Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 3587–3592
Bioorganic & Medicinal Chemistry

Synthesis and biological evaluation of α- and γ-carboxamide derivatives of 10-CF_3CO-DDACTHF

Youhoon Chong,[a,c] Inkyu Hwang,[a,c] Ali Tavassoli,[d] Yan Zhang,[b,c] Ian A. Wilson,[b,c] Stephen J. Benkovic[d] and Dale L. Boger[a,c,*]

[a]*Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
[b]*Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
[c]*The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*
[d]*Department of Chemistry, Pennsylvania State University, University Park, PA 16802, USA*

Received 13 September 2004; revised 24 November 2004; accepted 24 November 2004
Available online 2 April 2005

Abstract—Structurally-related, but non-polyglutamylatable, derivatives of 10-CF_3CO-DDACTHF (**1**), which incorporate L-glutamine (**2**) and L-isoglutamine (**3**) in place of L-glutamate, were prepared and evaluated as inhibitors of recombinant human (rh) GAR Tfase. While the L-glutamate α-carboxamide derivative **3** was much less effective as a rhGAR Tfase inhibitor (K_i = 4.8 μM) and inactive in cellular functional assays, the γ-carboxamide derivative **2** was found to be a potent and selective rhGAR Tfase inhibitor (K_i = 0.056 μM) being only 4-fold less potent than **1** (K_i = 0.015 μM). Moreover, **2** was effective in cellular functional assays exhibiting purine sensitive cytotoxic activity (IC_{50} = 300 nM, CCRF-CEM) only 20-fold less potent than **1** (IC_{50} = 16 nM), consistent with inhibition of de novo purine biosynthesis via selective inhibition of GAR Tfase. Like **1**, **2** is transported into the cell by the reduced folate carrier. Unlike **1**, the functional activity of **2** is not dependent upon FPGS polyglutamylation.

1. Introduction

Folylpolyglutamate synthetase (FPGS) plays a critical role in endogenous folate metabolism and retention and contributes to the cellular pharmacology of classical antifolates.[1] When folate or antifolates (as their α-monoglutamate) enter mammalian cells, they are converted by folylpolyglutamate synthetase (FPGS) to poly-γ-glutamates, containing between 2 and 9 additional γ-glutamate residues. Intracellularly formed γ-oligoglutamyl conjugates of reduced folates generally bind better than the parent monoglutamates to their cognate enzymes and are better retained in the cell.[2] For the same reasons, the potency of antifolates may be correlated with their state of polyglutamylation.[3] Recognition of the significance of polyglutamylation for the therapeutic efficacy of antifolates has led to drug discovery efforts to identify agents that are both inhibitors of folate-dependent enzymes[4,5] and are efficiently polyglutamylated.[6–10] For example, recent studies suggest that the polyglutamate metabolites of DDATHF are the principal species responsible for cell growth inhibition and that DDATHF itself may be a minor contributor to the cellular cytotoxic activity.[7] The pentaglutamate of DDATHF has been reported to be about 11 or 100 times more potent against human[8] and mouse[7] glycinamide ribonucleotide transformylase (GAR Tfase), respectively, than DDATHF. In addition, reduced polyglutamylation by down-regulation of FPGS has been described as a mechanism of inherent and acquired resistance to classical antifolates,[10] and deficient polyglutamylation renders cell lines resistant to both short and long term drug exposure.[11] Indeed, antifolate polyglutamylation and FPGS activity have been shown to be important determinants for both methotrexate cytotoxicity in vitro[12] and outcome in clinical studies.[12] Nonetheless, the long term and enhanced intracellular accumulation of antifolates that results from their polyglutamylation contributes to their cumulative toxicity. Thus, lack of polyglutamylation is potentially a therapeutic asset, especially in the treatment of antifolate-resistant tumors whose resistance is derived from a

* Corresponding author. Tel.: +1 858 784 7522; fax: +1 858 784 7550; e-mail: boger@scripps.edu

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.11.050

reduced capacity for polyglutamylation. In such instances, dose escalation should be tolerated better with non-polyglutamylatable compounds than with classical antifolates.

In previous studies,[13] we reported the design, synthesis, and biological evaluation of 10-trifluoroacetyl-DDACTHF (10-CF_3CO-DDACTHF, **1**, Fig. 1), which was shown to be a selective and exceptionally potent GAR Tfase inhibitor ($K_i = 0.015\ \mu M$, rhGAR Tfase) and an effective cytotoxic agent (CCRF-CEM cell line, $IC_{50} = 16$ nM). Thus, *gem*-diol binding of the electrophilic carbonyl of **1**, that mimics the tetrahedral intermediate of the formyl transfer reaction, conveys an approximate 100-fold enhancement in binding affinity for GAR Tfase accounting for its exquisite selectivity and exceptional potency over other folate-dependent enzymes. As such, **1** constitutes the most potent GAR Tfase inhibitor disclosed to date. Like other folate-based inhibitors incorporating the DDACTHF scaffold,[14] this inhibitor was not only an effective enzyme inhibitor, but it is also transported into the cell by the reduced folate carrier and polyglutamylated by FPGS. However, the polyglutamates of **1** exhibited only a modestly enhanced K_i against GAR Tfase (1- to 3-fold) implying that the FPGS and polyglutamylation requirement for observation of the potent, purine sensitive cytotoxic activity of **1** is the result of enhanced intracellular accumulation (retention) and not enhanced enzyme inhibitory potency.[15]

Thus, in conjunction with our interest in establishing the potential benefits of an antifolate incapable of polyglutamylation, the investigation of structurally-related, but non-polyglutamylatable, derivatives of **1** were pursued which would also provide valuable insight into the role of polyglutamylation on the activity of **1**. Herein, we report the synthesis and evaluation of two derivatives of the 10-CF_3CO-DDACTHF (**1**) that incorporate L-glutamine (**2**) and L-isoglutamine (**3**) in place of L-glutamate which preclude their ability to serve as substrates for FPGS. To our knowledge, the analogous monoamides of methotrexate are the only antifolates reported in which the L-glutamate side chain has been derivatized as the corresponding carboxamides.[16]

2. Chemistry

The syntheses of α- and γ-carboxamide derivatives of 10-CF_3CO-DDACTHF (**2** and **3**) were accomplished in a divergent manner as shown in Schemes 1 and 2. *N*,*N*-Dimethylhydrazone **4**[13] was alkylated to give the corresponding chloropropyl adduct **5** (NaHMDS, 3-chloro-1-iodopropane, THF, −78 °C, 63%). This alkylation procedure proved to be an improvement over that initially reported for the preparation of **1** enlisting 1,3-dibromopropane.[13] Although comparable conversions were observed, the desired monoalkylation was more dependably controlled with use of 3-chloro-1-iodopropane and the conditions detailed herein. The preformed sodium salt of ethyl cyanoacetate (NaH, DMF, 0 °C, 30 min) was alkylated with **5** (DMF, 60 °C, 8 h) to give **6** (82%), and its treatment with the free base of guanidine under basic conditions gave the desired pyrimidinone **7** (63%) as previously detailed.[13] Treatment of **7** with LiOH (3 equiv, 3:1 CH_3OH/H_2O, 25 °C, 48 h) followed by acidification to pH 4 with 1 N aqueous HCl

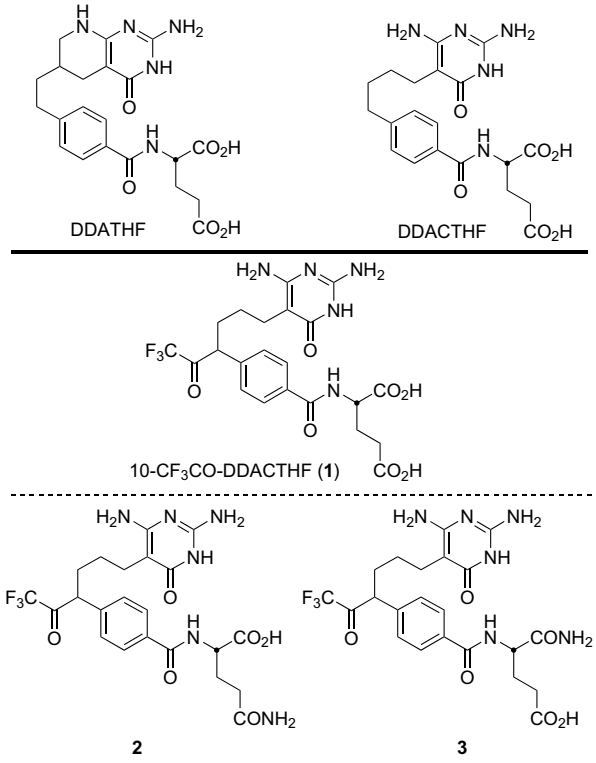
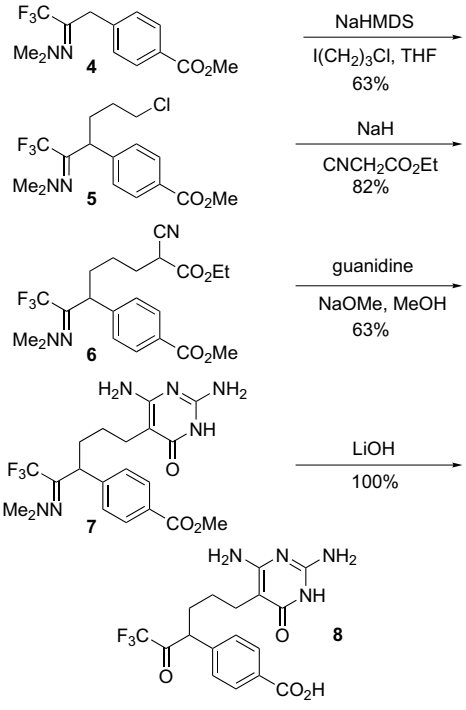
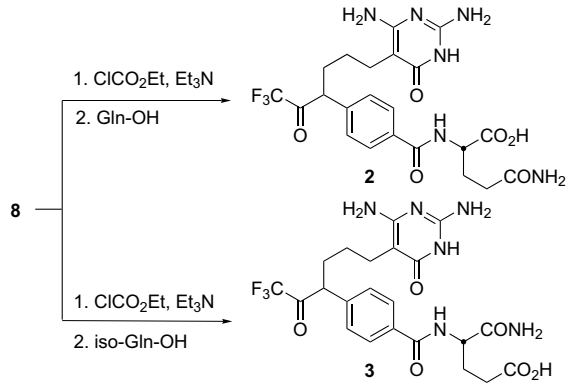


Figure 1.



Scheme 1.



Scheme 2.

cleanly hydrolyzed both the methyl ester and the dimethylhydrazone, providing the common intermediate **8**[13] (100%). In order to arrive at the target molecules, L-glutamine and L-isoglutamine[17] were coupled, under basic conditions (aq NaOH), with the mixed anhydride formed by the treatment of **8** with ethyl chloroformate ($ClCO_2Et$, Et_3N, DMF, −10 °C).[18] Acidification of the reaction mixture to pH 2 with 6 N HCl provided the HCl salts of **2** and **3** (Scheme 2).

3. GAR Tfase and AICAR Tfase inhibition

Compounds **2** and **3** were tested for inhibition of GAR Tfase and aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) and the results are presented in Table 1. Unlike 10-CF_3CO-DDACTHF (**1**)[13] which showed moderate inhibitory activity against *E. coli* GAR Tfase (K_i = 1.9 μM), the carboxamide derivatives **2** and **3** were not active against the bacterial enzyme. However, γ-carboxamido-10-CF_3-CO-DDACTHF (**2**) exhibited potent inhibitory activity (K_i = 56 nM) against recombinant human GAR Tfase comparable with that observed with **1** (K_i = 15 nM),[13] whereas the α-carboxamide derivative **3** exhibited a 320-fold decreased affinity (K_i = 4.8 μM). Thus, **2** exhibits the selective inhibition of human versus *E. coli* GAR Tfase analogous to **1** and is only 4-fold less potent than **1**. Moreover, **2** is roughly equipotent with lometrexol and 30-fold more potent than DDACTHF itself against rhGAR Tfase. This latter comparison indicates that the *gem* diol binding of the trifluoroacetyl group increases the enzyme binding affinity significantly (30-fold) consistent with expectations. The roughly 100-fold difference in inhibition observed between the γ-carboxamide (**2**) and α-carboxamide (**3**) derivatives clearly defines the role of the two glutamate carboxylates where the α-carboxylic acid contributes significantly to the binding affinity, whereas the γ-carboxylic acid does not.

Table 1. GAR and AICAR Tfase inhibition (K_i, μM)

Compd	*E. coli* GAR Tfase[a]	rhGAR Tfase[b]	rhAICAR Tfase[c]
1	1.9	0.015	>100
2	>100	0.056	>100
3	>100	4.8	>100
DDACTHF	5	1.7	nd[e]
Lometrexol	0.1	0.06[d]	nd[e]

[a] *E. coli* GAR Tfase.
[b] Recombinant human GAR Tfase.
[c] Recombinant human AICAR Tfase.
[d] Ref. 17.
[e] Not determined.

This result is consistent with the X-ray structure of compound **1** bound with human GAR Tfase[13] which defined a critical salt bridge between the glutamate α-carboxylate and Arg64 while the glutamate γ-carboxylate extends into solvent without any specific interactions with enzyme residues. None of the compounds tested showed inhibitory activity against rhAICAR Tfase.

4. Cytotoxic activity

Compounds **2** and **3** were examined for cytotoxic activity both in the presence (+) and absence (−) of added

Table 2. In vitro cytotoxic activity

Compound	CCRF-CEM (IC_{50}, μM)			
	(+) T, (+) H[a]	(−) T, (+) H	(+) T, (−) H	(−) T, (−) H
1	>100	>100	0.017	0.016
2	>100	>100	0.4	0.3
3	>100	>100	>100	>100
DDACTHF	>100	>100	3.6	2.7
Lometrexol	>100	>100	0.2	0.2

Compound	IC_{50}, μM [(−)T, (−)H]		
	CCRF-CEM	CCRF-CEM/FPGS$^-$	CCRF-CEM/MTX
1	0.016	>100	>100
2	0.3	0.5	40
3	>100	>100	nd[c]
DDACTHF	3	nd[c] (>100[b])	nd[c] (>100[b])
Lometrexol	0.2	25.0 (>100[b])	nd[c] (>100[b])

[a] T = Thymidine, H = Hypoxanthine.
[b] Ref. 15.
[c] Not determined.

hypoxanthine (purine) or thymidine (pyrimidine) against the CCRF-CEM cell line (Table 2). Like **1**, the γ-carboxamide derivative **2** exhibits potent cytotoxic activity (IC_{50} = 0.3 μM) against the CCRF-CEM cell line when purines (hypoxanthine) are absent in the medium, but was inactive (IC_{50} > 100 μM) in the presence of medium purines. This sensitivity to the presence of purines, but not pyrimidines (thymidine), indicates that the cytotoxic activity of **2**, like that of **1**, is derived from inhibition of an enzyme in the de novo purine biosynthetic pathway consistent with its potent and selective inhibition of rhGAR Tfase. In addition and in spite of the moderate differences in K_i (4-fold, Table 1), **2** is 20-fold less cytotoxic than **1** in this assay. This would imply that, along with the decreased affinity of **2** for the target enzyme, a decrease in intracellular accumulation of **2** derived from a lack of polyglutamylation may reduce the effective intracellular concentration of **2** and thereby reduce its cytotoxic activity. Importantly, assay of **1** and **2** against a FPGS-deficient CCRF-CEM cell line (CCRF-CEM/FPGS$^-$, Table 2) performed to establish the extent to which the cytotoxic activity of **1** and **2** was dependent on the FPGS-polyglutamylation indicates that **2** acts independent of the FPGS level. Unlike **1** and lometrexol which lost or lacked activity against this cell line, compound **2** remains equipotent against the FPGS-deficient cell line. In addition, the assay of **2** against a CCRF-CEM cell line with an impaired reduced folate carrier (CCRF-CEM/MTX) revealed that **2** is roughly 100-fold less active and that its cellular functional activity, like that of **1**, may benefit from reduced folate carrier transport into the cell. Thus, conversion of the glutamate γ-carboxylic acid of **1** to a carboxamide (**2**) precludes FPGS polyglutamylation, but does not appear to impact transport into the cell.

In contrast, the α-carboxamide derivative **3** was inactive against the CCRF-CEM cell line (Table 2). Thus, the L-glutamate α-carboxylate is critical and its derivatization to a carboxamide results in both low affinity for the target enzyme (K_i = 4.8 μM) and a loss in activity in cellular functional assays.

5. Conclusions

To address antifolate resistance derived from reduced polyglutamylation and to avoid the cumulative toxicity of polyglutamylatable antifolates, efforts have been directed at the development of novel antifolates that are designed to act irrespective of the level of FPGS expression in the cell.[19–22] For this purpose and in this study, non-polyglutamylatable γ-carboxamide (**2**) and α-carboxamide (**3**) derivatives of 10-CF_3CO-DDACTHF were prepared. Whereas the L-glutamate α-carboxamide derivative **3** was much less effective as a rhGAR Tfase inhibitor (K_i = 4.8 μM) and inactive in cellular functional assays, the γ-carboxamide derivative **2** was found to be a potent and selective rhGAR Tfase inhibitor (K_i = 0.056 μM) being only 4-fold less potent than **1** (K_i = 0.015 μM). Moreover, **2** was effective in cellular functional assays exhibiting purine sensitive cytotoxic activity (IC_{50} = 300 nM, CCRF-CEM) only 20-fold less potent than **1** (IC_{50} = 16 nM), consistent with inhibition of de novo purine biosynthesis via selective inhibition of GAR Tfase. Like **1**, **2** is transported into the cell by the reduced folate carrier. Unlike **1**, the functional activity of **2** is not dependent upon FPGS polyglutamylation. Thus, not only is **2** roughly equipotent with lometrexol and only slightly less active than **1**, but its functional activity is independent of FPGS levels and polyglutamylation providing a superb candidate for in vivo examination alongside **1**. Such studies are in progress and will be detailed in due course.

6. Experimental

6.1. Methyl 4-{4-chloro-1-[1-(dimethylhydrazono)-2,2,2-trifluoroethyl]butyl}-benzoate (5)

NaHMDS (2 M solution in THF, 0.17 mL, 0.35 mmol) was slowly added to a stirred solution of **4**[13] (100 mg, 0.35 mmol) in anhydrous THF (5 mL) at −78 °C. After stirring the reaction mixture for 15 min at −78 °C, 1-chloro-3-iodopropane (0.38 mL, 3.46 mmol) was added. The reaction mixture was slowly warmed to 23 °C and stirred for 10 h, before being quenched with the addition of saturated aqueous NH_4Cl. The aqueous layer was extracted three times with EtOAc, and the combined organic layers were washed with saturated aqueous NaCl, dried ($MgSO_4$), filtered, and concentrated. Column chromatography (SiO_2, 8% EtOAc–hexane) provided 80 mg (0.22 mmol, 63%) of a 2.8:1 mixture (*cis*/*trans* hydrazone) of **5** as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.99 (d, *J* = 8.2 Hz, 2H), 7.98 (d, *J* = 8.2 Hz, 0.7H), 7.38 (d, *J* = 8.5 Hz, 2H), 7.30 (d, *J* = 8.5 Hz, 0.7H), 4.79 (t, *J* = 7.6 Hz, 1H), 3.78 (t, *J* = 7.0 Hz, 0.3H), 3.59 (td, *J* = 6.4, 2.6 Hz, 2H), 3.49 (t, *J* = 6.4 Hz, 0.7H), 2.77 (s, 2.1H), 2.61 (s, 6H), 2.43–2.33 (m, 1H), 2.22–2.07 (m, 1.3H), 1.93–1.74 (m, 2.7H), 1.66–1.56 (m, 0.3H); MALDIFTHRMS (DHB) *m*/*z* 364.1242 ($M+H^+$, $C_{16}H_{20}ClF_3N_2O_2$ requires 364.1238).

6.2. Methyl 4-{5-cyano-1-[1-(dimethylhydrazono)-2,2,2-trifluoroethyl]-5-(ethoxycarbonyl)pentyl}benzoate (6)

A suspension of NaH (158 mg, 3.95 mmol) in anhydrous DMF (5 mL) at 0 °C was treated with ethyl cyanoacetate (0.42 mL, 3.95 mL) dropwise. The reaction mixture was stirred at 0 °C for 30 min, forming the sodium salt as a clear solution. This anion was treated with a solution of 5 (80 mg, 0.22 mmol) in anhydrous DMF (5 mL). The reaction mixture was stirred at 25 °C for 8 h and then warmed at 60 °C for 8 h before it was quenched by the addition of saturated aqueous NH_4Cl. The reaction mixture was diluted with EtOAc and washed with water and saturated aqueous NaCl. The organic layer was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The excess ethyl cyanoacetate was distilled off, and the residual product was purified by column chromatography (SiO_2, 12% EtOAc–hexanes) to give 80 mg (0.18 mmol, 82%) of a 2.4:1 mixture (*cis*/*trans* hydrazone) of **6** as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.99 (d, *J* = 8.2 Hz, 2H), 7.98 (d, *J* = 7.6 Hz, 0.8H), 7.36 (d, *J* = 8.0 Hz,

2H), 7.28 (d, J = 7.0 Hz, 0.8H), 4.78 (t, J = 7.6 Hz, 1H), 4.26 (t, J = 7.2 Hz, 0.4H), 3.76 (t, J = 7.4 Hz, 0.4H), 3.53 (td, J = 7.3, 2.6 Hz, 1H), 2.78 (s, 1.2H), 2.77 (s, 1.2H), 2.61 (s, 3H), 2.60 (s, 3H), 2.26–2.20 (m, 1H), 2.07–1.91 (m, 4.6H), 1.70–1.49 (m, 2.4H), 1.44–1.30 (m, 0.4H); MALDIFTHRMS (DHB) m/z 464.1768 ($M+Na^+$, $C_{21}H_{26}F_3N_3O_4Na$ requires 464.1773).

6.3. Methyl 4-{4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-[1-(dimethylhydrazono)-2,2,2-trifluoroethyl]butyl}benzoate (7)

A stirred solution of NaOMe (367 mg, 6.80 mmol) in MeOH (6 mL) was treated with guanidine·HCl (650 mg, 6.80 mmol) and the mixture was stirred for 30 min at 23 °C. A solution of **6** (1.0 g, 2.27 mmol) in MeOH (4 mL) was added and the mixture was stirred at reflux for 24 h. After concentration under reduced pressure, the residual product was purified by column chromatography (SiO_2, 5–10% MeOH–CH_2Cl_2) to give 650 mg (1.43 mmol, 63%) of **7** as a colorless syrup identical in all respects with that previously described:[13] ^1H NMR ($CDCl_3$, 400 MHz) δ 7.95 (d, J = 6.8 Hz, 2H), 7.42 (d, J = 6.6 Hz, 2H), 4.79 (t, J = 6.2 Hz, 1H), 3.88 (s, 3H), 2.62 (s, 6H), 2.48–2.34 (m, 2H), 2.31–2.27 (m, 1H), 2.02–1.95 (m, 1H), 1.59–1.56 (m, 1H), 1.52–1.46 (m, 1H); MALDIFTHRMS (DHB) m/z 455.2001 ($M+H^+$, $C_{20}H_{25}F_3N_6O_3$ requires 455.2013).

6.4. 4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoro-acetyl)butyl]benzoic acid (8)

A solution of **7** (111 mg, 0.24 mmol) in a 2:1 mixture of MeOH (2 mL) and H_2O (1 mL) was treated with LiOH-H_2O (31 mg, 7.33 mmol) and stirred for 24 h at 23 °C. After diluting the reaction mixture with water, the aqueous layer was washed with EtOAc two times and treated with 1 N aqueous HCl to adjust the pH to 4. After concentration under reduced pressure, the residue was treated with CH_3CN to remove traces of water to provide **8** (97 mg, 0.24 mmol, 100%), identical in all respects with material disclosed previously,[13] which was used for the next step without further purification: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.89 (d, J = 8.0 Hz, 2H), 7.30 (d, J = 8.0 Hz, 2H), 4.37 (t, J = 7.2 Hz, 1H), 2.25 (t, J = 7.4 Hz, 2H), 2.19–1.77 (m, 2H), 1.44–1.25 (m, 2H); MALDIFTHRMS (DHB) m/z 399.1275 ($M+H^+$, $C_{17}H_{17}F_3N_4O_4$ requires 399.1275).

6.5. 4-Carbamoyl-2-{4-[4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)butyl]benzoylamino}butyric acid (2)

A sample of **8** (45 mg, 0.11 mmol) and Et_3N (0.017 mL, 0.12 mmol) were dissolved in anhydrous DMF (5 mL) and cooled to −10 °C under a N_2 atmosphere. $ClCO_2Et$ (0.012 mL, 0.12 mmol) was added and, after 20 min, a solution of L-glutamine (33 mg, 0.23 mmol) in 0.3 N aqueous NaOH (0.88 mL, 0.27 mmol) was added. The solution was allowed to warm to 23 °C and stirred for 25 min at 23 °C. The DMF was removed in vacuo and the residue was dissolved in MeOH, cooled to 0 °C, and acidified with diluted aqueous HCl. After concentration under reduced pressure, the residue was purified by preparative TLC (reverse phase, 20% CH_3OH–H_2O) followed by trituation with ether to give 21 mg (0.04 mmol, 35%) of **2** as an off-white solid: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.85 (d, J = 8.2 Hz, 0.6H), 7.74 (d, J = 8.2 Hz, 1.6H), 7.39 (d, J = 7.6 Hz, 1.6H), 7.36 (d, J = 7.9 Hz, 0.6H), 4.59–4.48 (m, 2.2H), 3.10 (t, J = 5.2 Hz, 2.2H), 2.38 (t, J = 7.9 Hz, 2.2H), 2.42–2.20 (m, 2.2H), 2.30 (t, J = 8.2H, 1.6H), 2.22 (t, J = 8.2 Hz, 0.6H), 2.12–1.94 (m, 3.3H), 1.91–1.73 (m, 1.1H); MALDIFTHRMS (DHB) m/z 527.1872 ($M+H^+$, $C_{22}H_{25}F_3N_6O_6$ requires 527.186).

6.6. 4-Carbamoyl-4-{4-[4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)butyl]benzoylamino}butyric acid (3)

A sample of **8** (33 mg, 0.084 mmol) and Et_3N (0.013 mL, 0.093 mmol) were dissolved in anhydrous DMF (5 mL) and cooled to −10 °C under a N_2 atmosphere. $ClCO_2Et$ (0.0088 mL, 0.093 mmol) was added and, after 20 min, a solution of L-isoglutamine (25 mg, 0.17 mmol) in 0.3 N aqueous NaOH (0.7 mL, 0.20 mmol) was added. The solution was allowed to warm to 23 °C and stirred for 25 min at 23 °C. The DMF was removed in vacuo and the residue was dissolved in MeOH, cooled to 0 °C, and acidified with dilute aqueous HCl. After concentration under reduced pressure, the residue was purified by preparative TLC (reverse phase, 20% CH_3OH–H_2O) followed by trituation with ether to give 13 mg (0.025 mmol, 30%) of **3** as an off-white solid: ^1H NMR ($CDCl_3$, 400 MHz) δ 8.00 (d, J = 6.4 Hz, 0.6H), 7.90 (d, J = 6.4 Hz, 1.6H), 7.40 (d, J = 7.2 Hz, 1.6H), 7.36 (d, J = 6.4 Hz, 0.6H), 4.94–4.83 (m, 2.2H), 3.21 (t, J = 5.2 Hz, 0.6H), 3.20 (t, J = 5.2 Hz, 1.6H), 2.31 (t, J = 5.6 Hz, 2.2H), 2.25–2.20 (m, 2.2H), 2.24 (t, J = 5.6 Hz, 1.6H), 2.23 (t, J = 5.6 Hz, 0.6H), 2.05–1.98 (m, 3.3H), 1.89–1.80 (m, 1.1H); MALDIFTHRMS (DHB) m/z 527.1844 ($M+H^+$, $C_{22}H_{25}F_3N_6O_6$ requires 527.186).

6.7. GAR and AICAR Tfase assay

The K_i values for the folate analogs were measured as previously described.[13] For the GAR Tfase inhibition assay, each compound was dissolved in DMSO and then diluted in assay buffer using a concentration of DMSO that did not affect enzyme activity. Thus, all assays were conducted by mixing 10 μM of fDDF, 20 μM of inhibitor in total volume of 1 mL buffer (0.1 M HEPES, pH 7.5) at 26 °C, and the reaction initiated by the addition of 76 nM *E. coli* or rh GAR Tfase. The assay monitors the deformylation of fDDF ($\Delta\varepsilon$ = 18.9 mM^{-1} cm^{-1} at 295 nm) resulting from the transfer of the formyl group to GAR. If the inhibitor was found to be active, a series of $1/v_i$ versus 1/[GAR] at different, fixed concentrations of I (e.g., 1, 2, 4, 8, 12, 16, 20, 32 μM) were generated in order to determine K_i using the Michaelis–Menten equation for competitive inhibition. AICAR Tfase inhibition studies was conducted in the absence of 5 μM β-mercaptoethanol and screened with 10 nM enzyme, 25 μM inhibitor and 22.5 μM of cofactor. The results of the inhibition assays are summarized in Table 1.

6.8. Cytotoxic assay

The cytotoxic activity of the compounds was measured using the CCRF-CEM human leukemia cell lines as described previously.[13–15]

Acknowledgements

We gratefully acknowledge the financial support of the National Institutes of Health (CA 63536, to DLB, SJB and IAW), and the Skaggs Institute for Chemical Biology.

References and notes

1. Fabre, I.; Fabre, G.; Goldman, I. D. *Cancer Res.* **1984**, *44*, 3190; Pizzorno, G.; Mini, E.; Coronnello, M.; McGuire, J. J.; Moroson, B. A.; Cashmore, A. R.; Dreyer, R. N.; Lin, J. T.; Mazzeri, T.; Periti, P.; Bertino, J. R. *Cancer Res.* **1988**, *48*, 2149; Rumberger, B. G.; Barrueco, J. R.; Sirotnak, F. M. *Cancer Res.* **1990**, *50*, 4639.
2. Balinska, M.; Nimec, Z.; Galivan, J. *Arch. Biochem. Biophys.* **1982**, *216*, 466.
3. Synold, T. W.; Willits, E. M.; Barredo, J. C. *Leuk. Lymphoma* **1996**, *21*, 9.
4. McGuire, J. J.; Hsieh, P.; Franco, C. T.; Piper, J. R. *Biochem. Pharmacol.* **1986**, *35*, 2607; Rosowsky, A.; Freisheim, J. H.; Moran, R. G.; Solan, V. C.; Bader, H.; Wright, J. E.; Radike-Smith, M. *J. Med. Chem.* **1986**, *29*, 655.
5. Valiaeva, N.; Bartley, D.; Konno, T.; Coward, J. K. *J. Org. Chem.* **2001**, *66*, 5146; McGuire, J. J.; Haile, W. H.; Valiaeva, N.; Bartley, D.; Guo, J.; Coward, J. K. *Biochem. Pharmacol.* **2003**, *65*, 315.
6. Hanlon, M. H.; Ferone, R.; Mullin, R. J.; Keith, B. R. *Cancer Res.* **1990**, *50*, 3207.
7. Baldwin, S. W.; Tse, A.; Gossett, L. S.; Taylor, E. C.; Rosowsky, A.; Shih, C.; Moran, R. G. *Biochemistry* **1991**, *30*, 1997.
8. Habeck, L. L.; Leitner, T. A.; Shackelford, K. A.; Gossett, L. S.; Schultz, R. M.; Andis, S. L.; Shih, C.; Grindey, G. B.; Mendelsohn, L. G. *Cancer Res.* **1994**, *54*, 1021.
9. Shih, C.; Chen, V. J.; Gossett, L. S.; Gates, S. B.; MacKellar, W. C.; Habeck, L. L.; Sheckelford, K. A.; Mendelsohn, L. G.; Soose, D. J.; Patel, V. F.; Andis, S. L.; Bewley, J. R.; Rayl, E. A.; Moroson, B. A.; Beardsley, G. P.; Kohler, W.; Ratnam, M.; Schultz, R. M. *Cancer Res.* **1997**, *57*, 1116; Mendelsohn, L. G.; Shih, C.; Schultz, R. M.; Worzalla, J. F. *Invest. New Drugs* **1996**, *14*, 287.
10. McCloskey, D. E.; McGuire, J. J.; Russel, C. A.; Rowan, B. G.; Bertino, J. R.; Pizzorno, G.; Mini, E. *J. Biol. Chem.* **1991**, *266*, 6181; Jackman, A. L.; Kelland, I. R.; Kimbell, R.; Brown, M.; Gibson, W.; Aherne, G. W.; Hardcastle, A.; Boyle, F. T. *J. Cancer* **1995**, *71*, 914.
11. Takemura, Y.; Kobayashi, H.; Gibson, W.; Kimbell, R.; Miyachi, H.; Jackman, A. L. *Int. J. Cancer* **1996**, *66*, 29.
12. Whitehead, V. M.; Rosenblatt, D. S.; Vuchich, M.-J.; Shuster, J. J.; Witte, A.; Beaulieu, D. *Blood* **1990**, *76*, 44.
13. Zhang, Y.; Desharnais, J.; Marsilje, T. H.; Li, C.; Hedrick, M. P.; Gooljarsingh, L. T.; Tavassoli, A.; Benkovic, S. J.; Olson, A. J.; Boger, D. L.; Wilson, I. A. *Biochemistry* **2003**, *42*, 6043.
14. Bigham, E. C.; Hodson, S. J.; Mallory, W. R.; Wilson, D.; Duch, D. S.; Smith, G. K.; Ferone, R. *J. Med. Chem.* **1992**, *35*, 1399; Marsilje, T. H.; Labroli, M. A.; Hedrick, M. P.; Jin, Q.; Desharnais, J.; Baker, S. J.; Gooljarsingh, L. T.; Ramcharan, J.; Tavassoli, A.; Zheng, Y.; Wilson, I. A.; Beardsley, G. P.; Benkovic, S. J.; Boger, D. L. *Bioorg. Med. Chem.* **2002**, *10*, 2739.
15. Desharnais, J.; Hwang, I.; Zhang, Y.; Tavassoli, A.; Baboval, J.; Benkovic, S. J.; Wilson, I. A.; Boger, D. L. *Bioorg. Med. Chem.* **2003**, *11*, 4511.
16. Antonjuk, D. J.; Boadle, D. K.; Cheung, H. T. A.; Tran, T. Q. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1989.
17. Wang, Z.-F.; Xu, J.-C. *Tetrahedron* **1998**, *54*, 12597; Tsukida, T.; Moriyama, H.; Kurokawa, K.; Achiha, T.; Inoue, Y.; Kondo, H. *J. Med. Chem.* **1998**, *41*, 4279; Kaila, N.; Thomas, B. E., IV; Thakker, P.; Alvarez, J. C.; Camphausen, R. T.; Crommie, D. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 151; Kubasch, N.; Schmidt, R. R. *Eur. J. Org. Chem.* **2002**, 2710.
18. Wei, H.-X.; Truitt, C. L.; Paré, P. W. *Tetrahedron Lett.* **2003**, *44*, 831.
19. Kamen, B. A.; Eibl, B.; Cashmore, A.; Bertino, J. *Biochem. Pharmacol.* **1984**, *33*, 1697; Duch, D. S.; Edelstein, M. P.; Bowers, S. W.; Nichol, C. A. *Cancer Res.* **1982**, *42*, 3987; Webber, S.; Bartlett, C. A.; Boritzki, T. J.; Hilliard, J. A.; Howland, E. F.; Johnston, A. L.; Kosa, M.; Margosiak, S. A.; Morse, C. A.; Shetty, B. V. *Cancer Chemother. Pharmacol.* **1996**, *37*, 509.
20. Tsukamoto, T.; Kitazume, T.; McGuire, J. J.; Coward, J. K. *J. Med. Chem.* **1996**, *39*, 66.
21. Rosowsky, A.; Wright, J. E.; Vaidya, C. M.; Bader, H.; Rorsch, R. A.; Mota, C. E.; Pardo, J.; Chen, C. S.; Chen, Y.-N. *J. Med. Chem.* **1998**, *41*, 5310; Rosowsky, A.; Wright, J. E.; Vaidya, C. M.; Forsch, R. A.; Bader, H. *J. Med. Chem.* **2000**, *43*, 1620; Writht, J. E.; Rosowsky, A. *Bioorg. Med. Chem.* **2002**, *10*, 493; Vaidya, C. M.; Wright, J. E.; Rosowsky, A. *J. Med. Chem.* **2002**, *45*, 1690.
22. Jackman, A. L.; Kimbell, R.; Aherne, G. W.; Brunton, L.; Jansen, G.; Stephens, T. C.; Smith, M. N.; Wardleworth, J. M.; Boyle, F. T. *Clin. Cancer Res.* **1997**, *3*, 911; Kobayashi, H.; Takemura, Y.; Miyachi, H. *Cancer Chemother. Pharmacol.* **1998**, *42*, 105; Itoh, F.; Yukishige, K.; Wajima, M.; Ootsu, K.; Akimoto, H. *Chem. Pharm. Bull.* **1995**, *43*, 230.