Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 315–318
Rapid Communication
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Potent inhibition of human folylpolyglutamate synthetase by a phosphinic acid mimic of the tetrahedral reaction intermediate

John J. McGuire[a,*], William H. Haile[a], Nadya Valiaeva[b], David Bartley[b], Jianxia Guo[a], James K. Coward[b]

[a]*Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA*
[b]*Departments of Medicinal Chemistry and Chemistry, University of Michigan, Ann Arbor, MI 48109, USA*

Accepted 14 November 2002

Abstract

A phosphorous-containing pseudopeptide folate analog (Valiaeva *et al.*, J Org Chem 2001;66:5146–54) was designed to mimic the tetrahedral intermediate formed in the ATP-dependent reaction catalyzed by folylpolyglutamate synthetase (FPGS). This analog, methotrexate-phosphinate (MTX-phosphinate; 4-amino-4-deoxy-10-methylpteroyl-L-Glu-γ-[Ψ{P(O)(OH)–CH_2}]glutarate), is a highly potent (K_{is}, 3.1 ± 0.5 nM), competitive inhibitor of recombinant human cytosolic FPGS. Within experimental limits, FPGS inhibition was not time-dependent, and preincubation of FPGS, inhibitor, and ATP did not potentiate the inhibition. These results suggest that slow phosphorylation to produce a more potent inhibitor form is not involved. MTX-phosphinate was not growth inhibitory to human CCRF-CEM leukemia cells at 1 μM (70-fold above the concentration of MTX giving 50% growth inhibition), probably because of poor transport. Because of its exceedingly high potency as an FPGS inhibitor, MTX-phosphinate represents a lead structure from which cell-permeable analogs may be developed to test the hypothesis that FPGS inhibition is therapeutically efficacious.
© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Folylpolyglutamate synthetase; Phosphapeptide inhibitor; Tetrahedral mimic; Antifolate; Phosphinate; Methotrexate

1. Introduction

FPGS, the enzyme that synthesizes folate and antifolate poly(γ-glutamate) metabolites (reaction 1), is a target for antifolate drug design.

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

Cellular folate pools occur exclusively as reduced folylpoly(γ-glutamates) ($H_4PteGlu_n$) [2]. $H_4PteGlu_n$ primarily function to: (a) enhance retention of intracellular folates; and (b) act as kinetically preferred intracellular 1-carbon carriers. $H_4PteGlu_n$ are essential since mutational inactivation of FPGS induces auxotrophy for glycine, a purine, thymidine, and methionine [2].

FPGS-specific inhibition should induce the $FPGS^-$ phenotype and be lethal. Importantly for cancer chemotherapy, FPGS inhibition may be tumor selective [reviewed in Ref. [3]) since: (a) dietary folate deficiency selectively decreases tumor growth; and (b) tumor and normal cells may express different FPGS isozymes. FPGS inhibition might be useful in overcoming one clinical antifolate resistance mechanism, decreased FPGS activity, since such tumors are collaterally sensitive to FPGS inhibition [4].

Several groups (e.g. [5–7]) have attempted to develop folate analog FPGS inhibitors. Modification of the Glu moiety may result in FPGS inhibition, whereas changes in

* Corresponding author. Tel.: +1-716-845-8249; fax: +1-716-845-8857.
E-mail address: John.McGuire@RoswellPark.edu (J.J. McGuire).

Abbreviations: AMT, aminopterin (4-amino-4-deoxy-pteroylglutamic acid); DHFR, dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3); EC_{50}, drug concentration effective in inhibiting growth of cells by 50% relative to solvent-treated control cultures; FPGS, folylpolyglutamate synthetase (tetrahydrofolate:L-glutamate γ-ligase (ADP-forming), EC 6.3.2.17); rhcFPGS, recombinant human cytosolic FPGS; Glu, glutamic acid; $H_4PteGlu_n$, tetrahydrofolate poly(γ-glutamates) containing *n* total glutamate residues; IC_{50}, drug concentration effective in inhibiting enzyme activity by 50% relative to a solvent-containing control; MOI, multiplicity of infection; MTX, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamic acid); MTX-phosphinate, 4-amino-4-deoxy-10-methylpteroyl-L-Glu-γ-[Ψ{P(O)(OH)–CH_2}]glutarate; MTX-phosphonate, 4-amino-4-deoxy-10-methyl-pteroyl-L-Glu-γ-[Ψ{P(O)(OH)–O}]glutarate; Orn, ornithine; and the use of Ψ as a symbol for peptide surrogates is as described by the IUPAC-IUB Joint Commission on Biochemical Nomenclature [1].

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(02)01602-7

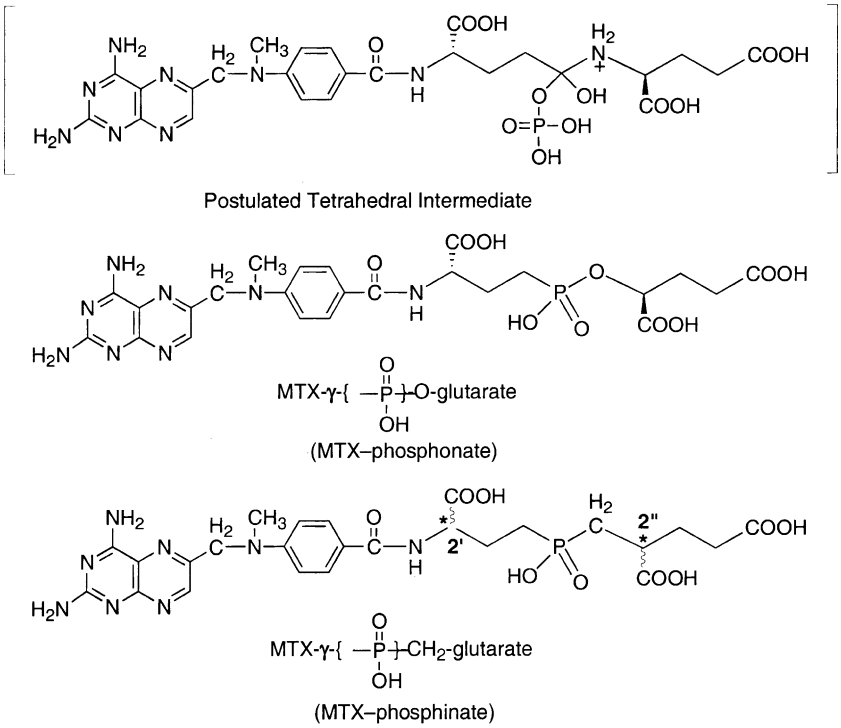

Fig. 1. Structures of the tetrahedral intermediate of the FPGS reaction and of MTX-phosphonate and MTX-phosphinate. Note that there are two chiral centers (*) at the 2′ and 2″ positions in MTX-phosphinate, and the synthetic method [13] yielded all four diastereomers.

the pteroyl moiety lead to increased inhibitory potency and/or specificity [3]. Shane and colleagues showed that substitution of Orn for Glu in PteGlu (i.e. PteOrn) leads to potent FPGS inhibition [6], but Orn substitution leads to poor cellular uptake because of the positively charged δ-amino group [8].

Another approach is the design of mechanism-based FPGS inhibitors. FPGS-catalyzed ligation of at least the first two γ-Glu residues proceeds through acyl-phosphate intermediates at the γ-COOH of the polyglutamate chain [9]. Attack by the amino group of the incoming Glu leads to transient formation of a tetrahedral intermediate at the γ-carbon (Fig. 1); such intermediates may be mimicked by stable phosphorous-containing structures (phosphonamidates, phosphonates, and phosphinates; discussed in Ref. [10]). Since the stability of phosphonamidates is a concern [11], our synthetic effort has focussed on MTX-phosphonate (Fig. 1), a potent competitive inhibitor of human FPGS (K_i, 46 nM) [12], and on MTX-phosphinate. MTX-phosphinate has been synthesized as a mixture of two racemic diastereomers (Fig. 1) [13]; its characterization as an inhibitor of human FPGS is presented below.

2. Materials and methods

2.1. Materials

Common chemicals were reagent grade or higher. MTX was a gift of Immunex. AMT was from the Sigma Chemical Co. MTX-phosphinate was synthesized as described previously [13]. Literature extinction coefficients [14] were used (that of MTX-phosphinate was assumed to be the same as that of MTX).

2.2. Enzymes and assays

A BaculoGold™ Transfection Kit (Pharmingen) was used to co-transfect *Sf*9 insect cells with pVL1392/cFPGS shuttle vector DNA (a gift of Dr. B. Shane), and BaculoGold™ DNA according to the instructions of the manufacturer to yield recombinant rhcFPGS-encoding baculovirus. After titering, recombinant virus was used to infect (MOI = 4) *Sf*9 monolayers, and cells were harvested at 72 hr. FPGS activity was extracted [15] and purified by $(NH_4)_2SO_4$ fractionation and BioGel A-0.5M chromatography (specific activity 6.4×10^6 pmol [^{3}H]Glu/hr/mg protein). K_m values for AMT and MTX as substrates are the same for this rhcFPGS as those reported for CCRF-CEM FPGS [12]. Inhibition constants K_{ii} and K_{is} [16] for rhcFPGS were determined [12] using MTX as the variable substrate and 2 and 8 nM MTX-phosphinate, levels yielding about 25 and 50% inhibition, respectively. Activity of and inhibitory potency (IC_{50}) against partially purified CCRF-CEM DHFR were assayed as described previously [15].

2.3. Cell culture

The human T-lymphoblastic leukemia cell line CCRF-CEM and its MTX transport-deficient subline R2, their

culture, and inhibition of growth under continuous (120-hr) drug exposure have been described previously [17]. Cell lines were negative for Mycoplasma (Mycoplasma Plus PCR primers).

3. Results and discussion

3.1. Inhibition of rhcFPGS

rhcFPGS did not use MTX-phosphinate as a substrate at 1 μM, the highest level tested (data not shown). When measured at a fixed pteroyl substrate (MTX) and enzyme concentration, MTX-phosphinate (IC_{50}, 8 ± 1 nM; average ± range, N = 2) was an even more potent FPGS inhibitor than MTX-phosphonate (IC_{50}, 120 nM) [12]. Kinetic constants for rhcFPGS inhibition using MTX as the variable substrate were: $K_{is} = 3.1 \pm 0.5$ nM and $K_{ii} = 56 \pm 5$ nM (average ± range; N = 2). Since $K_{is} \ll K_{ii}$, inhibition was essentially competitive; this is consistent with the Lineweaver-Burk replot of the kinetic data (not shown). Thus, MTX-phosphinate is the most potent FPGS inhibitor based on the MTX heterocycle that has been described to date. This high potency is even more impressive considering that binding of an antifolate FPGS inhibitor containing a Glu substitution (e.g. Orn) generally tracks the efficiency of the Glu-containing homolog to serve as an FPGS substrate [18], and MTX and MTX-γ-Glu are poor FPGS substrates [12]. If this relationship holds in the phosphinate series, it should be possible to substantially increase potency by replacing the 4-amino-4-deoxy-10-methylpteroyl heterocycle with one that, as a Glu-containing substrate, has a low K_m (e.g. DDATHF, K_m *ca.* 1 μM for human FPGS [19]).

Phosphinate inhibitors of ATP-dependent reactions may exhibit slow formation of a phosphorylated inhibitor that has much tighter binding (e.g. [20]). However, FPGS inhibition does not appear to be time-dependent, since at fixed levels of MTX-phosphinate (3 or 8 nM), FPGS activity was linear with respect to time for up to 2 hr. Also experiments in which MTX-phosphinate (10 or 20 nM) bound to FPGS was preincubated with ATP and L-Glu in the absence of pteroyl substrate (i.e. MTX) for 30 min showed no increase in inhibition relative to a control in which inhibitor and MTX were added simultaneously. However, the time period over which these reactions were observed (1–2 hr) might preclude kinetic observation of a phosphorylated inhibitor if it is synthesized over a few minutes [20].

MTX-phosphinate was synthesized as a diastereomeric mixture [13] to test the hypothesis that the phosphinate substructure imparts potent FPGS inhibition. Based on the known stereospecificity of FPGS [2], it is likely that only the 2′*S*, 2″*S* MTX-phosphinate diastereomer (Fig. 1) is highly active, and 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 pursued to explore this possibility.

3.2. Inhibition of human DHFR

Inhibitory potency against human DHFR (CCRF-CEM cells) was measured. MTX-phosphinate was slightly less potent than MTX (IC_{50}, 2.1 ± 0.1 vs 0.6 ± 0.1 nM, respectively; average ± range, N = 2) as a human DHFR inhibitor. The slightly lower potency of MTX-phosphinate may result from the presence of isomers (above) since the analog of MTX in which D-Glu replaces L-Glu is a much less potent DHFR inhibitor [21].

3.3. Cell growth inhibition

MTX-phosphinate and MTX were compared as growth inhibitors of CCRF-CEM human leukemia cells and a MTX transport-deficient subline, R2. MTX-phosphinate (cell growth 96.5 ± 0.5% of control at 1000 nM; average ± range; N = 2) was ≥70-fold less growth inhibitory to CCRF-CEM cells than was MTX (EC_{50}, 14.5 ± 0.5 nM; average ± range; N = 2). The subline R2 (EC_{50}, 2550 ± 50 nM; average ± range; N = 2) was 175-fold resistant to MTX and also did not respond to 1000 nM MTX-phosphinate (cell growth 104 ± 2% of control; average ± range; N = 2), the highest level tested. Despite the fact that MTX is a relatively poor (high K_m) mammalian FPGS substrate (e.g. [12]), we employed the 4-amino-4-deoxy-10-methyl heterocycle because it allows a simple way to separate uptake from FPGS inhibition [12]. If an analog containing this heterocycle is taken up, the potent inhibition of DHFR (above) will allow immediate and potent growth inhibition (FPGS inhibition causes a delayed effect); growth inhibition is thus a surrogate for uptake. Since MTX-phosphinate is not growth inhibitory, it is not taken up efficiently into cells. This is not surprising because of its negative charge and dipeptide structure [12]. Since we have shown that the phosphinate substructure imparts more potent FPGS inhibition than the phosphonate, our efforts will now turn to designing phosphapeptide prodrugs that are active in whole cells.

A potential complication to the development of therapeutically effective FPGS inhibitors has been noted. Human FPGS exists in cytosolic and mitochondrial isoforms [22–24]. It is unknown whether both FPGS isoforms must be inhibited to induce cell death. Inhibition of mitochondrial FPGS could be difficult because mitochondria are impermeable to many antifolates (e.g. [25]). However, since purine and thymidylate syntheses occur in the cytosol, cytosolic FPGS inhibition alone may be sufficient to inhibit these two processes that are critical for DNA synthesis and repair. The FPGS inhibitors now under development should allow us to answer this important question.

Acknowledgments

The authors thank Dr. Barry Shane (U.C. Berkeley) for supplying the pVL1392/cFPGS shuttle vector. These studies were supported by CA43500 (J.J.M.), RPCI CCSG CA16056, and CA28097 (J.K.C.).

References

[1] 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.

[2] McGuire JJ, Coward JK. Pteroylpolyglutamates: biosynthesis, degradation, and function. In: Blakley RL, Benkovic SJ, editors. Folates and pterins. Chemistry and biochemistry of folates, vol. 1. New York: Wiley; 1984. p. 135–90.

[3] McGuire JJ. Anticancer antifolates: current status and future directions. Curr Pharm Des, in press.

[4] McGuire JJ, Heitzman KJ, Haile WH, Russell CA, McCloskey DE, Piper JR. Cross-resistance studies of folylpolyglutamate synthetase-deficient, methotrexate-resistant CCRF-CEM human leukemia sublines. Leukemia 1993;7:1996–2003.

[5] McGuire JJ, Hsieh P, Franco CT, Piper JR. Folylpolyglutamate synthetase inhibition and cytotoxic effects of methotrexate analogs containing 2,ω-diaminoalkanoic acids. Biochem Pharmacol 1986;35: 2607–13.

[6] Cichowicz DJ, Cook J, George S, Shane B. Hog liver folylpolyglutamate synthetase: substrate specificity and regulation. In: Goldman ID, editor. Proceedings of the Second Workshop on Folyl- and Antifolylpolyglutamates. New York: Praeger; 1985. p. 7–13.

[7] Rosowsky A, Freisheim JH, Moran RG, Solan VC, Bader H, Wright JE, Radike-Smith M. 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.

[8] Tsukamoto T, Haile WH, McGuire JJ, Coward JK. The synthesis and biological evaluation of N^{α}-(4-amino-4-deoxy-10-methylpteroyl)-DL-4,4-difluoroornithine. J Med Chem 1996;39:2536–40.

[9] Banerjee RV, Shane B, McGuire JJ, Coward JK. Dihydrofolate synthetase and folylpolyglutamate synthetase: direct evidence for intervention of acyl phosphate intermediates. Biochemistry 1988;27: 9062–70.

[10] Malachowski WP, Coward JK. The chemistry of phosphapeptides: investigations on the synthesis of phosphonamidate, phosphonate, and phosphinate analogues of glutamyl-γ-glutamate. J Org Chem 1994;59: 7625–34.

[11] Chen S, Lin C-H, Kwon DS, Walsh CT, Coward JK. 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.

[12] Tsukamoto T, Haile WH, McGuire JJ, Coward JK. 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.

[13] Valiaeva N, Bartley D, Konno T, Coward JK. 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.

[14] Blakley RL. The biochemistry of folic acid and related pteridines. Amsterdam: Elsevier; 1969.

[15] McGuire JJ, Russell CA, Bolanowska WE, Freitag CM, Jones CS, Kalman TI. Biochemical and growth inhibition studies of methotrexate and aminopterin analogues containing a tetrazole ring in place of the γ-carboxyl group. Cancer Res 1990;50:1726–31.

[16] Cleland WW, Steady state kinetics. In: Boyer PD, editor. The enzymes. Kinetics and mechanism, vol. 2. New York: Academic Press; 1970. p. 1–65.

[17] McCloskey DE, McGuire JJ, Russell CA, Rowan BG, Bertino JR, Pizzorno G, Mini E. Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. J Biol Chem 1991;266:6181–7.

[18] McGuire JJ, Bolanowska WE, Piper JR. Structural specificity of inhibition of human folylpolyglutamate synthetase by ornithine-containing folate analogs. Biochem Pharmacol 1988;37:3931–9.

[19] Moran RG, Baldwin SW, Taylor EC, Shih C. The 6*S*- and 6*R*-diastereomers of 5,10-dideaza-5,6,7,8-tetrahydrofolate are equiactive inhibitors of *de novo* purine synthesis. J Biol Chem 1989;264: 21047–51.

[20] Lin CH, Chen S, Kwon DS, Coward JK, Walsh CT. Aldehyde and phosphinate analogs of glutathione and glutathionylspermidine: potent, selective binding inhibitors of the *E. coli* bifunctional glutathionylspermidine synthetase/amidase. Chem Biol 1997;4:859–66.

[21] Cramer SM, Schornagel JH, Kalghatgi KK, Bertino JR, Horvath C. Occurrence and significance of D-methotrexate as a contaminant of commercial methotrexate. Cancer Res 1984;44:1843–6.

[22] Lin B-F, Huang R-FS, 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.

[23] Freemantle SJ, Taylor SM, Krystal G, Moran RG. Upstream organization of and multiple transcripts from the human folylpoly-γ-glutamate synthetase gene. J Biol Chem 1995;270:9579–84.

[24] McGuire JJ, Balinska M, Russell CA. 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.

[25] Horne DW, Holloway RS, Said HM. Uptake of 5-formyltetrahydrofolate in isolated rat liver mitochondria is carrier-mediated. J Nutr 1992;122:2204–9.