Pergamon

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Bioorganic & Medicinal Chemistry 11 (2003) 4511–4521

BIOORGANIC & MEDICINAL CHEMISTRY

Design, Synthesis and Biological Evaluation of 10-CF_3CO-DDACTHF Analogues and Derivatives as Inhibitors of GAR Tfase and the De Novo Purine Biosynthetic Pathway

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Received 22 April 2003; accepted 22 April 2003

Abstract—The synthesis and evaluation of analogues and key derivatives of 10-CF_3CO-DDACTHF as inhibitors of glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide transformylase (AICAR Tfase) are reported. Polyglutamate analogues of **1** were evaluated as inhibitors of *Escherichia coli* and recombinant human (rh) GAR Tfase, and AICAR Tfase. Although the pentaglutamate **6** was found to be the most active inhibitor of the series tested against rhGAR Tfase ($K_i = 0.004$ μM), little distinction between the mono–pentaglutamate derivatives was observed ($K_i = 0.02$–0.004 μM), suggesting that the principal role of the required polyglutamation of **1** is intracellular retention. In contrast, **1** and its defined polyglutamates **3**–**6** were much less inactive when tested against rhAICAR Tfase ($K_i = 65$–0.120 μM) and very selective (≥ 100-fold) for rh versus *E. coli* GAR Tfase. Additional key analogues of **1** were examined (**7** and **8**) and found to be much less active (1000-fold) highlighting the exceptional characteristics of **1**.

Glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) are folate-dependent enzymes central to the de novo purine biosynthetic pathway. GAR Tfase utilizes the cofactor (6*R*)-N^{10}-formyltetrahydrofolate (Fig. 1) to transfer a formyl group to the primary amine of its substrate, glycinamide ribonucleotide (GAR, Fig. 1). This one carbon transfer incorporates the C-8 carbon of the purines and is the first of two formyl transfer reactions. The second formyl transfer reaction is catalyzed by the enzyme AICAR Tfase which also employs (6*R*)-N^{10}-formyltetrahydrofolate to transfer a formyl group to the C-5 amine of its substrate, aminoimidazole carboxamide ribonucleotide (AICAR, Fig. 1).[1] The discovery that (6*R*)-5,10-dideazatetrahydrofolate (Lometrexol, (6*R*)-DDATHF, Fig. 2) achieves its potent anticancer activity by selective GAR Tfase inhibition established GAR Tfase and the purine de novo biosynthetic pathway as viable targets for antineoplastic intervention.[2–4] Herein, we report the synthesis and evaluation of a series of analogues and key derivatives of **1** incorporating the DDACTHF scaffold.

Inhibitor Design

In previous studies, we examined folate-based inhibitors which incorporated electrophilic functional groups that could potentially interact either with active site nucleophiles or the GAR/AICAR substrate amines.[5–8] It was envisioned that the properly positioned nontransferable electrophilic carbonyl could potentially form an imine or a tetrahedral adduct with the active site or substrate nucleophiles or serve to stabilize gem diol formation of the electrophilic carbonyl and promote active site binding by mimicking the tetrahedral intermediate of the formyl transfer reactions. This latter effect was observed with folate-based inhibitors bearing a nontransferable formyl group and has provided potent, selective, and efficacious GAR Tfase inhibitors.[6,9]

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doi:10.1016/S0968-0896(03)00458-9

In the preceding articles, we examined folate-based inhibitors bearing alternative electrophilic carbonyls.[7,8] Embodied in these studies was the recognition that many such folate-based inhibitors based on the DDACTHF scaffold were not only effective enzyme inhibitors, but also effectively transported into the cell by the reduced folate carrier and polyglutamated by FPGS.[6] In conjunction with these studies, we also recently reported the

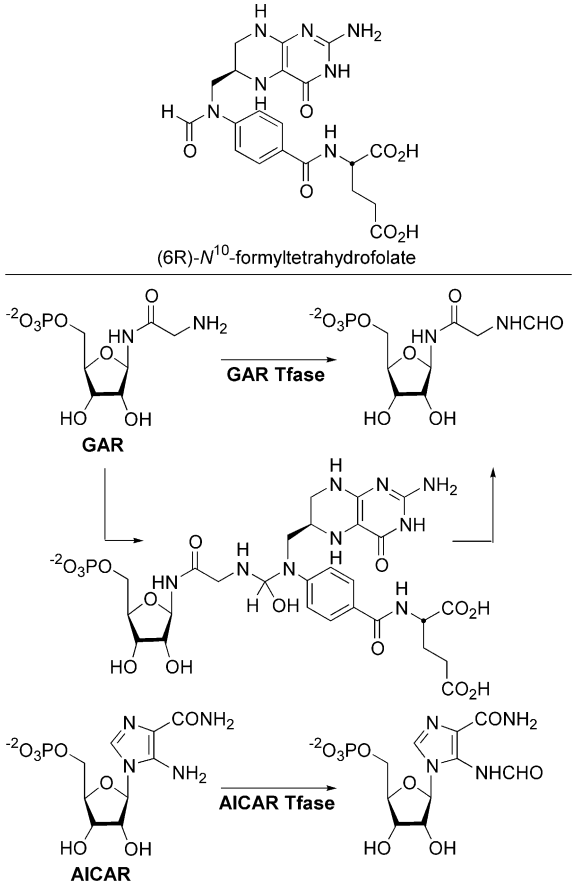


Figure 1.

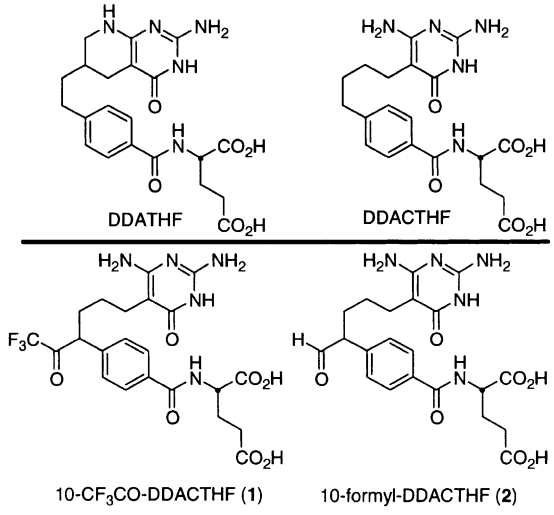


Figure 2.

design, synthesis, and the biological evaluation of 10-trifluoroacetyl-DDACTHF (10-CF_3CO-DDACTHF, **1**).[10] This analogue was shown to be a selective and potent GAR Tfase inhibitor ($K_i = 0.015$ μM against rhGAR Tfase) and an effective cytotoxic agent (CCRF–CEM $IC_{50} = 16$ nM).[10] This GAR Tfase inhibitor was shown not only to possess a potency similar to 10-formyl-DDACTHF (**2**, $K_i = 0.014$ μM against rhGAR Tfase and CCRF–CEM $IC_{50} = 60$ nM),[6] but to exhibit the necessary chemical stability for in vivo evaluation. Thus, a facile oxidative decarbonylation of the formyl group conveyed a chemical instability to **2** precluding consideration for in vivo use, while the presence of the trifluoromethyl ketone on the DDACTHF scaffold with **1** provides a stable compound suitable for in vivo evaluation.

A crystal structure of **1** with rhGAR Tfase was obtained and the overall structure is represented in Figure 3.[10] Interestingly, even though an inseparable and rapidly equilibrating diastereomeric mixture was used in the crystallization, only the *R* form of **1** was found in the folate-binding site. The inhibitor **1** binds as the gem diol mimicking the formyl transfer intermediate and making extensive interactions with the catalytic residues at the active site (Fig. 4).[10] The gem diol forms hydrogen bonds with several residues present in the active site, especially Asp144, His108, and Gly117. The terminal carboxylate of Asp144 forms two hydrogen bonds with each of the hydroxyls of the gem diol (2.5 and 2.7 Å), while one of the nitrogen atoms of the His108 imidazole also forms two hydrogen bonds with the gem diol (3.1 and 3.2 Å). In addition, the backbone carbonyl of Gly117 forms a 3.0 Å hydrogen bond with the gem diol. These hydrogen bonding interactions were found to be important for the activity since the alcohol variant of 10-CF_3CO-DDACTHF was 60-fold less potent, and DDACTHF, which lacks a C10 substituent and both alcohols of the gem diol, was 115 times less active.[10]

Herein, we report a series of analogues and derivatives of **1** that were synthesized and evaluated to further

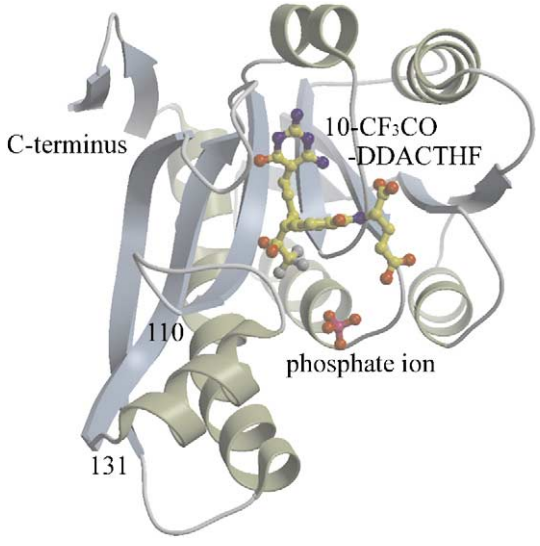


Figure 3.

define the details of the inhibition of GAR Tfase (Fig. 5). The full series of polyglutamated derivatives of **1** (from di- to pentaglutamate, **3–6**) were prepared since it has been observed that inhibitors like **1** are converted to polyglutamated homologues[11–15] by FPGS that are not only retained intracellularly,[15] but that were often more potent enzyme inhibitors than their monoglutamate counterpart.[12–14] Recent studies suggest that the polyglutamate metabolites of DDATHF are the principal species causative of cell growth inhibition, and that DDATHF itself may have minor cytotoxic activity.[12] The pentaglutamate of DDATHF has been reported to be about 11[13] or 100[12] times more potent against human and mouse GAR Tfase, respectively, than DDATHF.

We also describe two key analogues of **1**, one (**7**) that contains an additional carbon in the chain linking the benzoyl glutamate and the diaminopyrimidinone to establish the importance of the length of this spacer. The second (**8**) is an analogue where the trifluoromethyl ketone was replaced by a carboxylic acid. Since the hydrated form of **1** forms several hydrogen bonds with enzyme active site catalytic residues including His108, Asp144, and Gly117,[10] an analogue with a C10 carboxylic acid could similarly bind by forming multiple hydrogen bonds with the active site residues or even the substrate amine.

Chemistry

The di-, tri-, tetra- and pentaglumate analogues of **1** (**3–6**) were prepared from the common intermediate **9**,[10] as presented in Scheme 1. The carboxylic acid **9** was coupled with the appropriate di-*tert*-butyl L-polyglutamate hydrochloride **10**[16] to provide **11–14** (EDCI, $NaHCO_3$, DMF, 25 °C, 24 h, 14–34%). Acid-catalyzed deprotection of **11–14** (1:4 v/v TFA/$CHCl_3$, 24 h, 100%) provided the desired derivatives **3–6**. For comparative purposes, ketone **14** (pentaglutamate) was reduced with $NaBH_4$ (2.0 equiv, CH_3OH, −20 °C, 30 min) followed by acid-catalyzed deprotection (4 N HCl–dioxane, 0–25 °C, 3 h, 50% from **14**) to provide alcohol **15** (Scheme 2).

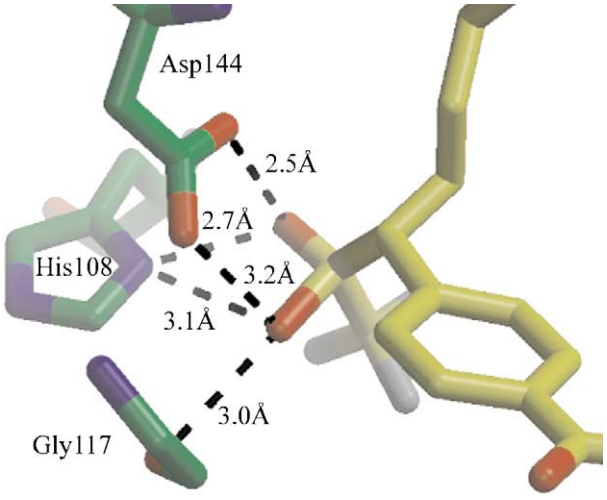
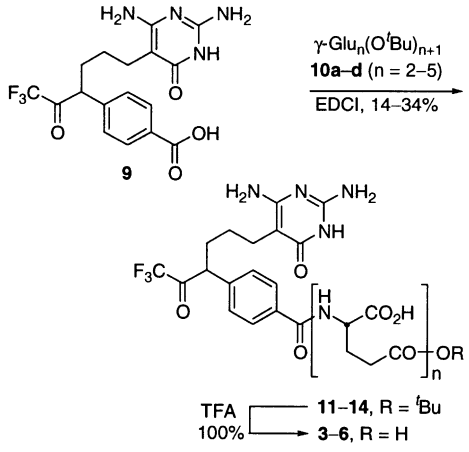


Figure 4.



Scheme 1.

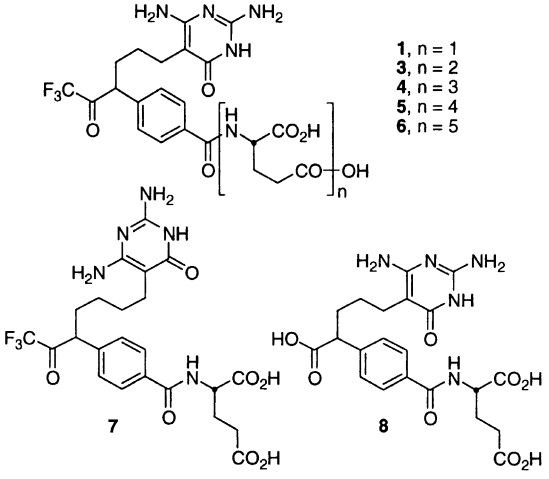
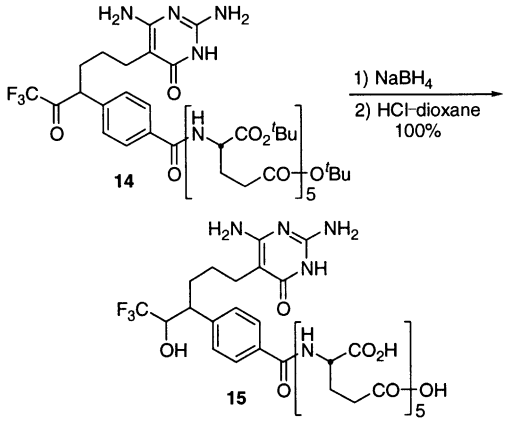


Figure 5.



Scheme 2.

The preparation of **7** follows closely that described for **1**.[10] NaH deprotonation of the known *N*,*N*-dimethylhydrazone **16**[10] (DMF, 0 °C, 15 min) and subsequent treatment with excess 1,4-dibromobutane (10 equiv, DMF, 25 °C, 2.5 h) provided the monoalkylation product **17**. The preformed sodium salt of ethyl cyanoacetate (NaH, DMF, 0 °C, 30 min) was alkylated with **17** to give **18** (DMF, 25 °C, 2 h, 28% from **16**). Cyclization with the free base of guanidine (CH_3OH, reflux, 16 h, 68%) under basic conditions gave the desired pyrimidinone **19**. Treatment of **19** with LiOH (2.1 equiv, 3:1 CH_3OH–H_2O, 25 °C, 24 h) cleanly provided the carboxylic acid **20** which was coupled with di-*tert*-butyl L-glutamate hydrochloride (EDCI, $NaHCO_3$, DMF, 25 °C, 48 h, 36%) to provide **21**. Deprotection of **21** was accomplished by treatment with trifluoroacetic acid (1:4 v/v TFA/$CHCl_3$, 25 °C, 16 h, 100%) to provide **7** (Scheme 3).

The synthesis of **8** was accomplished using a similar procedure. Protection of **22**[17] as a *tert*-butyl ester (^{t}BuOH, DCC, DMAP, 25 °C, 20 h, 44%), followed by NaH deprotonation of **23**[18] (DMF, 0 °C, 15 min) and subsequent treatment with excess 1,3-dibromopropane (10 equiv, DMF, 25 °C, 2.5 h, 58%) provided the monoalkylation product **24**. The preformed sodium salt of ethyl cyanoacetate (NaH, DMF, 0 °C, 30 min) was alkylated with **24** to give **25** (DMF, 25 °C, 2 h, 73%). Cyclization with the free base of guanidine (CH_3OH, reflux, 16 h, 51%) under basic conditions gave the desired pyrimidinone **26**. Treatment of **26** with LiOH (3.0 equiv, 3:1 CH_3OH–H_2O, 25 °C, 6 h) cleanly provided the carboxylic acid **27**, which was coupled with di-*tert*-butyl L-glutamate hydrochloride (EDCI, $NaHCO_3$, DMF, 25 °C, 48 h, 29%) to provide **28**. Deprotection of **28** was accomplished by treatment with trifluoroacetic acid (1:4 v/v TFA/$CHCl_3$, 25 °C, 16 h, 100%) to provide **8** (Scheme 4).

GAR Tfase and AICAR Tfase Inhibition

Compounds **3**–**8** and **15** were tested for inhibition of GAR Tfase and AICAR Tfase and the results are presented in Table 1 along with those derived from **1**, DDACTHF[19] lacking a C10 substituent (Fig. 2), and the key analogues **29**–**30** previously disclosed (Fig. 6).[7,10] The activity of the polyglutamated analogues of **1** against *Escherichia coli* GAR Tfase varied depending upon the length of the glutamate chain. For the derivatives with two (**3**), three (**4**) and four (**5**) glutamates, the K_i's were similar to the monoglutamate (5.6, 10 and 4.8 μM, respectively, vs 1.9 μM for **1**), while the pentaglutamate **6** was 7 times more active ($K_i = 0.27$ μM). The hydroxy derivative pentaglutamate **15** was 34-fold more potent than its monoglutamate **29** ($K_i = 20$ μM) and slightly more potent than the ketone monoglutamate **1** against *E. coli* GAR Tfase (0.58 μM for **15** vs 1.9 μM for **1**). The monoglutamate analogue **7** was only slightly active against *E. coli* GAR Tfase ($K_i = 24$ μM), whereas **8** was inactive in the assay ($K_i > 100$ μM). The same compounds were tested for inhibition of rhGAR Tfase and all of them showed an increase in activity (Table 1). Thus, the remarkable 100-fold selectivity for rhGAR Tfase versus *E. coli* GAR Tfase observed with **1**[10] and earlier with the corresponding aldehyde **2** (10-formyl-DDACTHF),[6] is also observed with the full series of polyglutamates. Importantly, it is this activity, not that of the *E. coli* enzyme, that correlates with the CCRF–CEM cytotoxic potency. Interestingly, each of the polyglutamates exhibited a similar activity against

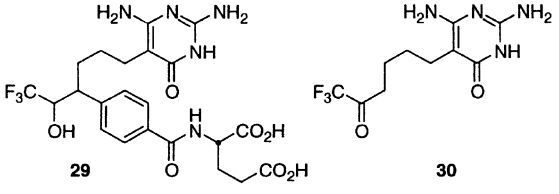
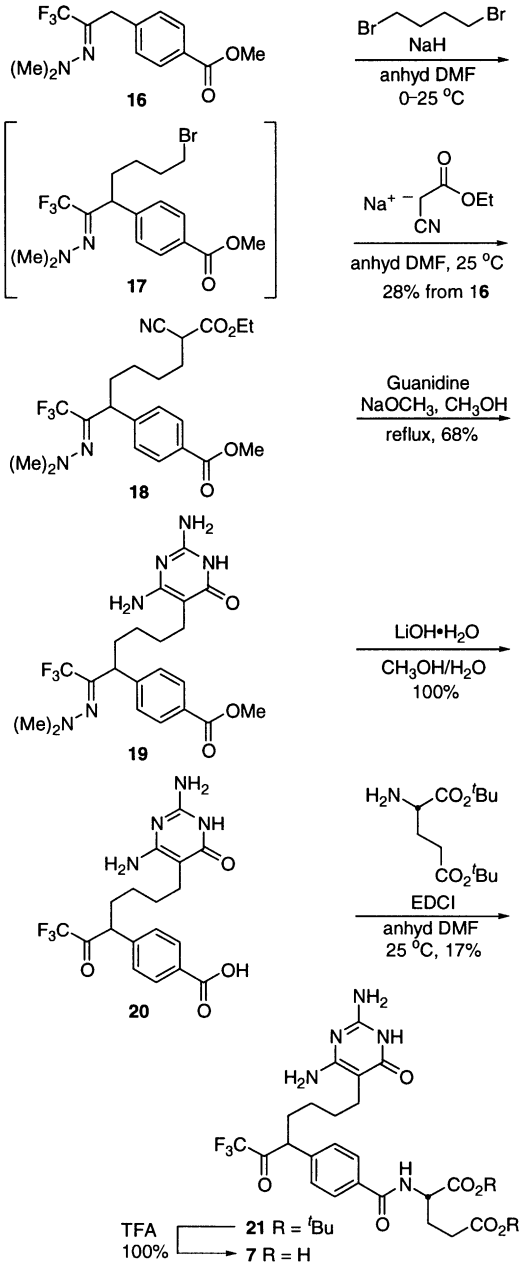
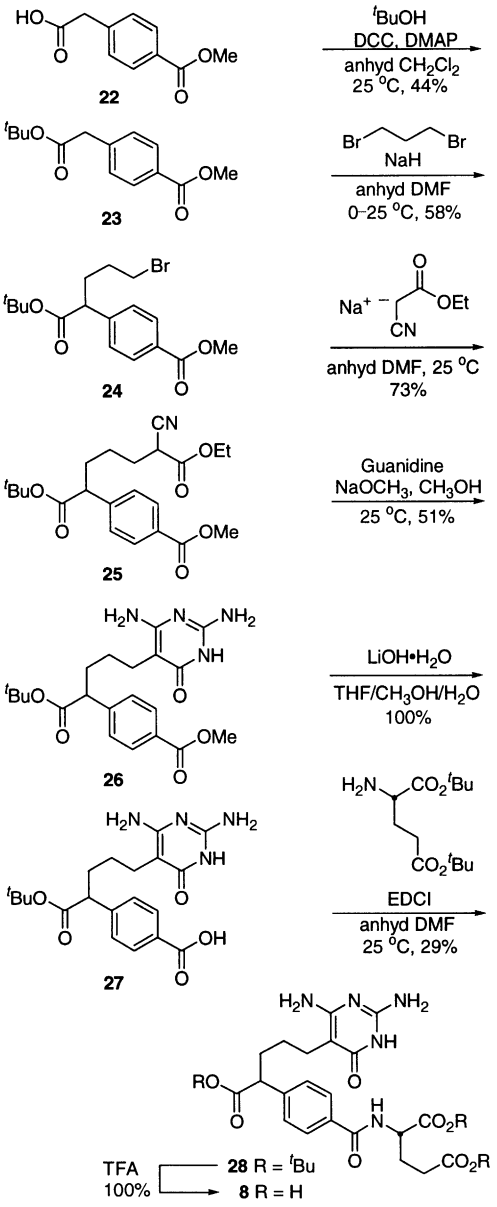


Figure 6.



Scheme 3.



Scheme 4.

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	65
3	5.6	0.019	1.6
4	10	0.010	1.0
5	4.8	0.019	0.18
6	0.27	0.004	0.12
15	0.58	0.04	0.8
29	20	0.9	>100
7	24	22	nd
8	>100	5.3	60
DDACTHF	4.6	1.7	20
Lometrexol	0.1	0.06[d]	1

[a]*E. coli* GAR Tfase.
[b]Recombinant human GAR Tfase.
[c]Recombinant human AICAR Tfase.
[d]ref. 13

rhGAR Tfase, where the di-, tri, tetra- and pentaglutamate had a K_i of 0.019, 0.01, 0.019 and 0.004 μM, respectively, similar to that of the monoglutamate **1** in a parallel assay ($K_i = 0.015$ μM). Thus, the trends follow a well-defined order of $5 > 3 > 1 > 2,4$ glutamates, although the distinctions are very small. The pentaglutamate **6** was 2-fold more potent than the triglutamate **4**, 3-fold more potent than the monoglutamate **1**, and 4–5-fold more potent than the di- and tetraglutamates. These observations do not follow those made with Lometrexol where a much more substantial increase in activity was seen with the addition of each glutamate side chain and where the pentaglutamate was established to be 11- or 100-fold more potent than the monoglutamate DDATHF.[12,13] Identical to trends observed in comparing **1** and **29** (monoglutamates, $K_i = 15$ and 900 nM),[10] the pentaglutamate **15** of the alcohol analogue was 10-fold less potent than the trifluoromethyl ketone pentaglutamate **6** (40 nM vs 4 nM) highlighting the importance of the active site gem diol versus alcohol interaction. Most impressive in this series, the trifluoromethyl ketone **1** ($K_i = 15$ nM) was 60-fold more potent than the corresponding alcohol **29** ($K_i = 900$ nM) and roughly 100-fold more potent than DDACTHF ($K_i = 1.7$ μM) which lacks a C10 substituent, indicating (like the comparison of **6** and **15**) that each hydroxyl group of the bound gem diol increases binding affinity roughly 10-fold. The importance of the benzoylglutamate subunit is defined by a comparison with the simplified trifluoromethyl ketone (**30**)[7] which was inactive against both human and *E. coli* GAR Tfase ($K_i > 100$ μM). Similarly, **9** and its corresponding methyl ester did not inhibit rhGAR Tfase ($K_i > 100$ μM) indicating that the potent inhibitory activity of **1** requires the intact benzoylglutamate including the glutamate subunit.[10] Compounds **1** and **29** were both inactive against rhAICAR Tfase ($K_i > 100$ μM), rhDHFR ($K_i > 100$ μM), and rhTS ($K_i > 100$ μM). The latter observations are consistent with the demonstrations below that **1** and **29** derive potent cytotoxic activity through inhibition of the purine, not pyrimidine, biosynthesis and at a step preceding the action of AICAR Tfase. Interestingly, the glutamate series ($n = 1$–5) did show a progressive increase in potency against rhAICAR Tfase, but each was 10–160 fold less potent for AICAR versus GAR Tfase.

Most surprising in the series of compounds examined was **7**. As observed with *E. coli* GAR Tfase ($K_i = 24$ μM), **7** was only slightly active against rhGAR Tfase ($K_i = 22$ μM) and displayed no selectivity between the two enzymes. This represents a remarkable 1000-fold loss in rhGAR Tfase activity with **7** relative to **1** resulting from the one carbon increased linker length even within a flexible linker. Clearly, the binding of **1** with the enzyme represents achievement of a near optimal interaction.

In the case of **8**, modest activity against rhGAR Tfase ($K_i = 5.3$ μM) was observed, whereas it was inactive against *E. coli* GAR Tfase ($K_i > 100$ μM). Although the activity of **8** is modest, like **1**, it was >20-fold selective for human versus *E. coli* GAR Tfase.

Table 2. In vitro cytotoxic activity

Compd	CCRF–CEM (IC_{50}, μM)			
	(+) T, (+) H[a]	(−) T, (+) H	(+) T, (−) H	(−) T, (−) H
1	>100	>100	0.017	0.016
3	>100	>100	0.5	0.5
4	>100	>100	2.0	1.5
5	>100	>100	2.5	3.0
6	>100	>100	2.0	4.0
15	nd	nd	nd	nd
29	>100	>100	1.4	1.1
7	>100	>100	50	50
8	>100	>100	40	50
DDACTHF	>100	>100	3.6	2.7
Lometrexol	>100	>100	0.52	0.23

[a]T, thymidine; H, hypoxanthine.

Cytotoxic Activity

Compounds **3**–**8** and DDACTHF were examined for cytotoxic activity both in the presence (+) and absence (−) of added thymidine (pyrimidine) and hypoxanthine (purine) against the CCRF–CEM cell line (Table 2). All the analogues of 10-CF_3CO-DDACTHF (**1**) were at least 40-fold less active against the CCRF–CEM cell line than **1**. This was expected for the polyglutamated analogues **3**–**6**, and is due to ineffective cellular penetration. Compound **1** exhibited very potent cytotoxic activity (IC_{50} = 16 nM) against the CCRF–CEM cell line when purines (hypoxanthine) are absent in the media.[10] Moreover, it is 14-fold more potent than Lometrexol (IC_{50} = 230 nM) and both were inactive (IC_{50} > 100 μM) in the presence of media purines. This sensitivity to the presence of purines, but not pyrimidines (thymine), indicates that the cytotoxic activity of **1** is derived from its inhibition of enzymes in the de novo purine biosynthetic pathway. The corresponding alcohol **29** and DDACTHF exhibited cytotoxic activity (IC_{50} = 1.1 and 2.7 μM, respectively) which were also sensitive to the presence of media purines. However, **29** and DDACTHF were ca. 70× and 170× less potent than ketone **1** indicating the potentiation of biological activity conveyed by the electrophilic carbonyl.

AICAR rescue experiments were performed using **1** and **29** in order to further elucidate the source of their cytotoxic activity (Table 3). In each case, the reversal of the cytotoxicity with hypoxanthine (100 μM) or AICAR monophosphate (100 μM) resulted in an ca. 10^3–10^4 increase in the IC_{50} value. This indicates that the activity is being observed through selective inhibition of purine biosynthesis prior to the AICAR Tfase enzymatic step, consistent with the inhibition of GAR Tfase. This selective sensitivity to GAR Tfase is the expected behavior of the inhibitors **1** and **29** based on their inactivity against AICAR Tfase in vitro.

Table 3. Cytotoxic activity in the presence of AICAR

Compd	CCRF–CEM (IC_{50}, μM)	
	(−) T, (−) H, (−) A[a]	(−) T, (−) H, (+) A
1	0.016	>150
29	1.1	>150
Lometrexol	0.23	>150

[a]T = thymidine; H, hypoxanthine, A = AICAR monophosphate (+100 μM).

Table 4. In vitro cytotoxic activity

Compd	CCRF–CEM/MTX (IC_{50}, μM)			
	(+) T, (+) H[a]	(−) T, (+) H	(+) T, (−) H	(−) T, (−) H
1	>100	nd	nd	>100
29	>100	nd	nd	>100
DDACTHF	>100	nd	nd	>100
Lometrexol	>100	nd	nd	>100
Compd	CCRF–CEM/FPGS$^-$ (IC_{50}, μM)			
	(+) T, (+) H[a]	(−) T, (+) H	(+) T, (−) H	(−) T, (−) H
1	>100	nd	nd	>100
29	87	nd	nd	25
DDACTHF	>100	nd	nd	>100
Lometrexol	>100	nd	nd	>100

[a]T = thymidine (+10 μM); H, hypoxanthine (+100 μM).

The extent to which the cytotoxic activity of **1** and **29** was dependent on folate active transport across the cellular membrane was established by assaying against a resistant CCRF–CEM cell line (CCRF–CEM/MTX) shown to have an impaired reduced folate active transport system[20] (Table 4). Like Lometrexol, **1** and **29** lost activity against CCRF–CEM/MTX indicating a functioning reduced folate carrier is required for functional activity and implying they are effective substrates for transport. Similarly, the importance of FPGS polyglutamation to the inhibitors cytotoxic activity was established by examining them against a CCRF–CEM cell line deficient in FPGS (CCRF–CEM/FPGS$^-$).[6] Like Lometrexol, **1** and **29** (to a lesser extent) lacked or lost activity against this cell line (Table 4) indicating polyglutamation is required for activity. Because of the comparable rhGAR Tfase inhibitory activity of **1** with its polyglutamates, this would seem to imply that the polyglutamation requirement serves to promote intracellular accumulation.

As for **7** and **8**, the addition of a carbon between the diaminopyrimidinone and the trifluoromethyl ketone (**7**) or the replacement of this ketone by a carboxylic acid (**8**) had a great impact on the activity. Both exhibited detectable purine sensitive cytotoxic activity that is derived from their GAR Tfase inhibitory properties, but at a level that is >1000-fold less potent than **1** and >10-fold less potent than DDACTHF.

Conclusions

Several derivatives and key analogues of 10-CF_3CO-DDACTHF (**1**) were prepared and evaluated as inhibitors of GAR Tfase and AICAR Tfase. Most prominent among the observations was the unusually selective

($\geq$100-fold) and potent (K_i = 4–20 nM) inhibition of human (vs *E. coli*) GAR Tfase analogous to that observed with **2**[6] and the lack of activity of the analogues against other representative folate dependent enzymes (rhTS, rhDHFR). The polyglutamates of **1** exhibited a well defined trend of $n = 5 > 3 > 1 > 2,4$ against rhGAR Tfase although the distinctions in potency were very modest (K_i = 4, 10, 15, 19 and 19 nM). Consequently, the requirement for FPGS and polyglutamation for observation of the potent, purine sensitive cytotoxic activity of **1**[10] is most likely the result of enhanced intracellular accumulation (retention) and not enhanced enzyme inhibitory potency. The $\geq$10-fold loss in activity resulting from reduction of the ketone to the alcohol **29** and the $\geq$100-fold loss in activity with its removal (DDACTHF) highlights the importance of the electrophilic carbonyl and each of the hydroxyls of the enzyme-bound gem diol.[10] Significantly, the key analogues lacking the benzoylglutamate (**30**) or glutamate (**9** or its methyl ester) subunits were inactive against rhGAR Tfase and CCRF–CEM, whereas the monoglutamate analogue of **1** with a one carbon extension in the flexible spacer (**7**) had a >1000-fold decrease in activity against both rhGAR Tfase (K_i = 22 μM) and CCRF–CEM growth inhibition (IC_{50} = 50 μM) indicating how unique the active site interaction of **1** may be. Similarly, the analogue **8** in which the electrophilic trifluoromethyl ketone was replaced with a carboxylic acid was >100-fold less active against rhGAR Tfase (K_i = 5 μM) and >1000-fold less cytotoxic (IC_{50} = 40 μM) further emphasizing the importance of the trifluoromethyl ketone (gem diol) for activity of **1**.

Experimental

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamic acid tetra-*tert*-butyl ester (11)**. A solution of **9**[10] (23.6 mg, 0.059 mmol) and **10a**[16] (34.3 mg, 0.077 mmol, 1.3 equiv) in DMF (0.5 mL) was treated with $NaHCO_3$ (19.6 mg, 0.23 mmol) followed by EDCI (16.4 mg, 0.09 mmol) and stirred at 25 °C for 48 h. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 9:1 $CHCl_3$/MeOH) provided **11** (13.1 mg, 27%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.72 (m, 2H), 7.39 (m, 2H), 4.47 (m, 1H), 4.27 (m, 2H), 3.86 (m, 1H), 2.31 (m, 17H), 1.42 (m, 23H); MALDIFTMS (DHB) *m*/*z* 825.4007 ($M + H^+$, $C_{39}H_{56}F_3N_6O_{10}$ requires 824.4004).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid hexa-*tert*-butyl ester (12)**. A solution of **9**[10] (24.4 mg, 0.061 mmol) and **10b**[16] (46.7 mg, 0.074 mmol, 1.2 equiv) in DMF (0.5 mL) was treated with $NaHCO_3$ (15.2 mg, 0.181 mmol) followed by EDCI (14.7 mg, 0.077 mmol) and stirred at 25 °C for 48 h. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 9:1 $CHCl_3$/MeOH) provided **12** (21.2 mg, 34%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.90 (m, 2H), 7.40 (m, 2H), 4.47 (m, 1H), 4.26 (m, 3H), 3.61 (m, 1H), 2.31 (m, 23H), 1.42 (m, 30H); MALDIFTMS (DHB) *m*/*z* 1032.4885 ($M + Na^+$, $C_{48}H_{70}F_3N_7O_{13}Na$ requires 1032.4876).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid octa-*tert*-butyl ester (13)**. A solution of **9**[10] (28.6 mg, 0.072 mmol) and **10c**[16] (83.9 mg, 0.10 mmol, 1.4 equiv) in DMF (0.5 mL) was treated with $NaHCO_3$ (20.0 mg, 0.24 mmol) followed by EDCI (20.1 mg, 0.10 mmol) and stirred at 25 °C for 48 h. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 9:1 $CHCl_3$/MeOH) provided **13** (11.9 mg, 14%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.87 (m, 2H), 7.39 (m, 2H), 4.52 (m, 1H), 4.18 (m, 4H), 3.60 (m, 1H), 2.31 (m, 29H), 1.42 (m, 37H); MALDIFTMS (DHB) *m*/*z* 1195.6106 ($M + Na^+$, $C_{66}H_{100}F_3N_9O_{19}Na$ requires 1195.6108).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid deca-*tert*-butyl ester (14)**. A solution of **9**[10] (24.2 mg, 0.061 mmol) and **10d**[16] (66.6 mg, 0.067 mmol, 1.0 equiv) in DMF (0.5 mL) was treated with $NaHCO_3$ (16.8 mg, 0.2 mmol) followed by EDCI (36.6 mg, 0.19 mmol) and stirred at 25 °C for 48 h. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. Chromatography (SiO_2, 9:1 $CHCl_3$/MeOH) provided **14** (13.6 mg, 16%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.82 (m, 2H), 7.40 (m, 2H), 4.59 (m, 1H), 4.31 (m, 5H), 3.60 (m, 1H), 2.31 (m, 34H), 1.42 (m, 42H); MALDIFTMS (DHB) *m*/*z* 1402.6959 ($M + Na^+$, $C_{66}H_{100}F_3N_9O_{19}Na$ requires 1402.6979).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamic acid (3)**. A solution of **11** (11.1 mg, 0.01 mmol) in $CHCl_3$ (1.0 mL), cooled to 0 °C, was treated with trifluoroacetic acid (0.3 mL). The solution was stirred 25 °C for 12 h before being concentrated under reduced pressure. The solid residue was triturated with ether and dried in vacuo to give **3**–CF_3CO_2H (10.0 mg, 100%) as a tan solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.54 (m, 2H), 7.18 (m, 2H), 4.36 (s, 1H), 4.22 (m, 2H), 2.21 (m, 15H); MALDIFTMS (DHB) *m*/*z* 679.1972 ($M + Na^+$, $C_{27}H_{31}F_3N_6O_{10}Na$ requires 679.1946).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid (4)**. A solution of **12** (20.1 mg, 0.02 mmol) in $CHCl_3$ (0.8 mL), cooled to 0 °C, was treated with trifluoroacetic acid (0.2 mL). The solution was stirred 25 °C for 12 h before being concentrated under reduced pressure. The solid residue was triturated

with ether and dried in vacuo to give **4**–CF_3CO_2H (17.9 mg, 100%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.76 (m, 2H), 7.40 (m, 2H), 4.61 (s, 1H), 4.42 (m, 3H), 2.22 (m, 19H); MALDIFTMS (DHB) *m/z* 786.2576 ($M+H^+$, $C_{32}H_{39}F_3N_7O_{13}$ requires 786.2552).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid (5)**. A solution of **13** (10.1 mg, 0.008 mmol) in $CHCl_3$ (0.8 mL), cooled to 0 °C, was treated with trifluoroacetic acid (0.2 mL). The solution was stirred 25 °C for 12 h before being concentrated under reduced pressure. The solid residue was triturated with ether and dried in vacuo to give **5**–CF_3CO_2H (8.7 mg, 100%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.88 (m, 2H), 7.37 (m, 2H), 4.61 (s, 1H), 4.39 (m, 4H), 2.26 (m, 22H); MALDIFTMS (DHB) *m/z* 937.2782 ($M+Na^+$, $C_{37}H_{45}F_3N_8O_{16}Na$ requires 937.2798).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid (6)**. A solution of **14** (13.6 mg, 0.001 mmol) in $CHCl_3$ (0.8 mL), cooled to 0 °C, was treated with trifluoroacetic acid (0.2 mL). The solution was stirred 25 °C for 12 h before being concentrated under reduced pressure. The solid residue was triturated with ether and dried in vacuo to give **6**–CF_3CO_2H (8.0 mg, 100%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.81 (m, 2H), 7.36 (m, 2H), 4.64 (s, 1H), 4.41 (m, 5H), 2.41 (m, 26H); MALDIFTMS (DHB) *m/z* 1044.3406 ($M+H^+$, $C_{42}H_{53}F_3N_9O_{19}$ requires 1044.3404).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoro-1-hydroxymethyl)but-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid (15)**. A solution of **14** (7.9 mg, 0.0057 mmol) in anhydrous CH_3OH (0.5 mL) at −20 °C was treated with $NaBH_4$ (1.5 mg, 0.004 mmol, 0.7 equiv). The reaction mixture was stirred at −20 °C for 30 min before it was quenched by the addition of H_2O (1 mL). The mixture was diluted with EtOAc (5 mL) and washed with H_2O (2×1 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The resulting product was treated with 4 N HCl–dioxane (2 mL) at 0 °C, and the solution was allowed to warm and stir at 25 °C for 3 h. The reaction was purged with N_2 and then concentrated under reduced pressure. Et_2O (1 mL) was added and a precipitate formed. The precipitate was collected, triturated with Et_2O (3×1 mL), and dried in vacuo to give **15**–HCl (6.6 mg, 100% from **14**) as a yellow solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.78 (m, 2H), 7.37 (m, 2H), 4.41 (m, 6H), 2.41 (m, 26H); MALDIFTMS (DHB) *m/z* 1046.3563 ($M+H^+$, $C_{42}H_{55}F_3N_9O_{19}$ requires 1046.3561).

Methyl 4-[5-bromo-1-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pent-2-yl]benzoate (17). NaH (60% dispersion, 0.13 g, 3.21 mmol, 1.0 equiv) was added to a stirred solution of **16**[10] (0.87 g, 3.03 mmol) in anhydrous DMF (15 mL) at 0 °C. The solution was stirred at 0 °C for 15 min. 1,4-Dibromobutane (2.20 mL, 18.4 mmol, 6.0 equiv) was added quickly to the reaction mixture and the cooling bath was removed. The reaction mixture was stirred at 25 °C for 2.5 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl (15 mL). The reaction mixture was partitioned between EtOAc (50 mL) and H_2O (40 mL). The organic layer was washed with H_2O (3×50 mL) and saturated aqueous NaCl (1×50 mL) followed by concentration under reduced pressure. Chromatography (SiO_2, 7:1 hexanes/EtOAc) afforded **17** and this product was used without further purification.

Methyl 4-[5-cyano-5-ethoxycarbonyl-1-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pent-2-yl]benzoate (18). A suspension of NaH (60% dispersion, 1.3 g, 32 mmol, 18 equiv) in anhydrous DMF (20 mL) at 0 °C was treated dropwise with ethyl cyanoacetate (3.9 mL, 37 mmol, 18 equiv). 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 **17** (crude, 0.76 g) in anhydrous DMF (20 mL). The reaction mixture was stirred at 25×°C for 2 h before being quenched by the addition of saturated aqueous NH_4Cl (5 mL). The reaction mixture was diluted with EtOAc (50 mL) and washed with H_2O (3×50 mL) and saturated aqueous NaCl (50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The excess ethyl cyanoacetate was distilled off and the residual product was purified by chromatography (SiO_2, 7:1 hexanes/EtOAc) affording **18** (0.39 g, 28% from **16**) as a yellow oil: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.98 (d, $J=8.5$ Hz, 2H), 7.35 (d, $J=8.2$ Hz, 2H), 4.47 (t, $J=7.7$ Hz, 1H), 4.35 (m, 2H), 3.86 (s, 3H), 3.49 (m, 2H), 2.59 (s, 6H), 1.96 (m, 9H), 1.37 (m, 5H); MALDIFTMS (DHB) *m/z* 456.2101 ($M+H^+$, $C_{22}H_{29}F_3N_3O_4$ requires 456.2105).

Methyl 4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pent-2-yl]benzoate (19). Sodium metal (0.027 g, 1.16 mmol, 2.0 equiv) was added to anhydrous CH_3OH (4 mL) and the reaction mixture was stirred at 25 °C for 10 min to generate $NaOCH_3$. Guanidine–HCl (0.055 g, 0.58 mmol, 1.0 equiv) was added and the reaction mixture was stirred at 25 °C for 30 min. Separately, **18** (0.39 g, 0.58 mmol) was dissolved in anhydrous CH_3OH (2 mL) and this solution was added quickly to the stirring reaction mixture. The resulting reaction mixture was stirred at reflux for 16 h. The reaction mixture was applied directly to a SiO_2 plug. Impurities were removed by washing with 3:1 hexanes/EtOAc. The product was subsequently eluted by washing with 10:1 $CHCl_3$/CH_3OH to afford **19** (0.18 g, 68%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.96 (d, $J=8.4$ Hz, 2H), 7.41 (d, $J=8.4$ Hz, 2H), 4.80 (t, $J=7.7$ Hz, 1H), 3.88 (s, 3H), 2.65 (s, 6H), 2.34 (m, 3H), 1.52 (m, 5H); MALDIFTMS (DHB) *m/z* 491.1996 ($M+Na^+$, $C_{21}H_{27}F_3N_6O_3Na$ requires 491.1989).

4-[5-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoro-acetyl)pent-2-yl]benzoic acid (20). A solution of **19** (0.18 g, 0.39 mmol) in 3:1 CH_3OH–H_2O (8 mL) was treated with LiOH–$1H_2O$ (0.054 g, 1.3 mmol, 3.3 equiv) and the reaction mixture was stirred at

25 °C for 24 h. The reaction mixture was diluted with H_2O (10 mL) and the aqueous layer was washed with EtOAc (10 mL). The aqueous layer was acidified to pH = 4 by the addition of 1 N aqueous HCl. The reaction mixture was concentrated under reduced pressure and the residue was treated with MeCN (3×10 mL) to remove traces of H_2O to provide **20** (0.16 g, 100%) which was used without further purification: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.95 (m, 2H), 7.32 (m, 2H), 4.26 (m, 1H), 2.15 (t, J = 6.8 Hz, 2H), 1.38 (m, 6H); MALDIFTMS (DHB) m/z 413.1453 ($M+H^+$, $C_{18}H_{20}F_3N_4O_4$ requires 413.1431).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(2,2,2-trifluoroacetyl)pent-2-yl]benzoyl}-L-γ-glutamic acid di-*tert*-butyl ester (21)**. A solution of **20** (0.24 g, 0.58 mmol) and di-*tert*-butyl L-glutamate hydrochloride (0.26 g, 0.88 mmol, 1.5 equiv) in DMF (8 mL) was treated with $NaHCO_3$ (0.30 g, 3.62 mmol, 6.0 equiv) followed by EDCI (0.36 g, 1.86 mmol, 3.2 equiv). The reaction mixture was stirred at 25 °C for 48 h. The reaction mixture was partitioned between EtOAc (20 mL) and H_2O (20 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (2×20 mL) and saturated aqueous NaCl (1×20 mL) followed by concentration under reduced pressure. Chromatography (SiO_2, 10:1 $CHCl_3$/CH_3OH) afforded **21** (0.066 g, 17%) as a yellow solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.71 (m, 2H), 7.37 (m, 2H), 4.50 (m, 1H), 3.83 (m, 1H), 2.42 (m, 8H), 1.43 (m, 18H); MALDIFTMS (DHB) m/z 676.2934 ($M+Na^+$, $C_{31}H_{42}F_3N_5O_7Na$ requires 676.2928).

***N*-{4-[5-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(2,2,2-trifluoroacetyl)pent-2-yl]benzoyl}-L-γ-glutamic acid (7)**. A solution of **21** (53.4 mg, 0.08 mmol) in $CHCl_3$ (4 mL) cooled to 0 °C was treated with trifluoroacetic acid (1 mL). The reaction mixture was allowed to warm and stirred at 25 °C for 16 h. The reaction was concentrated under reduced pressure. Et_2O (1 mL) was added and a precipitate formed. The precipitate was collected, triturated with Et_2O (3×1 mL) and dried in vacuo to give **7**–CF_3CO_2H (53.9 mg, 100%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.88 (m, 2H), 7.36 (m, 2H), 4.64 (m, 1H), 4.27 (m, 1H), 2.33 (m, 12H); MALDIFTMS (DHB) m/z 542.1853 ($M+H^+$, $C_{23}H_{27}F_3N_5O_7$ requires 542.1857).

Methyl 4-(*tert*-butylbutoxycarbonylethyl)benzoate (23)[18]. Known acid **22**[17] (1.03 g, 5.31 mmol) was dissolved in anhydrous CH_2Cl_2 (200 mL). *tert*-Butanol (1.87 g, 25.3 mmol) and *N*,*N*-dimethylaminopyridine (1.72 g, 8.34 mmol) were added slowly and the solution was stirred at 25 °C for 10 min. DCC (0.40 g, 3.30 mmol) was added at 0 °C and the reaction mixture was stirred at 25 °C for 20 h. The mixture was concentrated under reduced pressure and the product was purified by chromatography (SiO_2, 7:1 hexanes/EtOAc) afforded **23** (0.58 g, 44%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.06 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 3.98 (s, 3H), 3.66 (s, 2H), 1.49 (s, 9H).

Methyl 4-[4-bromo-1-(*tert*-butylbutoxycarbonylethyl)but-2-yl]benzoate (24). NaH (60% dispersion, 0.025 g, 0.63 mmol, 1.6 equiv) was added to a stirred solution of **23** (0.10 g, 0.40 mmol) in anhydrous DMF (2.5 mL) at 0 °C. The solution was stirred at 0 °C for 15 min. 1,3-Dibromopropane (0.25 mL, 2.5 mmol, 6.0 equiv) was added quickly to the reaction and the cooling bath was removed. The reaction mixture was stirred at 25 °C for 2.5 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl (15 mL). The reaction mixture was partitioned between EtOAc (50 mL) and H_2O (40 mL). The organic layer was washed with H_2O (3×50 mL) and saturated aqueous NaCl (1×50 mL) followed by concentration under reduced pressure. Chromatography (SiO_2, 9:1 hexanes/EtOAc) afforded **24** (0.086 g, 58%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98 (d, J = 7.9 Hz, 2H), 7.34 (d, J = 7.9 Hz, 2H), 3.90 (s, 3H), 3.50 (t, J = 7.1 Hz, 1H), 3.38 (t, J = 6.2 Hz, 2H), 2.19 (m, 2H), 1.83 (m, 2H), 1.37 (m, 9H); MALDIFTMS (DHB) m/z 371.0850 ($M+H^+$, $C_{17}H_{24}BrO_4$ requires 371.0852).

Methyl 4-[4-cyano-5-ethoxycarbonyl-1-(*tert*-butylbutoxycarbonylethyl)but-2-yl]benzoate (25). A suspension of NaH (60% dispersion, 29 mg, 0.72 mmol, 3.0 equiv) in anhydrous DMF (3 mL) at 0 °C was treated dropwise with ethyl cyanoacetate (75 μL, 0.70 mmol, 3.0 equiv). 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 **24** (86 mg, 0.23 mmol) in anhydrous DMF (3 mL). The reaction mixture was stirred at 25 °C for 2 h before being quenched by the addition of saturated aqueous NH_4Cl (1 mL). The reaction mixture was diluted with EtOAc (10 mL) and washed with H_2O (3×10 mL) and saturated aqueous NaCl (10 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The excess ethyl cyanoacetate was distilled off and the residual product was purified by chromatography (SiO_2, 4:1 hexanes/EtOAc) affording **25** (68 mg, 73%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98 (d, J = 8.3 Hz, 2H), 7.33 (d, J = 8.3 Hz, 2H), 4.26 (q, J = 7.7 Hz, 2H), 3.89 (s, 3H), 3.48 (m, 2H), 1.79 (m, 6H), 1.36 (s, 9H), 1.29 (t, J = 7.7 Hz, 3H); MALDIFTMS (DHB) m/z 426.1874 ($M+Na^+$, $C_{22}H_{29}NO_6Na$ requires 426.1887).

Methyl 4-[4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(*tert*-butylbutoxycarbonylethyl)but-2-yl]benzoate (26). Sodium methoxide (22 mg, 0.41 mmol, 2.4 equiv) was added to anhydrous CH_3OH (1 mL) and the reaction mixture was stirred at 25 °C for 10 min. Guanidine–HCl (20 mg, 0.21 mmol, 1.2 equiv) was added and the reaction mixture was stirred at 25 °C for 30 min. Separately, **25** (68 mg, 0.17 mmol) was dissolved in anhydrous CH_3OH (1 mL) and this solution was added quickly to the stirring reaction mixture. The resulting reaction mixture was stirred at reflux for 16 h. The reaction mixture was applied directly to a SiO_2 plug. Impurities were removed by washing with 3:1 hexanes/EtOAc. The product was subsequently eluted by washing with 10:1 $CHCl_3$/CH_3OH to afford **26** (36 mg, 51%) as a tan solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.96 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 3.90 (s, 3H), 3.63 (m, 1H), 2.33 (t, J = 7.4 Hz, 1H), 2.10 (s, 1H), 1.70 (m, 2H), 1.41 (m, 11H); MALDIFTMS

(DHB) m/z 439.1941 ($M+Na^+$, $C_{21}H_{28}N_4O_5Na$ requires 439.1952).

4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(*tert*-butylbutoxycarbonylethyl)but-2-yl]benzoic acid (27). A solution of **26** (10 mg, 0.02 mmol) in 3:1 THF–CH_3OH (0.4 mL) was treated with LiOH–$1H_2O$ (3.5 mg, 0.08 mmol, 3.0 equiv) dissolved in H_2O (0.1 mL) and the reaction mixture was stirred at 25 °C for 6 h. The aqueous layer was acidified by the addition of 1 N aqueous HCl (0.1 mL). The reaction mixture was partitioned between EtOAc (5 mL) and H_2O (5 mL). The organic layer was dried on Na_2SO_4 followed by concentration under reduced pressure to provide **27** as a tan solid (8.0 mg, 100%): ^1H NMR (CD_3OD, 400 MHz) δ 8.04 (m, 2H), 7.48 (m, 2H), 3.67 (m, 1H), 2.08 (m, 4H), 1.46 (m, 11H); MALDIFTMS (DHB) m/z 425.1779 ($M+Na^+$, $C_{20}H_{26}N_4O_5Na$ requires 425.1795).

***N*-{4-[4-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(*tert*-butylbutoxycarbonylethyl)but-2-yl]benzoyl}-L-γ-glutamic acid di-*tert*-butyl ester (28).** A solution of **27** (4.0 mg, 0.011 mmol) and di-*tert*-butyl L-glutamate hydrochloride (7.4 mg, 0.025 mmol, 2 equiv) in DMF (0.1 mL) was treated with $NaHCO_3$ (3.0 mg, 0.036 mmol, 3.0 equiv) followed by EDCI (4.4 mg, 0.023 mmol, 2.0 equiv). The reaction mixture was stirred at 25 °C for 48 h. The reaction mixture was partitioned between EtOAc (1.0 mL) and H_2O (1.0 mL). The organic layer was washed with saturated aqueous $NaHCO_3$ (2×1.0 mL) and saturated aqueous NaCl (1×1.0 mL) followed by concentration under reduced pressure. Chromatography (SiO_2, 10:1 $CHCl_3/CH_3OH$) afforded **28** (1.9 mg, 29%) as a yellow solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.71 (m, 2H), 7.36 (m, 2H), 4.18 (m, 2H), 2.33 (m, 9H), 1.49 (m, 28H); MALDIFTMS (DHB) m/z 666.3464 ($M+Na^+$, $C_{33}H_{49}N_5O_8Na$ requires 666.3473).

***N*-{4-[4-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(carboxyethyl)but-2-yl]benzoyl}-L-γ-glutamic acid (8).** A solution of **28** (2.8 mg, 0.0044 mmol) in $CHCl_3$ (0.3 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.1 mL). The reaction mixture was allowed to warm and stirred at 25 °C for 16 h. The reaction was concentrated under reduced pressure. Et_2O (0.5 mL) was added and a precipitate formed. The precipitate was collected, triturated with Et_2O (3×0.5 mL) and dried in vacuo to give **8**-CF_3CO_2H (2.8 mg, 100%) as a white solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.80 (m, 2H), 7.42 (m, 2H), 4.62 (m, 1H), 3.66 (m, 1H), 2.33 (m, 8H); MALDIFTMS (DHB) m/z 498.1590 ($M+Na^+$, $C_{21}H_{25}N_5O_8Na$ requires 498.1595).

Recombinant human GAR Tfase protein preparation. The recombinant human GAR Tfase construct includes residues 808–1010 from human trifunctional enzyme (purD-purM-purN). The gene was subcloned into pet22b vector using NdeI/XhoI cloning site with hexahistine tag at the C-terminus. The plasmid was transformed into the *E. coli* expression strain BL21 (DE3) Gold. The protein was expressed and purified as described previously.[21] The yield of the protein is greater than 30 mg per liter LB broth after purification with at lease 98% purity when assessed by SDS-PAGE. The purified protein was used in the inhibition assays, cytotoxic assays and crystallization experiments.

GAR and AICAR Tfase inhibition assay. The K_i values for the folate analogues were measured as previously described.[10] For the GAR Tfase inhibition assay, briefly, each compound was dissolved in DMSO and then diluted in assay buffer. The concentration of DMSO did not affect enzyme activity. Thus, all assays were conducted by mixing 10 μM of fDDF, 20 μM of inhibitor in total volumn 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 (4, 8, 12, 16, 20, 32 μM) were generated in order to determine K_i using the Michaelis–Menton equation for competitive inhibition. The results for the inhibition assays are summarized in Table 1.

Cytotoxic assay. The cytotoxic activity of the compounds was measured using CCRF–CEM human leukemia cells as described previously.[10]

Acknowledgements

We gratefully acknowledge the financial support of the National Institutes of Health (CA 63536, to D.L.B., I.A.W., and S.J.B.), the Skaggs Institute for Chemical Biology, and a Skaggs predoctoral fellowship (Y.Z.).

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