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10-Formyl-5,10-dideaza-acyclic-5,6,7,8-tetrahydrofolic Acid (10-Formyl-DDACTHF): A Potent Cytotoxic Agent Acting by Selective Inhibition of Human GAR Tfase and the De Novo Purine Biosynthetic Pathway

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Abstract—The synthesis of 10-formyl-DDACTHF (**3**) as a potential inhibitor of glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) is reported. Aldehyde **3**, the corresponding γ- and α-pentaglutamates **21** and **25** and related agents were evaluated for inhibition of folate-dependent enzymes including GAR Tfase and AICAR Tfase. The inhibitors were found to exhibit potent cytotoxic activity (CCRF-CEM IC_{50} for **3** = 60 nM) that exceeded their enzyme inhibition potency [K_i (**3**) = 6 and 1 μM for *Escherichia coli* GAR and human AICAR Tfase, respectively]. Cytotoxicity rescue by medium purines, but not pyrimidines, indicated that the potent cytotoxic activity is derived from selective purine biosynthesis inhibition and rescue by AICAR monophosphate established that the activity is derived preferentially from GAR versus AICAR Tfase inhibition. The potent cytotoxic compounds including aldehyde **3** lost activity against CCRF-CEM cell lines deficient in the reduced folate carrier (CCRF-CEM/MTX) or folylpolyglutamate synthase (CCRF-CEM/FPGS$^-$) establishing that their potent activity requires both reduced folate carrier transport and polyglutamation. Unexpectedly, the pentaglutamates displayed surprisingly similar K_i's versus *E. coli* GAR Tfase and only modestly enhanced K_i's versus human AICAR Tfase. On the surface this initially suggested that the potent cytotoxic activity of **3** and related compounds might be due simply to preferential intracellular accumulation of the inhibitors derived from effective transport and polyglutamation (i.e., ca. 100-fold higher intracellular concentrations). However, a subsequent examination of the inhibitors against recombinant human GAR Tfase revealed they and the corresponding γ-pentaglutamates were unexpectedly much more potent against the human versus *E. coli* enzyme (K_i for **3**, 14 nM against rhGAR Tfase versus 6 μM against *E. coli* GAR Tfase) which also accounts for their exceptional cytotoxic potency.

Glycinamide ribonucleotide transformylase (GAR Tfase) is an enzyme central to de novo purine biosynthesis.[1–12] Since purines are crucial components of DNA and RNA, inhibition of enzymes in the purine biosynthetic pathway has been proposed to be an effective approach for antineoplastic intervention.[13] The disclosure that (6*R*)-5,10-dideazatetrahydrofolate [Lometrexol, (6*R*)-DDATHF] 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.[14–23] GAR Tfase uses

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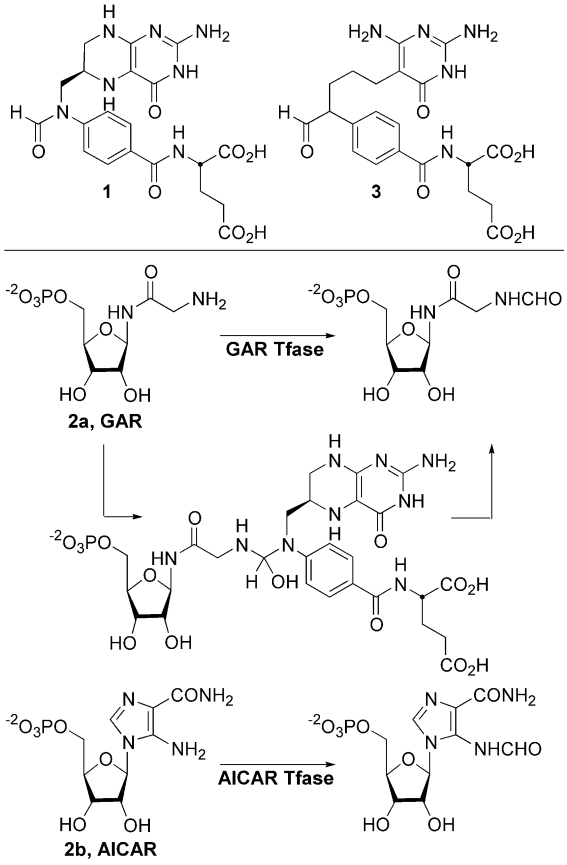


Figure 1.

(6*R*)-10-formyl-5,6,7,8-tetrahydrofolate (**1**) to transfer a formyl group to the primary amine of its substrate, glycinamide ribonucleotide (**2a**, GAR; Fig. 1). This one carbon transfer constitutes the incorporation of the C-8 carbon of the purines and is the first of two formyl transfer reactions. The second formyl transfer reaction is catalyzed by aminoimidazole carboxamide ribonucleotide transformylase (AICAR Tfase) which also employs **1** to transfer a formyl group to the C-5 amine of its substrate, aminoimidazole carboxamide ribonucleotide (**2b**, AICAR; Fig. 1).[1,24–27] Herein, we detail the preparation and evaluation of 10-formyl-DDACTHF (**3**) in our continued efforts to identify potent inhibitors of GAR Tfase and AICAR Tfase.[28]

Inhibitor Design

In previous studies, we examined aldehyde containing folate-based inhibitors incapable of transferring the formyl group.[28] Thus, replacement of N10 with a carbon atom prevents the transfer of the formyl group from the cofactor analogue providing unique opportunities for enzyme inhibition. This could entail either competitive inhibition of the enzymes through gem-diol binding of the aldehyde mimicking the formyl transfer tetrahedral intermediate or covalent trap of the substrate at the active site to provide enzyme-assembled tight binding inhibitors of GAR or AICAR Tfase.[28–32] Co-crystallization of GAR Tfase, β-GAR

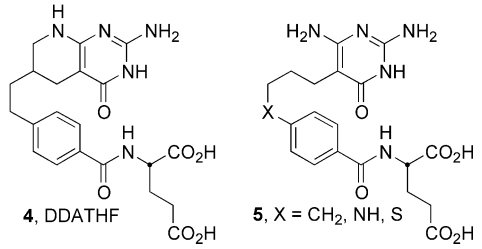


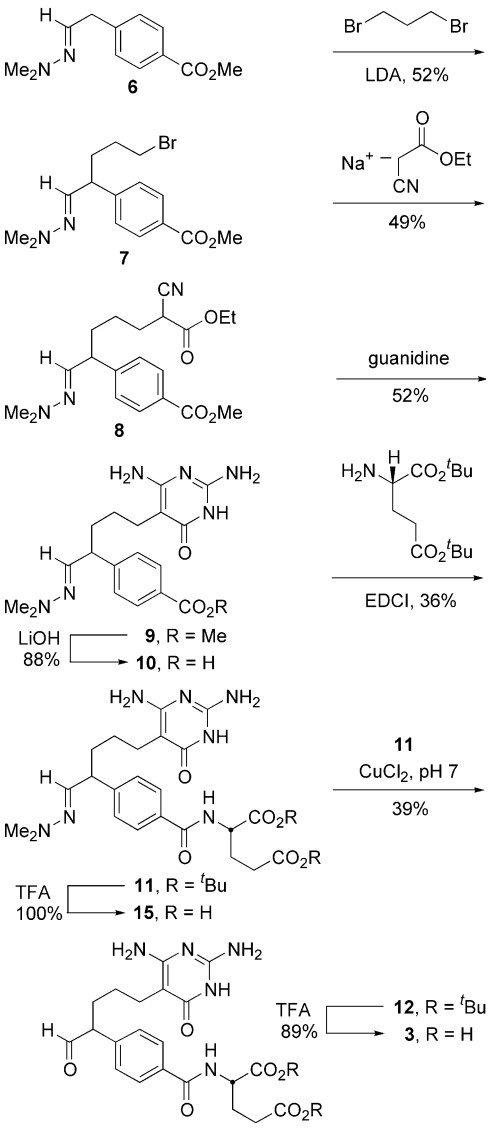
Figure 2.

and 10-formyl-5,8,10-trideazafolate (10-formyl-TDAF), the most potent of the inhibitors examined to date, revealed that the aldehyde inhibitor ($K_i = 260$ nM) binds in the active site as its hydrate mimicking the tetrahedral intermediate involved in formyl transfer.[32] Thus, no enzyme-assembled imine adduct with the substrate β-GAR or covalent adduct with nucleophiles of the GAR Tfase active site residues were observed, and the potent inhibitory activity could be attributed to the H-bonding interactions of the inhibitor aldehyde hydrate with the catalytically important residues of the enzyme active site. Despite these efforts, none of the potent GAR Tfase inhibitors in this series, including 10-formyl-TDAF, exhibited cytotoxic activity consistent with their level of enzyme inhibition potency; observations that could be attributed in part to their instability and ineffective transport by the reduced folate carrier.[28]

Numerous reports have described acyclic analogues of (6*R*)-5,10-dideazatetrahydrofolate [**4**, Lometrexol or (6*R*)-DDATHF, Fig. 2].[33–42] Several of these analogues, including the acyclic derivative **5** (Fig. 2, $X = CH_2$) of DDATHF,[33] have been shown to retain the potent cytotoxic and enzyme inhibitory properties of **4**. Additionally, several analogues of **4** with substituents at C-10 (e.g., 10-methyl and 10-hydroxymethyl) exhibit equivalent or increased biological activity relative to **4**.[36] Consequently, we were interested in establishing the properties of **3**, an acyclic analogue of DDATHF bearing a non-transferable C-10 formyl group.

Chemistry

The synthesis of 10-formyl-DDACTHF (**3**) was accomplished in a convergent manner through alkylation[43] of the known *N,N*-dimethylhydrazone **6**[28] with 1,3-dibromopropane. LDA deprotonation of **6** (THF, −78 °C, 30 min) and subsequent treatment with excess 1,3-dibromopropane (10 equiv, HMPA, −78 °C, 2 h, 52%) provided the key intermediate **7** (Scheme 1). The preformed sodium salt of ethyl cyanoacetate (NaH, DMF, 0 °C, 30 min) was alkylated with **7** (DMF, 25 °C, 2.5 h, 49%) providing **8**. Cyclization with the free base of guanidine (1.1 equiv, CH_3OH, 25 °C, 12 h, 52%) under basic conditions gave the desired pyrimidine **9**. Treatment of **9** with LiOH (3.0 equiv, 3:1 CH_3OH–H_2O, 25 °C, 12 h, 88%) cleanly provided the carboxylic acid **10** which was coupled with di-*tert*-butyl L-glutamate hydrochloride (EDCI, $NaHCO_3$, DMF, 25 °C, 12 h) to provide **11**. Subsequent hydrolysis of the dimethylhydrazone was

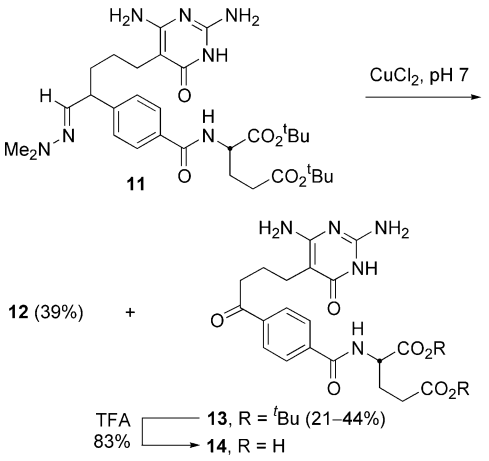


Scheme 1.

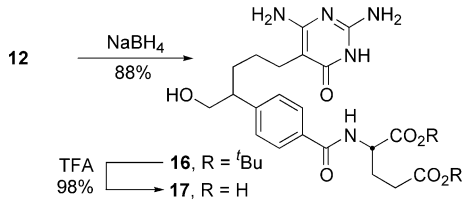
accomplished to provide the sensitive aldehyde **12** by treatment with $CuCl_2$ (5.0 equiv, 0 °C, 1 h, 39%) in THF–H_2O buffered to pH 7. In addition to obtaining aldehyde **12**, the oxidative deformylation product **13** (21–44%, Scheme 2) was also obtained. Deprotection of **12** was accomplished by treatment with trifluoroacetic acid (1:5 v/v TFA/$CHCl_3$, 12 h, 89%) to provide 10-formyl-DDACTHF (**3**).

Acid-catalyzed deprotection of **13** by treatment with trifluoroacetic acid (10 equiv, $CHCl_3$, 12 h, 83%) provided **14** (Scheme 2).

In addition, the stable *N*,*N*-dimethylhydrazone **11** was also converted to **15** by acid-catalyzed deprotection of the di-*tert*-butyl esters by treatment with trifluoroacetic acid (1:4 v/v TFA/$CHCl_3$, 12 h, quantitative) (Scheme 1). For comparative purposes, the aldehyde **12** was reduced to the alcohol **16** with $NaBH_4$ (3.0 equiv, CH_3OH, 4 h, 88%) followed by deprotection of **16** with trifluoroacetic acid (1:10 v/v TFA/$CHCl_3$, 12 h, 98%) to provide the known alcohol **17**[36] (Scheme 3).

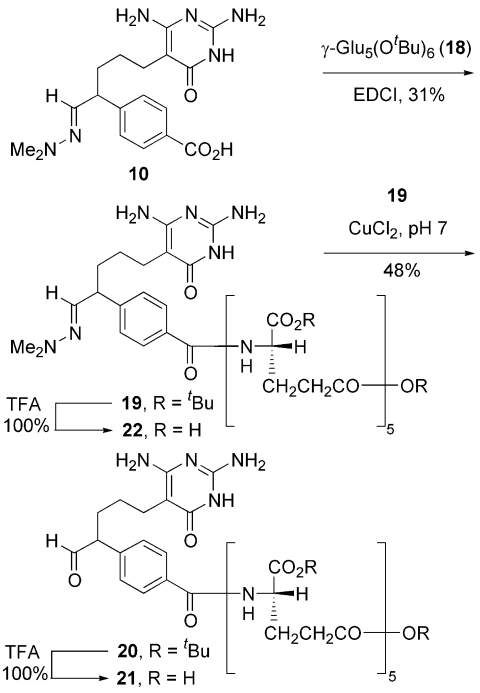


Scheme 2.

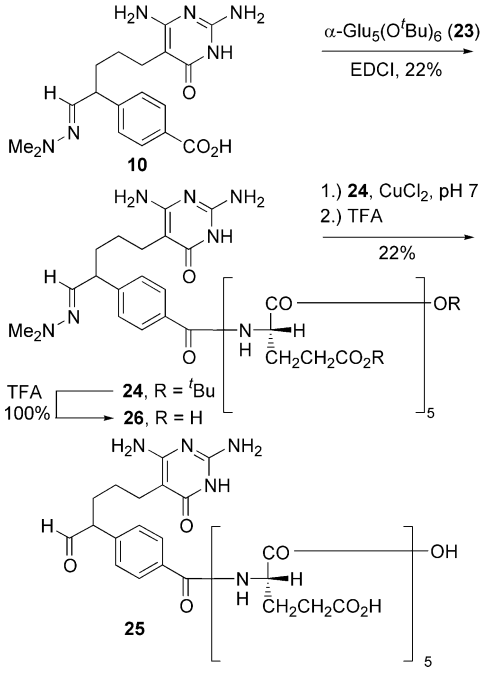


Scheme 3.

In efforts to establish the origin of the potent cytotoxic activity of **3** and related compounds, the corresponding γ- and α-pentaglutamates of **3** and **15** were also prepared. Whereas only γ-polyglutamates have been found in eurkaryotes, bacteria including *Escherichia coli* produce folate conjugates which contain two γ and subsequent α glutamate linkages [i.e., pAB(γ-Glu)$_2$-(α-Glu)$_n$].[46] To establish the importance of the nature of the linkage, both the γ- and α-pentaglutamates were prepared. The carboxylic acid **10** was coupled with the known free amine of the *tert*-butyl ester protected γ-pentaglutamate **18**[44] (EDCI, $NaHCO_3$, DMF, 25 °C, 12 h, 31%) to provide **19** (Scheme 4) as well as with the known free amine of the *tert*-butyl ester protected α-pentaglutamate **23**[45] (EDCI, $NaHCO_3$, DMF, 25 °C, 48 h, 22%) to provide **24** (Scheme 5). Subsequent hydrolysis of the dimethylhydrazone in **19** was accomplished to generate the sensitive aldehyde **20** by treatment with $CuCl_2$ (5.0 equiv, 0 °C, 1.5 h, 48%) in THF–H_2O buffered to pH 7. Subsequent deprotection of the *tert*-butyl esters was accomplished by treatment with trifluoroacetic acid (1:4 v/v TFA/$CHCl_3$, 12 h, 100%) to provide the 10-formyl-DDACTHF γ-pentaglutamate **21**. In a similar manner, hydrolysis of the dimethylhydrazone in **24** was accomplished to generate the corresponding sensitive aldehyde by treatment with $CuCl_2$ (5.0 equiv, 0 °C, 1 h) in THF–H_2O buffered to pH 7. Subsequent deprotection of the *tert*-butyl esters was accomplished by treatment with trifluoroacetic acid (1:4 v/v TFA/$CHCl_3$, 12 h, 22% from **24**) to provide the 10-formyl-DDACTHF α-pentaglutamate **25**. Similarly, the *N*,*N*-dimethylhydrazones **19** and **24** were converted to **22** and **26**, respectively, by acid-catalyzed deprotection of the di-*tert*-butyl esters (1:4 v/v TFA/$CHCl_3$, 12 h, 100%) for direct comparison (Schemes 4 and 5).



Scheme 4.



Scheme 5.

GAR Tfase, AICAR Tfase, and DHFR inhibition

Compounds **3**, **9**–**12**, **14**, **15**, **17**, **21**, **22**, **25**, and **26** were tested initially for inhibition of *E. coli* GAR Tfase, human AICAR Tfase, and *E. coli* DHFR, and the results are presented in Table 1. With the exception of **11**, all compounds demonstrate inhibition of GAR Tfase within one order of magnitude K_i range (1.9–48 μM). Compounds **3** and **12** were also found to be effective inhibitors of AICAR Tfase with identical K_i values of 1 μM, surprisingly comparable to the K_i's observed with GAR Tfase. This lack of potentiation by the glutamate (**3** vs **12**) suggests that the enzyme inhibition properties are being dominated by the presence of the aldehyde in **3** and **12** and we will return to this unusual observation when we discuss human GAR Tfase. A more conventional glutamate potentiation of the dimethylhydrazone **15** (vs **11**) was observed. Although the GAR Tfase inhibition by **15** proved comparable to that of the aldehyde **3** ($K_i = 6$ μM), **15** was 30-fold less effective than **3** against AICAR Tfase. Also representative of this importance of the aldehyde in **3**, the corresponding alcohol **17** and the norketone **14** were significantly less active against GAR Tfase and inactive against AICAR Tfase. Similar observations have been made with related series of aldehyde-based inhibitors ($CHO > HC{=}NNMe_2 > CH_2OH > C{=}O$)[28] with the exception that the inhibition of GAR Tfase has generally been greater than AICAR Tfase. While this was observed with **14**, **15**, and **17**, the aldehyde **3** was found to be slightly more potent against AICAR Tfase.

Interestingly, the aldehyde γ-pentaglutamate **21** and dimethylhydrazone γ-pentaglutamate **22** did not exhibit as large an increase in affinity for GAR Tfase as expected. Both γ-pentaglutamate derivatives only exhibit a 2–3× higher binding affinity for *E. coli* GAR Tfase as compared to the monoglutamate inhibitors. A similar modest 4-fold increase in potency was observed with the aldehyde γ-pentaglutamate **21** versus **3** against AICAR Tfase, whereas the dimethylhydrazone **22** exhibited a much more substantial 140-fold increase relative to **15**. Moreover, both γ-pentaglutamate derivatives exhibit an ca. 10× higher binding affinity for AICAR Tfase than GAR Tfase.

Table 1. GAR Tfase, AICAR Tfase, and DHFR inhibition (K_i, μM)[a]

Compd	K_i GAR Tfase	K_i AICAR Tfase	K_i DHFR
9	17	>100	>100
10	48	>100	>100
11	>100	>100	>100
12	5	1	>100
3	6	1	>100
14	24	>100	>100
15	6	28	>100
17	16	>100	>100
21	2.7	0.26	25
22	1.9	0.20	62
25	16	16	>200
26	23	7.1	>200
Lometrexol	0.1	nd[b]	nd[b]

[a]*E. coli* GAR Tfase, human AICAR Tfase, and *E. coli* DHFR.
[b]nd, not done.

Since it has been shown previously that there are two folylpolyglutamate synthetase activities (both α and γ) in *E. coli*,[46] the α-pentaglutamate derivatives **25** and **26** were also synthesized and evaluated. The aldehyde α-pentaglutamate **25** was 3–16× less potent than the aldehyde monoglutamate **3** against GAR Tfase and AICAR Tfase, respectively. Likewise, the dimethylhydrazone α-pentaglutamate **26** was 4× less potent than the hydrazone monoglutamate **15** against GAR Tfase, whereas it was 4× more potent against AICAR Tfase

Table 2. In vitro cytotoxic activity

Compd	CCRF-CEM (IC_{50}, μM)			
	(+) T, (+) H[a]	(−) T, (+) H	(+) T, (−) H	(−) T, (−) H
9	225	>250	80	90
10	>250	>250	>250	>250
11	50	50	50	40
12	50	50	40	50
3	150	170	0.06	0.07
14	80	80	0.20	0.10
15	>200	>200	0.04	0.03
17	>200	160	0.04	0.03
21	>100	>100	>100	>100
22	>100	>100	>100	>100
25	80	60	9	7
26	>100	>100	7	6
Lometrexol	>250	>250	0.20	0.15

[a]T, thymidine; H, hypoxanthine.

and both were 1–2 orders of magnitude less potent than the corresponding γ-pentaglutamate **22**. Thus, the γ-pentaglutamates were notably more potent than the α-pentaglutamates. However, with the exception of **22** versus AICAR Tfase, the γ-pentaglutamates were not significantly more potent enzyme inhibitors than the corresponding monoglutamates. While interesting, this behavior toward *E. coli* GAR Tfase proved not to be consistent with the functional potency of the compounds and we will return to this point later.

None of the compounds tested for inhibition of DHFR exhibited activity, establishing a selectivity for GAR Tfase and AICAR Tfase versus DHFR.

Cytotoxic activity

Compounds **3**, **9**–**12**, **14**, **15**, **17**, **21**, **22**, **25**, and **26** were examined for cytotoxic activity both in the presence (+) and absence (−) of added hypoxanthine against the CCRF-CEM cell line (Table 2). The cytotoxic activity of the precursor agents (**9**–**12**) was relatively nonpotent and uniform against the CCRF-CEM cell line regardless of whether the assay was conducted in the presence or absence of a media purine (hypoxanthine) or pyrimidine (thymidine). In contrast, aldehyde **3**, like Lometrexol, exhibits no activity against the CCRF-CEM cell line cultured in media supplemented with a purine. However, both Lometrexol and aldehyde **3** exhibit potent cytotoxic activity ($IC_{50} = 0.15$ and 0.06–0.07 μM, respectively) when purines are absent in the media. This sensitivity to the presence of purines, but not pyrimidines, indicates that the activity of aldehyde **3** is derived from its inhibition of enzymes in the de novo purine biosynthetic pathway.

Significantly, the degradation product **14**, obtained by oxidative deformylation of **3**, proved to be only slightly less potent or roughly equivalent in potency ($IC_{50} = 0.10$ μM) to **3** and it also exhibited the selective purine rescue. Even more significantly, both the stable *N*,*N*-dimethylhydrazone **15** and the alcohol **17** exhibited a purine sensitive cytotoxic potency ($IC_{50} = 0.03$ and 0.03 μM, respectively) that was at least as great or exceeded that of the aldehyde **3** or ketone **14**. In each case with **3**, **14**, **15**, or **17**, the reversal of the cytotoxicity with hypoxanthine (100 μM) resulted in a $\geq 10^3$–10^4 change in the IC_{50} value indicating that the activity was being observed through selective inhibition of purine biosynthesis.

Table 3. In vitro cytotoxic activity in the presence of AICAR

Compd	CCRF-CEM (IC_{50}, μM)			
	(−)T, (−) H, (−) A[a]	(+) T, (−) H, (−) A	(−) T, (+) H, (−) A	(−) T, (−) H, (+) A
3	0.07	0.06	>150	>150
14	0.10	0.20	>200	>200
15	0.03	0.04	>200	>200
17	0.03	0.04	>200	>200
Lometrexol	0.15	0.20	>200	>200

[a]T, thymidine; H, hypoxanthine; A, AICAR monophosphate.

Table 4. In vitro cytotoxic activity

Compd	(+) T, (+) H[a]	(−) T, (+) H	(+) T, (−) H	(−) T, (−)H
	CCRF-CEM/MTX (IC_{50}, μM)			
3	130	>200	140	>200
14	>100	nd	nd	>100
15	>200	>200	>200	>200
17	>100	nd	nd	>100
Lometrexol	>200	>200	>200	>200
	CCRF-CEM/FPGS$^-$ (IC_{50}, μM)			
3	>100	nd	nd	>100
14	>100	nd	nd	>100
15	>100	nd	nd	>100
17	25	nd	nd	55
Lometrexol	>100	nd	nd	>100

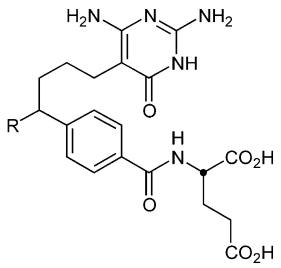
[a]T, thymidine; H, hypoxanthine.

The aldehyde pentaglutamate derivatives **21** and **25** and dimethylhydrazone pentaglutamate derivatives **22** and **26** exhibited little or no cytotoxic activity presumably due to difficulty in traversing the cellular membrane.

AICAR rescue experiments were performed using **3**, **14**, **15**, and **17** in order to further elucidate the source of their cytotoxic activity (Table 3). In each case, the reversal or rescue of the cytotoxicity with hypoxanthine (100 μM) or AICAR monophosphate (100 μM) resulted in a $\geq 10^3$–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, presumably through inhibition of GAR Tfase. This selective sensitivity to GAR Tfase is the expected behavior of the inhibitors **14**, **15**, and **17**, whereas the aldehyde **3** and the corresponding γ-pentaglutamates **21** (from **3**) and **22** (from **15**) would be expected to be more effective or at least as effective at acting on AICAR Tfase.

The extent to which the potent cytotoxic activity of **3**, **14**, **15**, and **17** was dependent on reduced folate transport across the cellular membrane was established by assaying against a mutant CCRF-CEM cell line (CEM/MTX) (Table 4). This cell line has been shown to have an impaired reduced folate carrier.[47] All the potent inhibitors including **3** and **15** lost cytotoxic activity against this mutant CCRF-CEM/MTX cell line ($IC_{50} > 100$ μM), indicating that reduced folate carrier transport is essential for their biological activity.

Table 5. *E. coli* and rhGAR Tfase inhibition (K_i, μM)



Compound	K_i *E. coli* GAR Tfase	K_i rhGAR Tfase
3 R = CHO	6	0.014
14 R = O=	24	13
15 R = CH=NNMe$_2$	6	0.17
17 R = CH$_2$OH	16	1.7
21(γGlu$_5$-**3**)	2.7	0.013
22 (γGlu$_5$-**15**)	1.9	0.032
25 (αGlu$_5$-**3**)	16	0.034
26 (αGlu$_5$-**15**)	23	0.12
Lometrexol	0.1	nd

Finally, the extent to which the potent cytotoxic compounds were dependent upon polyglutamation was established by assaying against a mutant CCRF-CEM cell line (CEM/FPGS$^-$) that lacks folylpolyglutamate synthase (FPGS)[48] (Table 4). All the potent inhibitors including **3** and **15** lost cytotoxic activity against this cell line indicating that inhibitor polyglutamation is essential for their biological activity.

Human GAR Tfase inhibition

In the preceding studies, the four key inhibitors **3**, **14**, **15**, and **17** exhibited exceptionally potent cytotoxic activity (e.g., **3** $IC_{50} = 60$ nM) that proved sensitive to media purines and AICAR, that required reduced folate carrier transport into the cells, and that required polyglutamation. This level of functional potency surpassed the enzyme inhibition activity approximately 100-fold ($K_i = 6$ μM for **3** vs *E. coli* GAR Tfase) suggesting that the cytotoxic potency enhancement might rest with intracellular accumulation of the inhibitors. However, human AICAR Tfase was found to be more potently inhibited by the γ-pentaglutamates inconsistent with GAR Tfase being the target suggesting that intracellular accumulation by transport and polyglutamation might be only part of the answer. Consequently, we examined the inhibitors against recombinant human GAR Tfase (rhGAR Tfase) and found a remarkable and unprecedented sensitivity to the aldehyde inhibitor **3**.

The results of the examination of **3**, **14**, **15**, and **17** as well as the corresponding α- and γ-pentaglutamates of **3** and **15** (**21**, **22**, **25** and **26**) against recombinant human GAR Tfase are summarized in Table 5 alongside the results against *E. coli* GAR Tfase. Remarkably, the inhibitors displayed trends against rhGAR Tfase that might be expected of the original design. For the monoglutamates, the aldehyde **3** proved to be an exceptionally potent inhibitor of rhGAR Tfase ($K_i = 14$ nM) and it was approximately 10-fold more potent than the corresponding dimethylhydrazone **15** ($K_i = 170$ nM), 100-fold more potent than the corresponding alcohol **17** ($K_i = 1.7$ μM), and 1000-fold more potent than the degradation ketone **14** ($K_i = 13$ μM). The γ-pentaglutamates of **3** and **15** (**21** and **22**) were roughly 3–4× more potent than the corresponding α-pentaglutamates **25** and **26**. Whereas the γ-pentaglutamate of aldehyde **3** did not enhance its remarkable potency against rhGAR Tfase (**3** vs **21**), the γ-pentaglutamate of the hydrazone **15** did increase the potency 5-fold (**15** vs **22**). Most notably, this potency of the γ-pentaglutamates against rhGAR Tfase is comparable to the cytotoxic potencies observed with **3** and **15** and consistent with the studies indicating that their target is GAR Tfase and not AICAR Tfase.

To our knowledge, this striking difference in the behavior of the *E. coli* versus human GAR Tfase toward the inhibitors represents the first such demonstration of an unexpectedly selective inhibition of the human enzyme. In total, there are 20 enzyme residues that constitute the core of the folate binding site and 12 enlist their side chains to stabilize folate binding. These 12 are identical in the human and *E. coli* enzymes except for one conservative Leu-143 (*E. coli*) versus Val-143 (human) substitution. This has led to the expectation that little inhibitor distinction between the *E. coli* and human enzymes might be observed. The results with **3**, which is over 400-fold more potent against the human versus *E. coli* enzyme, indicate that this need not be the case.

Conclusions

A series of compounds were synthesized and evaluated as potential inhibitors of GAR Tfase and AICAR Tfase. Four compounds (**3**, **14**, **15**, and **17**) were identified as having potent biological activity (IC_{50} values ≤ 0.20 μM) in the absence of media purines, indicating selective cytotoxicity through the inhibition of the purine de novo biosynthetic pathway. Purine and AICAR rescue experiments indicate that they exhibit their potent cytotoxic activity specifically through intracellular GAR Tfase inhibition even though none of the compounds examined demonstrated sub-micromolar in vitro inhibition of *E. coli* GAR Tfase or human AICAR Tfase.

Subsequent assays were performed in order to determine if polyglutamation and/or reduced folate carrier transport were responsible for the significant increase in cellular biological activity compared to in vitro enzymatic activity. The lack of cytotoxic activity of agents (**3**, **14**, **15**, and **17**) against CCRF-CEM cells with impaired reduced folate active transport (CCRF-CEM/MTX) indicates that these agents require the reduced folate carrier for biological activity and their inactivity against CCRF-CEM/FPGS$^-$ lacking folylpolyglutamate synthase establishes that their polyglutamation is also required for activity. The γ-pentaglutamate derivatives **21** and **22** demonstrated only marginal enhanced binding affinity for *E. coli* GAR Tfase, and a more significant 4× (**21**) and 140× (**22**) enhanced binding affinity for human AICAR Tfase resulting in inhibitors with a

10× higher affinity for human AICAR Tfase over *E. coli* GAR Tfase in vitro. These observations on the pentaglutamates, while interesting, were inconsistent with GAR Tfase as a primary site of action. Subsequent examination of the inhibitors against human GAR Tfase revealed that they and the corresponding γ-pentaglutamates were unexpectedly much more potent against the human versus *E. coli* enzyme which also contributes to their exceptional cytotoxic potency.

Experimental

Methyl 4-[1-dimethylhydrazono-5-bromopent-2-yl]benzoate (7). A solution of diisopropylamine (1.82 mL, 13.0 mmol, 1.5 equiv) in THF (13 mL) cooled to 0 °C was treated with *n*-BuLi (2.5 M in hexanes, 4.50 mL, 11.2 mmol, 1.3 equiv) and stirred at 0 °C for 30 min and at −78 °C for 20 min. A solution of **6** (1.91 g, 8.65 mmol, 1.0 equiv) in THF (3.9 mL) was added dropwise and the resulting solution was stirred at −78 °C for 30 min. A solution of 1,3-dibromopropane (8.79 mL, 86.5 mmol, 10.0 equiv) in HMPA (5.5 mL) was added and the mixture was stirred at −78 °C for 2 h. The reaction mixture was quenched by the dropwise addition of saturated aqueous NH_4Cl (10 mL) and allowed to warm to 25 °C. The reaction mixture was diluted with EtOAc (200 mL) and washed successively with H_2O (2×50 mL) and saturated aqueous NaCl (50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Chromatography (SiO_2, 5×15 cm, 70% hexanes–EtOAc) provided **7** (1.55 g, 52%) as a yellow oil: ^1H NMR ($CDCl_3$, 250 MHz) δ 7.96 (d, J = 8.8 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 6.65 (d, J = 5.8 Hz, 1H), 3.89 (s, 3H), 3.58–3.49 (m, 1H), 3.38 (t, J = 6.2 Hz, 2H), 2.74 (s, 6H), 2.08–1.78 (m, 4H); MALDIFTMS (DHB) m/z 341.0856 ($M + H^+$, $C_{15}H_{21}BrN_2O_2$ requires 341.0859).

Methyl 4-[6-carboethoxy-6-cyano-1-(dimethylhydrazono)hex-2-yl]benzoate (8). A suspension of NaH (60% dispersion, 0.211 g, 5.28 mmol, 1.2 equiv) in anhydrous DMF (25 mL) at 0 °C was treated dropwise with ethyl cyanoacetate (0.61 mL, 5.7 mmol, 1.3 equiv). The solution was stirred at 0 °C for 30 min, forming the sodium salt as a clear solution. This solution was treated with a solution of **7** (1.50 g, 4.40 mmol) in anhydrous DMF (10 mL). The resulting reaction mixture was stirred at 25 °C for 2.5 h. The reaction mixture was diluted with EtOAc (200 mL) and washed successively 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. Chromatography (SiO_2, 4×15 cm, 60% hexanes–EtOAc) provided **8** (0.798 g, 49%) as a yellow oil: ^1H NMR ($CDCl_3$, 250 MHz) δ 7.97 (d, J = 8.0 Hz, 2H), 7.28 (d, J = 8.1 Hz, 2H), 6.61–6.55 (m, 1H), 4.23 (q, J = 7.0 Hz, 2H), 3.89 (s, 3H), 3.58–3.42 (m, 2H), 2.74 (s, 6H), 2.01–1.70 (m, 4H), 1.61–1.42 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H); MALDIFTMS (DHB) m/z 374.2066 ($M + H^+$, $C_{20}H_{27}N_3O_4$ requires 374.2074).

Methyl 4-[5-(2,4-diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)-pent-2-yl]benzoate (9). A solution of Na metal (0.035 g, 1.54 mmol, 2.2 equiv) in CH_3OH (0.87 mL) at 25 °C was treated with guanidine hydrochloride (0.073 g, 0.769 mmol, 1.1 equiv). The solution was stirred at 25 °C for 30 min and then treated with a solution of **8** (0.261 mg, 0.698 mmol) in CH_3OH (0.87 mL). The solution was stirred at 25 °C for 12 h. The excess $NaOCH_3$ was neutralized by the addition of HOAc (0.045 mL). Chromatography (SiO_2, 3×15 cm, 10% CH_3OH–$CHCl_3$) provided **9** (0.140 g, 52%) as a white solid: ^1H NMR (CD_3OD, 250 MHz) δ 7.94 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 6.81 (d, J = 7.1 Hz, 1H), 3.88 (s, 3H), 3.59 (dd, J = 14.7, 7.4 Hz, 1H), 2.70 (s, 6H), 2.30 (t, J = 7.7 Hz, 2H), 1.94–1.78 (m, 2H), 1.55–1.29 (m, 2H); MALDIFTMS (DHB) m/z 409.1963 ($M + Na^+$, $C_{19}H_{26}N_6O_3$ requires 409.1964).

4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoic acid (10). A solution of **9** (0.063 g, 0.163 mmol) in 3:1 CH_3OH–H_2O (1.63 mL) was treated with LiOH–H_2O (0.021 g, 0.489 mmol, 3.0 equiv) and the mixture was stirred at 25 °C for 12 h. The mixture was diluted with H_2O (10 mL) and the aqueous layer was washed with EtOAc (3×3 mL). The aqueous layer was acidified to pH = 4 by the addition of 1 M aqueous HCl. The solution was concentrated under reduced pressure and the residue was treated with toluene (3×5 mL) to remove traces of H_2O to provide **10** (0.053 g, 88%): ^1H NMR (CD_3OD, 400 MHz) δ 7.90 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.1 Hz, 2H), 6.83 (d, J = 7.3 Hz, 1H), 3.51 (dd, J = 15.0, 7.3 Hz, 1H), 2.31 (t, J = 7.3 Hz, 2H), 1.92–1.82 (m, 2H), 1.52–1.28 (m, 2H); MALDIFTMS (DHB) m/z 395.1824 ($M + Na^+$, $C_{18}H_{24}N_6O_3$ requires 395.1824).

Di-*tert*-butyl *N*-{4-[5-(2,4-diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-glutamate (11). A solution of **10** (0.029 g, 0.078 mmol) and di-*tert*-butyl L-glutamate hydrochloride (0.034 g, 0.117 mmol, 1.5 equiv) in DMF (0.31 mL) was treated with $NaHCO_3$ (0.020 g, 0.234 mmol, 3.0 equiv) followed by EDCI (0.045 g, 0.234 mmol, 3.0 equiv). The reaction mixture was stirred at 25 °C for 12 h before the solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (5 mL) and extracted with saturated aqueous $NaHCO_3$ (2 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 2 mm plate, 10% CH_3OH–$CHCl_3$) provided **11** (0.017 g, 36%) as a white solid: ^1H NMR (CD_3OD, 250 MHz) δ 7.78 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 6.82 (d, J = 7.1 Hz, 1H), 4.51–4.45 (m, 1H), 3.53 (dd, J = 14.4, 7.0 Hz, 1H), 2.71 (s, 6H), 2.42–2.12 (m, 5H), 2.08–1.89 (m, 5H), 1.48 (s, 9H), 1.43 (s, 9H); MALDIFTMS (DHB) m/z 614.3678 ($M + H^+$, $C_{31}H_{47}N_7O_6$ requires 614.3666).

Di-*tert*-butyl *N*-{4-[4-(2,4-diamino-6(1*H*)-pyrimidinon-5-yl)-1-formyl-but-2-yl]benzoyl}-L-glutamate (12). A solution of **11** (30 mg, 0.049 mmol) in THF (0.9 mL) and pH 7 aqueous phosphate buffer (0.02 mL) cooled to 0 °C was treated with a solution of $CuCl_2$ (33 mg, 0.244 mmol, 5.0 equiv) in H_2O (0.3 mL). The solution

was stirred at 0 °C for 1 h before it was quenched by the dropwise addition of a pH 8 saturated aqueous NH_4Cl–NH_4OH solution (20 mL). The solution was extracted with $CHCl_3$ (3×20 mL), purged with N_2, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 1 mm plate, 20% CH_3OH–$CHCl_3$) provided **12** (11 mg, 39%; typically 21–44%) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.69 (s, 1H), 9.65 (s, 1H), 8.56 (d, J = 7.3 Hz, 1H), 7.84 (d, J = 8.1 Hz, 2H), 7.33 (d, J = 8.1 Hz, 2H), 5.86 (br s, 2H), 5.60 (br s, 2H), 4.34–4.30 (m, 1H), 3.78–3.70 (m, 1H), 2.33 (t, J = 6.6 Hz, 2H), 2.17 (t, J = 7.0 Hz, 2H), 2.04–2.01 (m, 3H), 1.93–1.88 (m, 2H), 1.69–1.62 (m, 1H), 1.41 (s, 9H), 1.39 (s, 9H); MALDIFTMS (DHB) m/z 594.2904 ($M + Na^+$, $C_{29}H_{41}N_5O_7$ requires 594.2904).

Di-*tert*-butyl *N*-{4-[4-(2,4-diamino-6(1*H*)-pyrimidinon-5-yl)-1-oxo-but-1-yl]benzoyl}-L-glutamate (13). Obtained as the higher R_f spot from the reaction that provided **12**. PCTLC (SiO_2, 1 mm plate, 20% CH_3OH–$CHCl_3$) provided **13** (12 mg, 44%) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.75 (s, 1H), 8.79 (d, J = 7.6 Hz, 1H), 8.02 (d, J = 8.5 Hz, 2H), 7.96 (d, J = 8.5 Hz, 2H), 5.92 (br s, 2H), 5.76 (br s, 1H), 4.37–4.31 (m, 1H), 3.02 (t, J = 7.3 Hz, 2H), 2.35 (t, J = 7.0 Hz, 2H), 2.23 (t, J = 7.0 Hz, 2H), 2.07–2.00 (m, 1H), 1.97–1.88 (m, 1H), 1.73–1.59 (m, 2H), 1.41 (s, 9H), 1.39 (s, 9H); MALDIFTMS (DHB) m/z 580.2730 ($M + Na^+$, $C_{28}H_{39}N_5O_7$ requires 580.2747).

***N*-{4-[4-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-formyl-but-2-yl]benzoyl}-L-glutamic acid (3).** A solution of **12** (2.9 mg, 0.0051 mmol) in $CHCl_3$ (0.20 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.04 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. Et_2O (1 mL) was added and a precipitate formed. The precipitate was triturated with Et_2O (3×1 mL) and dried in vacuo to give **3**-CF_3CO_2H (2.6 mg, 89%) as a tan solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.91 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 4.70–4.59 (m, 2H), 4.45–4.38 (m, 1H), 2.51–2.43 (m, 2H), 2.38–2.25 (m, 2H), 2.19–1.95 (m, 3H), 1.94–1.79 (m, 1H); MALDIFTMS (DHB) m/z 482.1652 ($M + Na^+$, $C_{21}H_{25}N_5O_7$ requires 482.1670).

***N*-{4-[4-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-oxo-but-1-yl]benzoyl}-L-glutamic acid (14).** A solution of **13** (3.3 mg, 0.0059 mmol) in $CHCl_3$ (0.12 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.01 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. Et_2O (1 mL) was added and a precipitate formed. The precipitate was triturated with Et_2O (3×1 mL) and dried in vacuo to give **14**-CF_3CO_2H (2.7 mg, 83%) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.81 (d, J = 7.6 Hz, 1H), 8.01 (d, J = 8.8 Hz, 2H), 7.98 (d, J = 8.8 Hz, 2H), 4.44–4.37 (m, 1H), 3.05 (t, J = 7.0 Hz, 2H), 2.36 (t, J = 7.4 Hz, 2H), 2.30 (t, J = 7.4 Hz, 2H), 2.13–2.06 (m, 1H), 2.00–1.87 (m, 2H), 1.72–1.66 (m, 1H); MALDIFTMS (DHB) m/z 446.1674 ($M + H^+$, $C_{20}H_{23}N_5O_7$ requires 446.1676).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-glutamic acid (15).** A solution of **11** (7.5 mg, 0.0122 mmol) in $CHCl_3$ (0.20 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.05 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. The reaction was concentrated under reduced pressure. The product was triturated with Et_2O (1×1 mL) and dried in vacuo to give **15**-CF_3CO_2H (7.5 mg, 100%) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.83 (d, J = 8.2 Hz, 2H), 7.37 (d, J = 8.2 Hz, 2H), 4.68–4.55 (m, 1H), 3.68–3.61 (m, 1H), 2.87 (s, 6H), 2.85 (t, J = 7.4 Hz, 2H) 2.49 (t, J = 7.7 Hz, 2H), 2.40–2.30 (m, 2H), 2.26–1.90 (m, 4H); MALDIFTMS (DHB) m/z 524.2248 ($M + Na^+$, $C_{23}H_{31}N_7O_6$ requires 524.2233).

Di-*tert*-butyl *N*-{4-[5-(2,4-diamino-6(1*H*)-pyrimidinon-5-yl)-1-hydroxypent-2-yl]benzoyl}-L-glutamate (16). A solution of **12** (6.1 mg, 0.0107 mmol) in CH_3OH (0.11 mL) at 0 °C was treated with $NaBH_4$ (1.2 mg, 0.032 mmol, 3.0 equiv). The solution was stirred at 0 °C for 2 h and 25 °C for 2 h before the solvent was removed under reduced pressure. The residue was diluted with $CHCl_3$ (2 mL) and washed successively with saturated aqueous NH_4Cl (1 mL) and saturated aqueous NaCl (1 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 1 mm plate, 8% CH_3OH–$CHCl_3$) provided **16** (5.4 mg, 88%) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.68 (s, 1H), 8.50 (d, J = 7.6 Hz, 1H), 7.75 (d, J = 7.9 Hz, 2H), 7.26 (d, J = 8.2 Hz, 2H), 5.86 (br s, 2H), 5.57 (br s, 2H), 4.58 (d, J = 5.0 Hz, 1H), 4.34–4.28 (m, 1H), 3.04–2.95 (m, 2H), 2.71–2.66 (m, 1H), 2.32 (t, J = 7.4 Hz, 2H), 2.10 (t, J = 7.0 Hz, 2H), 2.04–1.97 (m, 1H), 1.95–1.87 (m, 1H), 1.76–1.69 (m, 2H), 1.40 (s, 9H), 1.38 (s, 9H); 1.17–1.09 (m, 2H); MALDIFTMS (DHB) m/z 574.3263 ($M + H^+$, $C_{29}H_{43}N_5O_7$ requires 574.3241).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-hydroxypent-2-yl]benzoyl}-L-glutamic acid (17).** A solution of **16** (4.2 mg, 0.0073 mmol) in $CHCl_3$ (0.20 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.02 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. Et_2O (1 mL) was added and a precipitate formed. The precipitate was triturated with Et_2O (3×1 mL) and dried in vacuo to give **17**-CF_3CO_2H (4.1 mg, 98%) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.72 (br s, 1H), 8.59 (d, J = 7.9 Hz, 1H), 7.80 (d, J = 8.2 Hz, 2H), 7.36 (d, J = 8.2 Hz, 2H), 6.04 (br s, 2H), 5.67 (br s, 1H), 4.56 (d, J = 6.2 Hz, 1H), 4.41–4.35 (m, 1H), 3.16–3.10 (m, 1H), 3.05–2.97 (m, 1H), 2.76 (t, J = 7.3 Hz, 2H), 2.14–2.06 (m, 3H), 1.96–1.89 (m, 1H), 1.75–1.58 (m, 2H), 1.23–1.13 (m, 2H); MALDIFTMS (DHB) m/z 484.1824 ($M + Na^+$, $C_{21}H_{27}N_5O_7$ requires 484.1808).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid hexa-*tert*-butyl ester (19).** A solution of **10** (90 mg, 0.24 mmol) and **18**[44] (242 mg, 0.24 mmol, 1.0 equiv) in anhydrous DMF (1.0 mL) was treated with $NaHCO_3$ (61 mg, 0.73 mmol, 3.0 equiv) followed by EDCI (139 mg, 0.73 mmol, 3.0 equiv) and stirred at 25 °C for 12 h. The reaction mixture was diluted with EtOAc (50 mL) and washed with saturated aqueous $NaHCO_3$ (2×20 mL)

followed by saturated aqueous NaCl (20 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Chromatography (SiO_2, 3×15 cm, 10% CH_3OH–$CHCl_3$) provided **19** (102 mg, 31%) as a white solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.83 (d, $J=8.2$ Hz, 2H), 7.36 (d, $J=8.2$, 2H), 6.82 (d, $J=7.0$ Hz, 1H), 4.58–4.49 (m, 1H), 4.40–4.25 (m, 4H), 3.57–3.51 (m, 1H), 2.70 (s, 6H), 2.45–1.72 (m, 26H), 1.51–1.41 (m, 54H); MALDIFTMS (DHB) m/z 1354.7844 ($M+H^+$, $C_{67}H_{107}N_{11}O_{18}$ requires 1354.7868).

***N*-{4-[4-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-formylbut-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid hexa-*tert*-butyl ester (20).** A solution of **19** (41 mg, 0.030 mmol) in THF (0.43 mL) and pH 7 aqueous phosphate buffer (0.09 mL) cooled to 0 °C was treated with a solution of $CuCl_2$ (20.4 mg, 0.15 mmol, 5.0 equiv) in H_2O (0.15 mL). The solution was stirred at 0 °C for 1.5 h before it was quenched by the dropwise addition of a pH 8 saturated aqueous NH_4Cl–NH_4OH solution (5 mL). The solution was extracted with $CHCl_3$ (3×10 mL), purged with N_2, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 1 mm plate, 8% CH_3OH–$CHCl_3$) provided **20** (19 mg, 48%) as a white solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.79 (d, $J=7.6$ Hz, 2H), 7.36 (d, $J=7.9$ Hz, 2H), 4.63–4.51 (m, 2H), 4.38–4.30 (m, 5H), 2.49–1.70 (m, 26H), 1.49–1.41 (m, 54H); MALDIFTMS (DHB) m/z 1312.7312 ($M+H^+$, $C_{65}H_{101}N_9O_{19}$ requires 1312.7286).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid (22).** A solution of **19** (25 mg, 0.019 mmol) in $CHCl_3$ (1.00 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.25 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. The solution was concentrated under reduced pressure. The solid residue was triturated with Et_2O (3×5 mL) and dried in vacuo to give **22**-CF_3CO_2H (21 mg, 100%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.85 (d, $J=7.4$ Hz, 2H), 7.41 (bs, 1H), 7.36 (d, $J=8.2$ Hz, 2H), 4.68–4.60 (m, 1H), 4.48–4.39 (m, 5H), 2.88 (s, 6H), 2.51–1.82 (m, 26H); MALDIFTMS (DHB) m/z 1018.4102 ($M+H^+$, $C_{43}H_{59}N_{11}O_{18}$ requires 1018.4112).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-formylbut-2-yl]benzoyl}-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamyl-L-γ-glutamic acid (21).** A solution of **20** (16 mg, 0.012 mmol) in $CHCl_3$ (1.00 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.25 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. The solution was concentrated under reduced pressure. The solid residue was triturated with Et_2O (3×5 mL) and dried in vacuo to give **21**-CF_3CO_2H (13 mg, 100%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.86 (d, $J=8.2$ Hz, 2H), 7.50 (d, $J=8.5$ Hz, 2H), 4.69–4.61 (m, 2H), 4.48–4.40 (m, 5H), 2.52–1.83 (m, 26H); MALDIFTMS (DHB) m/z 976.3570 ($M+H^+$, $C_{41}H_{53}N_9O_{19}$ requires 976.3530).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-α-glutamyl-L-α-glutamyl-L-α-glutamyl-L-α-glutamyl-L-α-glutamic acid hexa-*tert*-butyl ester (24).** A solution of **10** (7.5 mg, 0.020 mmol) and **23**[45] (20.2 mg, 0.020 mmol, 1.0 equiv) in DMF (0.1 mL) was treated with $NaHCO_3$ (5.1 mg, 0.060 mmol, 3.0 equiv) followed by EDCI (11.6 mg, 0.060 mmol, 3.0 equiv) and stirred at 25 °C for 48 h. The reaction was diluted with EtOAc (20 mL) and washed with saturated aqueous $NaHCO_3$ (5 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 2 mm plate, 10% CH_3OH–$CHCl_3$) provided **24** (6.0 mg, 22%) as a white solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.86 (d, $J=8.5$ Hz, 2H), 7.36 (d, $J=8.2$ Hz, 2H), 6.80 (d, $J=4.4$ Hz, 1H), 4.49–4.30 (m, 5H), 4.08–4.00 (m, 1H), 2.70 (s, 6H), 2.50–2.31 (m, 12H), 2.21–1.83 (m, 14H), 1.49–1.38 (m, 54H); MALDIFTMS (DHB) m/z 1376.7730 ($M+Na^+$, $C_{67}H_{107}N_{11}O_{18}$ requires 1376.7687).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-(dimethylhydrazono)pent-2-yl]benzoyl}-L-α-glutamyl-L-α-glutamyl-L-α-glutamyl-L-α-glutamyl-L-α-glutamic acid (25).** A solution of **24** (19 mg, 0.014 mmol) in THF (0.2 mL) and pH 7 aqueous phosphate buffer (0.04 mL) cooled to 0 °C was treated with a solution of $CuCl_2$ (9.4 mg, 0.070 mmol, 5.0 equiv) in H_2O (0.07 mL). The solution was stirred at 0 °C for 1 h before it was quenched by the dropwise addition of a pH 8 saturated aqueous NH_4Cl–NH_4OH solution (5 mL). The solution was extracted with $CHCl_3$ (3×5 mL), purged with N_2, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. PCTLC (SiO_2, 1 mm plate, 8% CH_3OH–$CHCl_3$) removed baseline impurities. The isolated product was dissolved in $CHCl_3$ (1.00 mL), cooled to 0 °C and treated with trifluoroacetic acid (0.25 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. The solution was concentrated under reduced pressure. The solid residue was triturated with Et_2O (3×5 mL) and dried in vacuo to give **25**-CF_3CO_2H (3.4 mg, 22% over two steps from **24**) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.84 (d, $J=8.5$ Hz, 2H), 7.48 (d, $J=8.5$ Hz, 2H), 4.54–4.32 (m, 5H), 2.78–2.70 (m, 2H), 2.55–2.36 (m, 14H), 2.28–2.10 (m, 5H), 2.09–1.92 (m, 5H); MALDIFTMS (DHB) m/z 976.3517 ($M+H^+$, $C_{41}H_{53}N_9O_{19}$ requires 976.3530).

***N*-{4-[5-(2,4-Diamino-6(1*H*)-pyrimidinon-5-yl)-1-formylbut-2-yl]benzoyl}-L-α-glutamyl-L-α-glutamyl-L-α-glutamyl-L-α-glutamyl-L-α-glutamic acid (26).** A solution of **24** (5.4 mg, 0.0040 mmol) in $CHCl_3$ (1.00 mL) cooled to 0 °C was treated with trifluoroacetic acid (0.25 mL). The solution was stirred at 0 °C for 2 h and 25 °C for 12 h. The solution was concentrated under reduced pressure. The solid residue was triturated with Et_2O (3×5 mL) and dried in vacuo to give **26**-CF_3CO_2H (4.5 mg, 100%) as a tan solid: ^1H NMR (CD_3OD, 400 MHz) δ 7.85 (d, $J=8.5$ Hz, 2H), 7.35 (d, $J=8.5$ Hz, 2H), 7.18–7.14 (m, 1H), 4.52–4.33 (m, 5H), 4.09–4.02 (m, 1H), 2.79 (s, 6H), 2.52–2.38 (m, 16H), 2.23–2.08 (m, 5H), 2.05–1.92 (m, 5H); MALDIFTMS (DHB) m/z 1018.4157 ($M+H^+$, $C_{43}H_{59}N_{11}O_{18}$ requires 1018.4112).

GAR Tfase, AICAR Tfase, and DHFR inhibition

GAR and AICAR Tfase inhibition studies were conducted as previously detailed[28] with the exception that the AICAR Tfase inhibition 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 DHFR inhibition study was conducted as previously detailed[49] with 10 nM enzyme, 30 μM H_2F, 100 μM NADPH and 30 μM inhibitor.

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