York 1984, 307-27.

26. Galivan, J., Ryan, T.J., Chave, K., Rhee, M., Yao, R., Yin, D. *Glutamyl hydrolase: Pharmacological role and enzymatic characterization.* Pharmacol Ther 2000, 85: 207-15.

27. McGuire, J.J., Haile, W.H. *Potent inhibition of human folylpolyglutamate synthetase by suramin.* Arch Biochem Biophys 1996, 335: 139-44.

28. Horne, D.W., Holloway, R.S., Said, H.M. *Uptake of 5-formyltetrahydrofolate in isolated rat liver mitochondria is carrier-mediated.* J Nutr 1992, 122: 2204-9.

29. Kim, J.S., Shane, B. *Role of folylpolyglutamate synthetase in the metabolism and cytotoxicity of 5-deazaacyclotetrahydrofolate, an anti-purine drug.* J Biol Chem 1994, 269: 9714-20.

30. Qi, H., Atkinson, I., Xiao, S., Choi, Y.J., Tobimatsu, T., Shane, B. *Folylpoly-γ-glutamate synthetase: Generation of isozymes and the role in one-carbon metabolism and antifolate cytotoxicity.* Adv Enzyme Regul 1999, 39: 263-73.

31. Smith, S.G., Lehman, N.L., Moran, R.G. *Cytotoxicity of antifolate inhibitors of thymidylate and purine synthesis to WiDr colonic carcinoma cells.* Cancer Res 1993, 53: 5697-706.

32. McGuire, J.J., Hsieh, P., Franco, C.T., Piper, J.R. *Folylpolyglutamate synthetase inhibition and cytotoxic effects of methotrexate analogs containing 2,omega-diaminoalkanoic acids.* Biochem Pharmacol 1986, 35: 2607-13.

33. Cichowicz, D.J., Cook, J., George, S., Shane, B. *Hog liver folylpolyglutamate synthetase: Substrate specificity and regulation.* In: Proc Second Workshop Folyl- and Antifolylpolyglutamates, I.D. Goldman (Ed.). Praeger: New York 1985, 7-13.

34. Rosowsky, A., Freisheim, J.H., Moran, R.G. et al. *Methotrexate analogues. 26. Inhibition of dihydrofolate reductase and folylpolyglutamate synthetase activity and in vitro tumor cell growth by methotrexate and aminopterin analogues containing a basic amino acid side chain.* J Med Chem 1986, 29: 655-60.

35. Gangjee, A., Dubash, N.P., Zeng, Y., McGuire, J.J. *Recent advances in the chemistry and biology of folypoly-γ-glutamate synthetase substrates and inhibitors.* Curr Med Chem Anti-Cancer Agents 2002, 2: 331-55.

36. McGuire, J.J., Bolanowska, W.E., Piper, J.R. *Structural specificity of inhibition of human folylpolyglutamate synthetase by ornithine-containing folate analogs.* Biochem Pharmacol 1988, 37: 3931-9.

37. Rosowsky, A., Bader, H., Cucchi, C.A., Moran, R.G., Kohler, W., Freisheim, J.H. *Methotrexate analogues. 33. N^{δ}-Acyl-N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine derivatives: Synthesis and in vitro antitumor activity.* J Med Chem 1988, 31: 1332-7.

38. Tsukamoto, T., Haile, W.H., McGuire, J.J., Coward, J.K. *Synthesis and biological evaluation of N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-DL-4,4-difluoroornithine.* J Med Chem 1996, 39: 2536-40.

39. Banerjee, R.V., Shane, B., McGuire, J.J., Coward, J.K. *Dihydrofolate synthetase and folylpolyglutamate synthetase: Direct evidence for intervention of acyl phosphate intermediates.* Biochemistry 1988, 27: 9062-70.

40. Tang, K.-C., Coward, J.K. *Synthesis of acyl phosphonate analogues of biologically important acyl phosphates: N-(2-Amino-10-methylpteroyl)-5-amino-2-oxopentane phosphonic acid.* J Org Chem 1983, 48: 5001-6.

41. Malachowski, W.P., Coward, J.K. *The chemistry of phosphapeptides: Investigations on the synthesis of phosphonamidate, phosphonate, and phosphinate analogues of glutamyl-gamma-glutamate.* J Org Chem 1994, 59: 7625-34.

42. Chen, S.J., Lin, C.H., Kwon, D.S., Walsh, C.T., Coward, J.K. *Design, synthesis, and biochemical evaluation of phosphonate and phosphonamidate analogs of glutathionylspermidine as inhibitors of glutathionylspermidine synthetase/amidase from Escherichia coli.* J Med Chem 1997, 40: 3842-50.

43. McGuire, J.J., Haile, W.H., Valiaeva, N., Bartley, D., Guo, J., Coward, J.K. *Potent inhibition of human folylpolyglutamate synthetase by a phosphinic acid mimic of the tetrahedral reaction intermediate.* Biochem Pharmacol 2003, 65: 315-8.

44. Tsukamoto, T., Haile, W.H., McGuire, J.J., Coward, J.K. *Mechanism-based inhibition of human folylpolyglutamate synthetase: Design, synthesis, and biochemical characterization of a phosphapeptide mimic of the tetrahedral intermediate.* Arch Biochem Biophys 1998, 355: 109-18.

45. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). *Nomenclature and symbolism for amino acids and peptides. Recommendations 1983.* Eur J Biochem 1984, 138: 9-37.

46. Valiaeva, N., Bartley, D., Konno, T., Coward, J.K. *Phosphinic acid pseudopeptides analogous to glutamyl-γ-glutamate: Synthesis and coupling to pteroyl azides leads to potent inhibitors of folylpoly-γ-glutamate synthetase.* J Org Chem 2001, 66: 5146-54.

47. McGuire, J.J., Hsieh, P., Coward, J.K., Bertino, J.R. *In vitro methotrexate polyglutamate synthesis by rat liver folylpolyglutamate synthetase and its inhibition by bromosulfophthalein.* Adv Exp Biol Med 1983, 163: 199-214.

48. Rhee, M.S. *Inhibition of γ-glutamyl hydrolase by levamisole and suramin.* In: Chemistry and Biology of Pteridines and Folates. W. Pfleiderer, H. Rokos (Eds.). Blackwell Science: Berlin 1997, 467-70.

49. Stein, C.A. *Suramin: A novel antineoplastic agent with multiple potential mechanisms of action.* Cancer Res 1993, 53: 2239-48.