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Synthesis and biological evaluation of *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoyl}-L-glutamic acid as a potential inhibitor of GAR Tfase and the de novo purine biosynthetic pathway

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

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

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Abstract—The synthesis and evaluation of *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoyl}-L-glutamic acid (**2**) as an inhibitor of glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) are reported. The inhibitor **2** was prepared in a convergent synthesis involving C-alkylation of methyl 4-(4,4,4-trifluoro-3-dimethylhydrazonobutyl)benzoate with 1-chloro-3-iodopropane followed by construction of the pyrimidinone ring. Compound **2** was found to be an effective inhibitor of recombinant human GAR Tfase (K_i = 0.50 μM), whereas it was inactive (K_i > 100 μM) against *E. coli* GAR Tfase as well as recombinant human AICAR Tfase. Compound **2** exhibited modest, purine-sensitive growth inhibitory activity against the CCRF-CEM cell line (IC_{50} = 6.0 μM).

1. Introduction

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 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] Since purines are required components of DNA and RNA, inhibition of enzymes in the purine biosynthetic pathway has been found to be an effective approach for antineoplastic intervention. Notably, the disclosure that (6*R*)-5,10-dideazatetrahydrofolate (Lometrexol, (6*R*)-DDATHF, Fig. 2) is an efficacious antitumor agent that acts as an effective inhibitor of GAR Tfase (K_i = 0.1 μM) established inhibition of purine biosynthesis and GAR Tfase as viable targets for antineoplastic intervention.[2–4]

Herein, we report the synthesis and evaluation of *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoyl}-L-glutamic acid (**2**), an analogue of the potent and selective GAR Tfase inhibitor 10-CF_3CO-DDACTHF (**1**) (Fig. 2).

2. Inhibitor design

In previous studies, we have disclosed studies that examined folate-based inhibitors which incorporate

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

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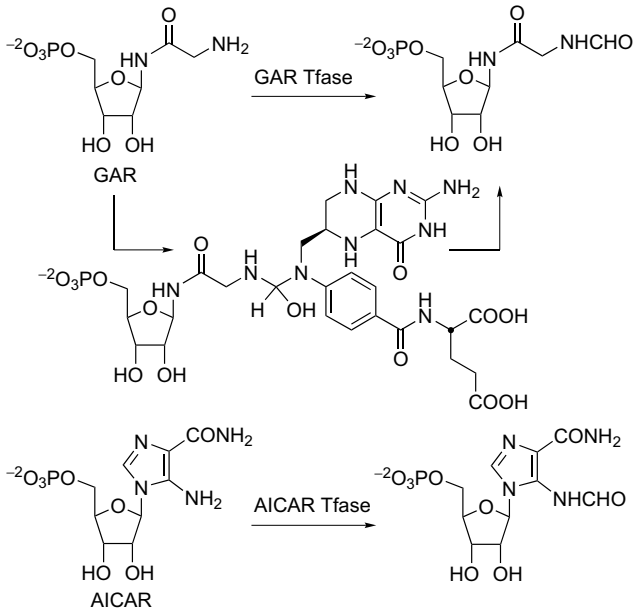


Figure 1.

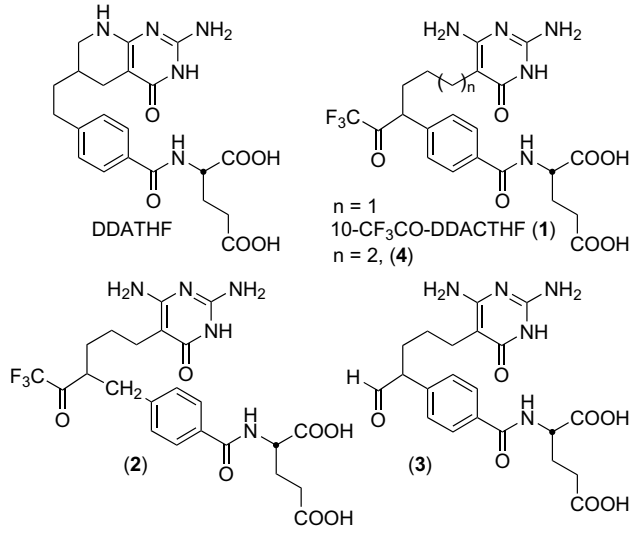


Figure 2.

electrophilic functional groups that could potentially interact either with active site nucleophiles or the GAR/AICAR substrate amines.[5–8] The most significant of these were the folate-based inhibitors 10-formyl-DDACTHF (**3**)[8] and 10-CF_3CO-DDACTHF (**1**)[9,10] (Fig. 2), bearing a nontransferable formyl or trifluoroacetyl group, which both proved to be potent GAR Tfase inhibitors. X-ray and NMR studies of the inhibitor–enzyme complexes revealed that both inhibitors bound as their *gem*-diols.[10,11] The formation of the *gem*-diol mimics the formyl transfer reaction tetrahedral intermediate and provides strong hydrogen bond interactions between the inhibitor and protein.

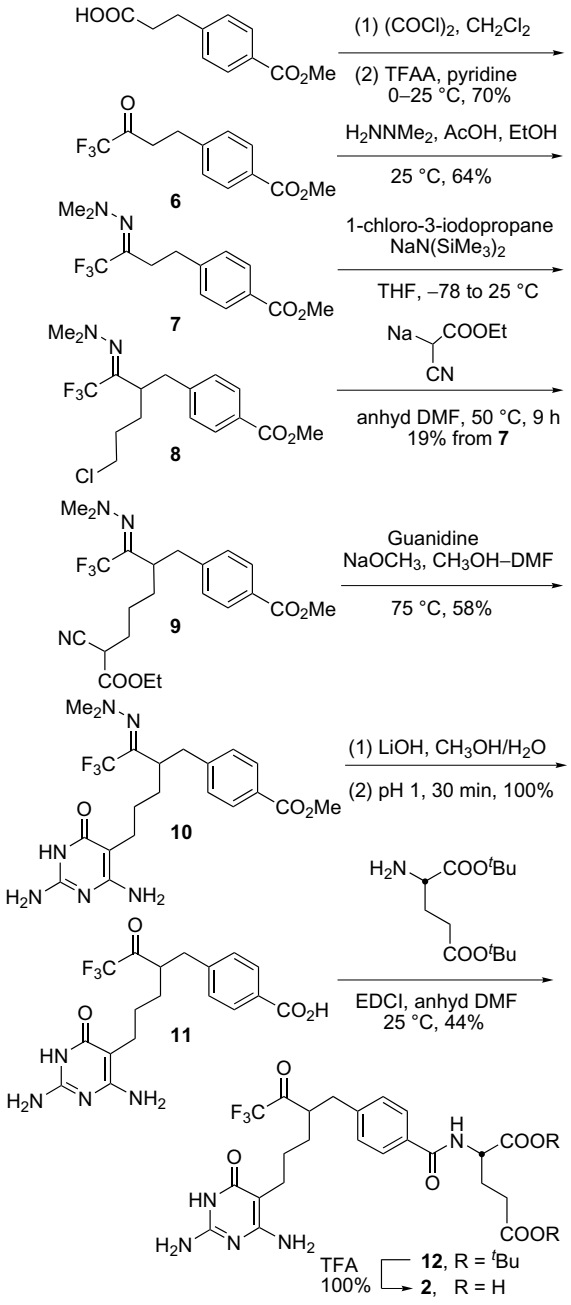
Both 10-formyl-DDACTHF (**3**) and 10-CF_3CO-DDACTHF (**1**) were shown to be selective and potent GAR Tfase inhibitors (**3**, K_i = 0.014 μM against rhGAR Tfase; **1**, K_i = 0.015 μM against rhGAR Tfase).[8–11] Both were found to be effectively transported into the cell by the reduced folate carrier and polyglutamated by FPGS, which contributes to their cytotoxic activity by enhancing their intracellular accumulation (**3**, CCRF-CEM IC_{50} = 60 nM; **1**, CCRF-CEM IC_{50} = 16 nM).[8–11] Notably, 10-CF_3CO-DDACTHF (**1**) is the most potent and selective GAR Tfase disclosed to date, being >10-fold more potent than Lometrexol, and, unlike **3**, is suitably stable for in vivo evaluation.[10]

A preceding analogue of **1**, compound **4** which contains an additional carbon in the chain linking the trifluoromethyl ketone and the diaminopyrimidinone, was recently reported.[9] The analogue **4** with this one carbon extension in the flexible spacer exhibited a >1000-fold decrease in activity against both rhGAR Tfase (K_i = 22 μM) and CCRF-CEM cell growth inhibition (IC_{50} = 50 μM). Herein, we describe the synthesis and evaluation of *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)-pentyl]benzoyl}-L-glutamic acid (**2**, Fig. 2), an analogue of **1** which contains an additional carbon between C10 and the benzoyl glutamate.

3. Chemistry

The synthesis of **2** was accomplished in a convergent manner as presented in Scheme 1.[8–11] 3-(4-Carbomethoxyphenyl)propanic acid[12] was treated with oxalyl chloride in CH_2Cl_2 to afford the corresponding acid chloride. Without further purification, the acid chloride was converted to trifluoromethyl ketone **6** by reaction with trifluoroacetic anhydride (pyridine, CH_2Cl_2, 0 °C, 20 min) followed by an aqueous quench (70%).[13] Reaction of **6** with *N*,*N*-dimethylhydrazine (glacial AcOH, anhydrous EtOH, 25 °C, 24 h, 64%) provided the key *N*,*N*-dimethylhydrazone **7**. Sodium bis(trimethylsilyl)amide deprotonation of **7** (THF, −78 °C) and subsequent treatment with excess 1-chloro-3-iodopropane (10 equiv, THF, 25 °C, 1 h) provided the monoalkylation product **8**, inseparable from **7**. The use of 1,3-dibromopropane in place of 1-chloro-3-iodopropane gave only elimination versus alkylation product. α-Alkylation of the dimethylhydrazone versus alkylation at the acidic benzylic position was confirmed by ^1H COSY NMR of **8** and related intermediates. The preformed sodium salt of ethyl cyanoacetate (NaH, DMF, 0 °C, 30 min) was alkylated with **8** (DMF, 50 °C, 9 h, 19% for two steps from **7**) to give **9**, and treatment with free base guanidine (1.2 equiv, CH_3OH–DMF, 75 °C, overnight, 58%) under basic conditions gave the desired pyrimidinone **10**.

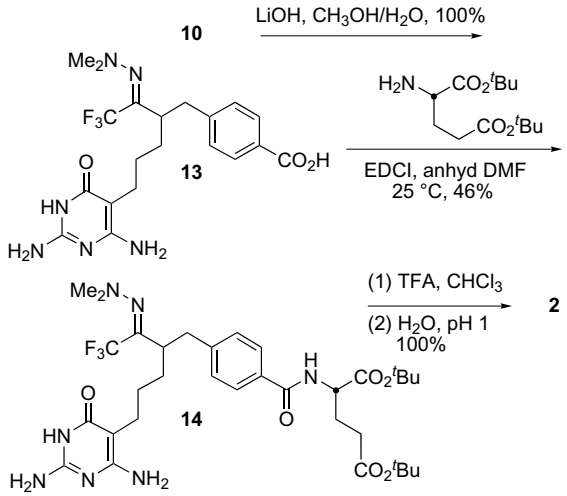
Methyl ester hydrolysis and cleavage of the hydrazone was accomplished by treatment of **10** with LiOH (3 equiv, CH_3OH–H_2O 3:1, 25 °C, overnight) followed by acidification with aqueous 1 N HCl to pH 1 (30 min, 100%) to provide **11**, which was coupled with di-*tert*-butyl L-glutamate hydrochloride (1.5 equiv, EDCI, $NaHCO_3$, DMF, 25 °C, overnight, 44%) to pro-



Scheme 1.

vide **12** as a 1:1 mixture of the inseparable diastereomers. Deprotection of **12** was accomplished by treatment with trifluoroacetic acid (TFA–$CHCl_3$, 25 °C, overnight, 100%) to provide *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)-pentyl]benzoyl}-L-glutamic acid (**2**).

The synthesis of **2** was accomplished by an alternative route as presented in Scheme 2 that also provided access to additional intermediates that still contain the *N*,*N*-dimethylhydrazone. Treatment of **10** with lithium hydroxide (3 equiv, CH_3OH–H_2O 3:1, 25 °C, overnight, 100%) provided the carboxylic acid **13**, which was coupled with di-*tert*-butyl L-glutamate hydrochloride (1.5 equiv, EDCI, $NaHCO_3$, DMF, 25 °C, overnight,



Scheme 2.

46%) to provide **14** as a 1:1 mixture of the inseparable diastereomers. Deprotection of **14** was accomplished by treatment with trifluoroacetic acid (TFA–$CHCl_3$, 25 °C, overnight) followed by acidification to pH 1 (30 min, 100%) to provide **2**.

4. GAR Tfase and AICAR Tfase inhibition

Compound **2** was tested for inhibition of *E. coli* GAR Tfase, recombinant human GAR Tfase, and recombinant human AICAR Tfase, and the results are presented in Table 1. Compound **2** exhibited selective and potent inhibition of recombinant human GAR Tfase (K_i = 0.50 μM), whereas it was inactive against *E. coli* GAR Tfase (K_i > 100 μM). This unusual selective inhibition of human GAR Tfase was also observed with **1** and **3**. Compound **2** was roughly 10-fold less potent than Lometrexol, and 30-fold less potent than **1** against rhGAR Tfase. Compound **2** was inactive against rhAICAR Tfase (K_i > 100 μM).

5. Cell growth inhibition

Compounds **2**, as well as **10**–**14**, were examined for cell growth inhibition both in the presence (+) and absence (−) of added hypoxanthine against the CCRF-CEM cell line (Table 2). Compound **2** exhibited modest inhibitory activity against the CCRF-CEM cell line (**2**, IC_{50} = 6.0 μM) when purines (hypoxanthine) were

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

Compound	*E. coli* GAR Tfase[a]	rhGAR Tfase[b]	rhAICAR Tfase[c]
2	>100	0.50	>100
1	1.9	0.015	>100
Lometrexol	0.1	0.06[d]	1

[a] *E. coli* GAR Tfase.
[b] Recombinant human GAR Tfase.
[c] Recombinant human AICAR Tfase.
[d] Ref. 14.

Table 2. In vitro cytotoxic activity, CCRF-CEM (IC_{50}, μM)

Compound	(−)T (−)H[a]	(+)T (−)H	(−)T (+)H
2	6	10	>100
10	60	60	>100
11	>100	>100	>100
12	20	20	>100
13	>100	>100	>100
14	30	20	>100
1	0.08 (0.016)[b]	0.08 (0.016)[b]	>100
Lometrexol	0.3	0.2	>100

[a] T, thymidine; H, hypoxanthine.
[b] Ref. 10.

Table 3. In vitro cytotoxic activity (IC_{50}, μM)[a]

Compd	CCRF-CEM	CCRF-CEM/MTX	CCRF-CEM/FPGS$^-$
2	6	>100	6

[a] (−)T, thymidine; (−) H, hypoxanthine.

absent in the media. This sensitivity to the presence of purines, but not pyrimidines (thymidine), indicates that the cell growth inhibitory activity of **2** is derived from its inhibition of enzymes (i.e., GAR Tfase) in the de novo purine biosynthetic pathway. Compound **2** was 20-fold less potent than Lometrexol, and >75-fold less potent than **1** against the CCRF-CEM cell line. In addition, compounds **10**, **12**, and **14** exhibited weak cell growth inhibitory activity against the CCRF-CEM cell line (**10**, IC_{50} = 60 μM; **12**, IC_{50} = 20 μM; **14**, IC_{50} = 30 μM), while compounds **11** and **13** were inactive (IC_{50} > 100 μM) highlighting the importance of the trifluoroacetyl and glutamate subunits of **2** for effective cell growth inhibitory activity.

Compound **2** was also examined for cell growth inhibitory activity in CCRF-CEM cell lines that lack the reduced folate carrier (CCRF-CEM/MTX) and folyl polyglutamate synthase (CCRF-CEM/FPGS$^-$). Its inactivity against CCRF-CEM/MTX, like that of **1**, indicates that **2** is actively transported into the cell by the reduced folate carrier, Table 3. Unlike **1**, which is also inactive against CCRF-CEM/FPGS$^-$, the cytotoxic activity of **2** is unaltered with this cell line indicating that polyglutamation of **2** does not contribute to its cellular activity and likely that it is not an effective FPGS substrate. This may contribute to the relative potency differences between **1** and **2** in the cellular functional assays where polyglutamation of **1**, but not **2**, increases intracellular retention and concentration. In contrast and as expected, the activity of **10**–**14** was unaffected in both the CCRF-CEM/MTX and CCRF-CEM/FPGS-cell lines indicating that their modest, purine-sensitive cell growth inhibitory activity is not dependent on either reduced folate carrier transport or FPGS polyglutamation (data not shown).

6. Conclusions

Compound **2**, an analogue of the potent antineoplastic compound **1**, which contains an additional methylene between the trifluoromethyl ketone and the benzoylglutamate, was examined and found to be an effective GAR Tfase inhibitor, albeit 30-fold less potent than **1**. It was established to possess modest, purine-sensitive cell growth inhibitory activity against cultured cells consistent with functional inhibition of GAR Tfase and, like **1**, was found to benefit from reduced folate carrier transport into the cell. Unlike **1**, the functional activity of **2** was not dependent on FPGS (i.e., not a substrate for FPGS) suggesting that the 75–400-fold loss in cellular activity with **2** versus **1** results from both its reduced enzymatic inhibition and less effective intracellular retention attributable to polyglutamation. Regardless of the origin of the effects, the comparison of **2** (and **4**)[9] with **1** highlights the unique combination of properties embodied in **1**, which provides an unusually potent and effective antineoplastic candidate.

7. Experimental

7.1. Methyl 4-(4,4,4-trifluoro-3-oxobutyl)benzoate (6)

A solution of 3-(4-carbomethoxyphenyl)propionic acid (9.81 g, 47.1 mmol) in anhydrous CH_2Cl_2 (167 mL) was treated with oxalyl chloride (12.4 mL, 0.141 mol, 3.0 equiv), and the resulting solution was stirred at 25 °C for 2 h. After evaporation of the solvent and excess oxalyl chloride, a solution of the resulting residue and trifluoroacetic anhydride (40.7 mL, 0.394 mol, 6.1 equiv) in CH_2Cl_2 (294 mL) was treated with pyridine (31.9 mL, 0.394 mol, 8.4 equiv) at 0 °C for 20 min. Water (72 mL) was added cautiously, and the reaction mixture was stirred at 25 °C for 1 h. The reaction mixture was poured into water (300 mL), and extracted with CH_2Cl_2 (3 × 150 mL). The combined organic layers were washed with saturated aqueous NaCl (250 mL), dried (Na_2SO_4), filtered, and concentrated. Column chromatography (SiO_2, 25% EtOAc–hexanes) afforded **6** (8.57 g, 70%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.92 (d, J = 8.2 Hz, 2H), 7.21 (d, J = 8.2 Hz, 2H), 3.85 (s, 3H), 3.01 (t, J = 4.7 Hz, 4H); IR (neat) ν_{max} 3415, 2941, 1767, 1713, 1611, 1435, 1286, 1178, 1110 cm^{-1}; ESITOF-HRMS m/z 259.0589 (M−H$^+$, $C_{12}H_{11}F_3O_3$ requires 259.0587).

7.2. Methyl 4-(4,4,4-trifluoro-3-dimethylhydrazonobutyl)-benzoate (7)

A solution of **6** (4.95 g, 19.0 mmol), *N*,*N*-dimethylhydrazine (3.28 mL, 43.2 mmol, 2.27 equiv) in anhydrous EtOH (79 mL) was treated with acetic acid (1.11 mL, 19.4 mmol, 1.02 equiv), and the reaction solution was stirred at 25 °C overnight. The reaction solution was concentrated under reduced pressure, and column chromatography (SiO_2, 8–25% EtOAc–hexanes) afforded **7** (3.67 g, 64%) as a yellow oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98 (d, J = 8.2 Hz, 2H), 7.28 (d, J = 8.2 Hz, 2H), 3.91 (s, 3H), 2.96–2.92 (m, 2H), 2.77 (s, 6H), 2.79–2.75 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.9, 145.6, 130.0, 128.7, 128.2, 121.4 (q, J = 275 Hz), 51.9, 47.1, 31.6, 28.8; IR (neat) ν_{max} 2941, 2860, 1719, 1604, 1428, 1354, 1279, 1185, 1103,

1022 cm^{-1}; MALDI-FTHRMS (DHB) *m/z* 303.1320 ($M+H^+$, $C_{14}H_{17}F_3N_2O_2$ requires 303.1315).

7.3. Methyl 4-[5-chloro-2-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pentyl]benzoate (8)

A solution of sodium bis(trimethylsilyl)amide (1.0 M in THF, 1.88 mL, 1.88 mmol, 1.05 equiv) was treated with a solution of **7** (0.54 g, 1.79 mmol) in freshly distilled THF (8.1 mL) at −78 °C, and 1-chloro-3-iodopropane (1.92 mL, 17.9 mmol, 10 equiv) was added quickly to the reaction mixture. The cooling bath was removed, and the reaction mixture was stirred at 25 °C for 1 h before being quenched by addition of saturated aqueous NH_4Cl (100 mL). The resulting aqueous solution was extracted with EtOAc (5 × 30 mL). The combined organic layers were washed with saturated aqueous NaCl (60 mL), dried (Na_2SO_4), filtered, and concentrated. Column chromatography (SiO_2, 7–25% EtOAc–hexanes) afforded **8** mixed with **7** as a yellow oil, which was used directly in the next reaction. For **8**: MALDI-FTHRMS (DHB) *m/z* 379.1398 ($M+H^+$, $C_{17}H_{22}ClF_3N_2O_2$ requires 379.1395).

7.4. Methyl 4-[6-cyano-6-ethoxycarbonyl-2-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)hexyl]benzoate (9)

A suspension of sodium hydride (60% dispersion, 85.9 mg, 2.15 mmol, 1.2 equiv) in freshly distilled DMF (1.69 mL) was treated with ethyl cyanoacetate (0.248 mL, 2.33 mmol, 1.3 equiv) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, forming the sodium salt as a clear solution. A solution of **8** (**8**, 0.68 g, 1.79 mmol, mixed with **7**) in freshly distilled DMF (1.69 mL) was added at 0 °C. The reaction mixture was allowed to warm to 50 °C, and stirred for 9 h before being quenched by addition of saturated aqueous NH_4Cl (50 mL). The resulting aqueous solution was extracted with EtOAc (5 × 15 mL). The combined organic layers were washed with saturated aqueous NaCl (50 mL), dried (Na_2SO_4), filtered, and concentrated. Column chromatography (SiO_2, 8–33% EtOAc–hexanes) afforded **9** (0.57 g, 19% from **7**) as a yellow oil: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.97 (d, J = 7.9 Hz, 2H), 7.27 (d, J = 7.9 Hz, 2H), 4.24 (q, J = 7.0 Hz, 2H), 3.91 (s, 3H), 3.82–3.74 (m, 1H), 3.46–3.40 (m, 1H), 2.98–2.90 (m, 2H), 2.43 (s, 3H), 2.42 (s, 3H), 1.97–1.87 (m, 1H), 1.75–1.69 (m, 1H), 1.68–1.55 (m, 3H), 1.49–1.43 (m, 1H), 1.30 (t, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.8, 165.8, 163.0, 144.2, 129.8, 129.0, 128.6, 121.1 (q, J = 278 Hz), 116.3, 113.1, 62.9, 62.8, 52.0, 47.5, 40.2, 38.1, 37.3, 30.4, 29.5, 25.1, 24.9, 24.7, 13.9; IR (neat) ν_{max} 2941, 2868, 1745, 1715, 1678, 1611, 1434, 1282, 1178, 1105, 1014 cm^{-1}; MALDI-FTHRMS (DHB) *m/z* 456.2107 ($M+H^+$, $C_{22}H_{28}F_3N_3O_4$ requires 456.2105).

7.5. Methyl 4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pentyl]benzoate (10)

A solution of sodium methoxide prepared by dissolving sodium (60.4 mg, 2.63 mmol, 2.1 equiv) in anhydrous MeOH (1.58 mL) was treated with guanidine hydrochloride (0.143 g, 1.5 mmol, 1.2 equiv) at ambient temperature. After the reaction mixture was stirred at ambient temperature for 30 min, **9** (0.57 g, 1.25 mmol) in freshly distilled DMF (1.58 mL) was added. The reaction mixture was warmed to 75 °C and stirred for 16 h. The reaction mixture was cooled and quenched by addition of acetic acid (79.7 μL, 1.5 mmol, 1.1 equiv). Column chromatography (SiO_2, 8–25% MeOH–$CHCl_3$) afforded **10** (0.32 g, 58%) as a light yellow solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.67 (d, J = 8.2 Hz, 2H), 7.09 (d, J = 8.2 Hz, 2H), 3.63 (s, 3H), 3.56–3.52 (m, 1H), 2.75 and 2.66 (AB in ABX system, J = 7.0, 13.8 Hz, 2H), 2.12 (s, 6H), 2.10–1.97 (m, 2H), 1.48–1.37 (m, 2H), 1.24–1.16 (m, 2H); IR (neat) ν_{max} 3330, 3180, 2941, 1709, 1604, 1492, 1440, 1328, 1275, 1178, 1126, 1014, 753 cm^{-1}; MALDI−FTHRMS (DHB) *m/z* 469.2155 ($M+H^+$, $C_{21}H_{27}F_3N_6O_3$ requires 469.2169).

7.6. 4-[5-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoic acid (11)

A solution of **10** (30 mg, 64.0 μmol) in MeOH (0.87 mL) was treated with LiOH monohydrate (8.1 mg, 0.192 mmol, 3 equiv) in water (0.30 mL), and the reaction solution was stirred at ambient temperature for 24 h. The reaction solution was diluted with water (10 mL), washed with EtOAc (2 × 10 mL), acidified to pH 1 by addition of 1 N aqueous HCl, stirred for 30 min, and concentrated. Removal of traces of water by treatment of the residue with benzene (3 × 5 mL) provided **11** (26.4 mg, 100%) as a white solid: ^1H NMR (D_2O, 400 MHz) δ 7.68 (d, J = 8.2 Hz, 2H), 7.13 (d, J = 8.2 Hz, 2H), 2.98–2.93 (m, 1H), 2.37 (t, J = 12.3 Hz, 1H), 2.04–2.00 (m, 1H), 1.90–1.84 (m, 2H), 1.36–1.26 (m, 2H), 1.08–1.02 (m, 1H), 0.87–0.79 (m, 1H); ESITOF-HRMS *m/z* 413.1436 ($M+H^+$, $C_{18}H_{19}F_3N_4O_4$ requires 413.1431).

7.7. Di-*tert*-butyl *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoyl}-L-glutamate (12)

A solution of **11** (26.4 mg, 64.0 μmol), di-*tert*-butyl L-glutamate hydrochloride (28.4 mg, 0.096 mmol, 1.5 equiv) and $NaHCO_3$ (16.1 mg, 0.19 mmol, 3 equiv) in DMF (0.62 mL) was treated with EDCI (36.8 mg, 0.19 mmol, 3 equiv) at 0 °C. The reaction mixture was stirred at ambient temperature overnight before the addition of $CHCl_3$ (10 mL). The resulting solution was washed with saturated aqueous $NaHCO_3$ (2 × 10 mL), dried (Na_2SO_4), filtered, and concentrated. PCTLC (SiO_2, 1 mm plate, 14% MeOH–$CHCl_3$) afforded **12** (18.4 mg, 44%) as a light yellow solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.50 (d, J = 7.6 Hz, 1H), 7.44 (d, J = 7.6 Hz, 1H), 7.02 (d, J = 7.6 Hz, 2H), 4.28–4.22 (m, 1H), 3.41–3.38 (m, 0.5H), 3.19–3.15 (m, 0.5H), 2.94–2.88 (m, 0.5H), 2.81–2.75 (m, 0.5H), 2.68–2.61 (m, 0.5H), 2.50–2.48 (m, 0.5H), 2.17–2.01 (m, 2H), 1.99–1.78 (m, 2H), 1.48–1.36 (m, 2H), 1.24 (s, 9H), 1.19 (s, 9H), 1.08–1.04 (m, 2H), 0.72–0.60 (m, 2H); IR (neat) ν_{max} 3340, 3215, 2921, 1724, 1616, 1446, 1355, 1151 cm^{-1}; ESITOF-HRMS *m/z* 654.3109 ($M+H^+$, $C_{31}H_{42}F_3N_5O_7$ requires 654.3109).

7.8. *N*-{4-[5-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoyl}-L-glutamic acid (2)

A solution of **12** (10 mg, 15.3 μmol) in $CHCl_3$ (0.2 mL) was treated with trifluoroacetic acid (1 mL) at 0 °C. The reaction solution was allowed to warm to 25 °C, and stirred overnight. The solution was concentrated, and triturated with Et_2O (3 × 1 mL) to give **2**-CF_3COOH (10.0 mg, 100%) as a white solid: ^1H NMR (D_2O, 500 MHz) δ 7.54 (d, J = 8.1 Hz, 1H), 7.52 (d, J = 8.1 Hz, 1H), 7.19 (d, J = 8.1 Hz, 2H), 4.51–4.48 (m, 1H), 3.02–2.99 (m, 1H), 2.46–2.41 (m, 3H), 2.23–2.18 (m, 1H), 2.06–1.99 (m, 2H), 1.93–1.89 (m, 2H), 1.35–1.27 (m, 2H), 1.10–1.04 (m, 1H), 0.88–0.81 (m, 1H); ESITOF-HRMS m/z 542.1858 ($M+H^+$, $C_{23}H_{26}F_3N_5O_7$ requires 542.1857).

7.9. 4-[5-(2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pentyl]benzoic acid (13)

A solution of **10** (30 mg, 64.0 μmol) in MeOH (0.87 mL) was treated with LiOH monohydrate (8.1 mg, 0.192 mmol, 3 equiv) in water (0.30 mL), and the reaction solution was stirred at ambient temperature for 24 h. The reaction solution was diluted with water (10 mL), washed with EtOAc (2 × 10 mL), acidified to pH 4 by addition of 1 N aqueous HCl, and concentrated. Removal of traces of water by treatment of the residue with benzene (3 × 5 mL) provided **13** (29.1 mg, 100%) as a white solid: ^1H NMR (D_2O, 500 MHz) δ 7.61 (d, J = 8.1 Hz, 2H), 7.10 (d, J = 8.1 Hz, 2H), 3.45–3.40 (m, 1H), 2.77 (d, J = 7.8 Hz, 2H), 2.11 (s, 6H), 2.07 (t, J = 6.6 Hz, 2H), 1.49–1.43 (m, 2H), 1.25–1.20 (m, 2H); ESITOF-HRMS m/z 455.2017 ($M+H^+$, $C_{20}H_{25}F_3N_6O_3$ requires 455.2013).

7.10. Di-*tert*-butyl *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoro-1-dimethylhydrazonoethyl)pentyl]benzoyl}-L-glutamate (14)

A solution of **13** (29.1 mg, 64.0 μmol), di-*tert*-butyl L-glutamate hydrochloride (28.4 mg, 0.096 mmol, 1.5 equiv) and $NaHCO_3$ (16.1 mg, 0.19 mmol, 3 equiv) in DMF (0.62 mL) was treated with EDCI (36.8 mg, 0.19 mmol, 3 equiv) at 0 °C. The reaction mixture was stirred at ambient temperature overnight before the addition of $CHCl_3$ (10 mL). The resulting solution was washed with saturated aqueous $NaHCO_3$ (2 × 10 mL), dried (Na_2SO_4), filtered, and concentrated. PCTLC (SiO_2, 1 mm plate, 14% MeOH–$CHCl_3$) afforded **14** (20.5 mg, 46%) as a light yellow solid: ^1H NMR (CD_3OD, 500 MHz) δ 7.53 (d, J = 8.1 Hz, 2H), 7.09 (d, J = 8.1 Hz, 2H), 4.26–4.24 (m, 1H), 3.58–3.54 (m, 1H), 2.76 and 2.68 (AB in ABX system, J = 7.8, 13.6 Hz, 2H), 2.16 (s, 6H), 2.11–2.06 (m, 1H), 2.04–1.94 (m, 2H), 1.82–1.76 (m, 1H), 1.51–1.40 (m, 2H), 1.25 (s, 9H), 1.21 (s, 9H), 1.13–1.10 (m, 2H), 0.74–0.68 (m, 2H); IR (neat) ν_{max} 3333, 3186, 2915, 2847, 2349, 1727, 1710, 1614, 1450, 1371, 1280, 1252, 1145 cm^{-1}; ESITOF-HRMS m/z 696.3678 ($M+H^+$, $C_{33}H_{48}F_3N_7O_6$ requires 696.3691).

7.11. *N*-{4-[5-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-2-(2,2,2-trifluoroacetyl)pentyl]benzoyl}-L-glutamic acid (2)

A solution of **14** (10 mg, 14.4 μmol) in $CHCl_3$ (0.2 mL) was treated with trifluoroacetic acid (1 mL) at 0 °C. The reaction solution was allowed to warm to 25 °C, and stirred overnight. The solvent was evaporated, and water (0.5 mL) was added. The resulting solution (pH = 1) was stirred at 25 °C for 30 min, before the solvent was evaporated to give **2**-CF_3COOH (9.4 mg, 100%) as a white solid identical to the sample described previously.

7.12. GAR and AICAR Tfase assay

The K_i values for the folate analogues were measured as previously described.[7] 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 rhGAR 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-Menton 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 for the inhibition assays are summarized in Table 1.

7.13. Cytotoxic assay

The cytotoxic activity of the compounds was measured using the CCRF-CEM human leukemia cell lines as described previously.[10]

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