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Design, Synthesis, and Biological Evaluation of Fluoronitrophenyl Substituted Folate Analogues as Potential Inhibitors of GAR Transformylase and AICAR Transformylase

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Abstract—The examination results of a novel series of potential inhibitors of glycinamide ribonucleotide transformylase (GAR Tfase) and aminoimidazole carboxamide transformylase (AICAR Tfase) are reported. These agents incorporate an electrophilic fluoronitrophenyl group that can potentially react with an active site nucleophile or the substrate GAR/AICAR amine via nucleophilic aromatic substitution.

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 and AICAR Tfase catalyze the transfer of the N^{10}-formyl group from (6*R*)-N^{10}-formyltetrahydrofolate to their respective substrates: GAR and AICAR.[1] The discovery that (6*R*)-5,10-dideazatetrahydrofolate (Lometrexol, DDATHF) 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.[2,3] In previous studies, we examined numerous folate-based inhibitors that incorporated electrophilic functional groups, which could potentially interact either with active site nucleophiles or the GAR/AICAR substrate amine.[4] Herein, we report the synthesis and evaluation of a novel class of 5,8-dideazafolate (DDAF) and monocyclic 5-deazatetrahydrofolate (DACTHF) GAR Tfase and AICAR Tfase inhibitors that incorporate an electrophilic fluoronitrophenyl group.

Fluoronitroaromatic compounds are known to be unusually susceptible to nucleophilic attack by soft nucleophiles. Thus, while water and hydroxide fail to displace fluoride, soft nucleophiles including phenols, imidazoles, amines, thiols, and selected amides are able to undergo rapid aromatic nucleophilic displacement reactions even at room temperature.[5] In our own studies, we have observed that fluoronitroaromatics stable to LiOH capture imidazole at room temperature in the presence of a tertiary amine base.[6] Fluoronitroaromatics have gained wide use in protein sequencing and protein cross-linking due to their reactivity toward amino acids with nucleophilic side chains.[7] Despite this potential, there have been few reports of the use of fluoronitroaromatic-based enzyme inhibitors.[8]

Our initial efforts entailed the examination of two DDAF-based inhibitors (Fig. 1). In contrast to expectations, neither exhibited time-dependent or irreversible GAR or AICAR Tfase inhibition. Rather, they served as simple competitive inhibitors, displaying K_i's comparable to those observed with typical DDAF or TDAF-based inhibitors.[4] Encouragingly, this indicated that the large fluoronitrophenyl groups were not hindering or destabilizing inhibitor binding at the two enzyme active sites, just that they were not participating in an aromatic nucleophilic substitution reaction within the active sites. Consequently, these studies were extended to the fluoronitroaromatic inhibitors shown in Table 1. The acyclic pyrimidinone analogue of the tetrahydrofolate ring system, 5,10 - dideaza - acyclic - 5,6,7,8 - tetrahydrofolic (DACTHF) has been shown to retain potent cytotoxic and enzyme inhibitory properties of the DDATHF ring system exemplified by Lometrexol.[9] The DACTHF core

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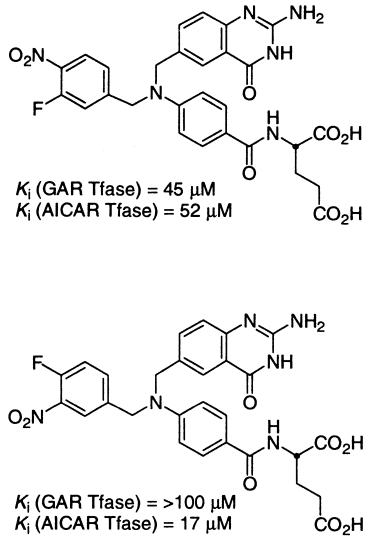


Figure 1.

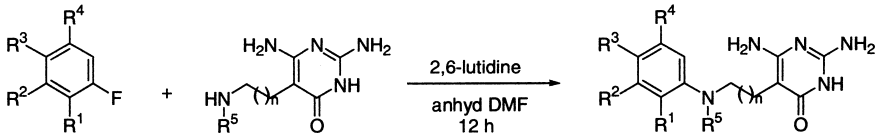
ring system was chosen in order to provide the inhibitors greater flexibility facilitating the active site placement of the fluoronitrophenyl group for nucleophilic attack. The simplest inhibitors were obtained by reaction of the Cbz-protected bromoalkyl amine[10] with ethyl cyanoactetate (1.5 equiv, K_2CO_3 (3 equiv), anhyd DMF, 70 °C, 2 h) followed by condensation with guanidine (1.2 equiv, NaOMe (2 equiv), anhyd CH_3OH, 25 °C, 2 h) to furnish the pyrimidinone core. Deprotection of the primary amine (H_2, Pd–C, CH_3OH, 25 °C, 6 h) followed by arylation of the free amine with the appropriate fluoronitrobenzene (1.5 equiv, 2,6-lutidine (2 equiv), anhyd DMF, 25 °C, 12 h) generated the inhibitor structures **1**–**6**. The more complex agents incorporating the benzoate and benzoyl glutamate groups were obtained similarly. The same alkylamine pyrimidinones generated above were alkylated (0.75 equiv, anhyd. DMF, 60 °C, 8 h) with either methyl 4-(bromomethyl)benzoate or the benzylic bromide derived from the coupling of α-bromo-*p*-toluic acid with di-*t*-butyl glutamate via its acid chloride intermediate (oxalyl chloride, (1.1 equiv), cat. anhyd DMF, anhyd. THF, 25 °C, 1 h followed by *i*Pr_2NEt (3 equiv), di-*t*-butyl glutamate (1.1 equiv), anhyd DMF, 25 °C, 12 h). Arylation of the secondary amine produced with the appropriate fluoronitrobenzene (1.5 equiv, 2,6-lutidine (2 equiv), anhyd DMF, 25 °C, 12 h) generated the inhibitor structures **7**–**28**. Deprotection of the glutamate side chain (1:1 v/v TFA–CH_2Cl_2, 25 °C, 1.5 h) provided inhibitors **29**–**40**.[11]

The activity of inhibitors **1**–**40** against GAR and AICAR Tfase as well as their cytotoxic activity (CCRF-CEM) is shown in Table 2. Two separate GAR Tfase inhibition experiments were performed to determine if the time-dependent inhibition was due to inhibitor adduct formation with either GAR or the enzyme itself. As shown in Table 2, a number of the inhibitors examined exhibited time-dependant inhibition of the GAR Tfase at a concentration of 250 μM. Agent **2** was the only compound examined which showed significantly higher potency in the presence of GAR than in its absence. This indicates that compound **2** probably gains its inhibitory activity via direct adduct formation with the substrate GAR. With the exception of **12** and **17**, which were equally effective in the presence or absence of GAR, the remaining inhibitors were more potent when pre-incubated with only GAR Tfase than when they were pre-incubated with both GAR Tfase and GAR. This indicates that the inhibitors examined probably derive their activity via direct adduct formation with GAR Tfase itself and the pre-incubation conducted with GAR may protect the enzyme from inactivation. A number of compounds (**2**, **7**, **9**, **11**, **12**, **14**–**17**, **22**, **27**, **35** and **40**) inactivated the enzyme entirely after 6–8 h of pre-incubation at 250 μM. The remainder of the inhibitors examined show only moderate activity or were inactive. With the simplest inhibitors (**1**–**6**), only one (**2**) showed strong activity. It appears that without the added binding interaction of an appended benzyl side chain (as seen in **7**–**40**), the potency relies strongly on the substitution pattern of the fluoronitrophenyl portion of the molecule. The most potent inhibitor within this group was **2** which is also the only inhibitor that had two activating nitro groups enhancing the electrophilicity of the fluoronitroaromatic. This requirement for two activating nitro groups was only seen in the simplest inhibitors (**1**–**6**). Inhibitors **7**–**16** all share an appended methyl benzoate group. The structural requirement for activity appears to be a fluorine *meta* to the amino attachment point with a nitro group *para* to the fluorine. Other factors including alkyl chain length appear to be largely inconsequential. Inhibitors **7**–**16** are the most consistently active sub-class of inhibitors examined. Notably, after only 1 min of incubation, compound **14** inhibited GAR Tfase 94% and 75% in the absence and presence of GAR respectively. The rapid and potent inhibitory activity of **14** is especially interesting since **14** only contains one activating nitro group. The di-*t*-butyl protected glutamate inhibitors **17**–**28** appear to have much more stringent structural requirements for activity with only three inhibitors of this class (**17**, **22** and **27**) inactivating the GAR Tfase enzyme after 6–8 h of pre-incubation. All three contain two nitro activating groups and the less effective behavior of this subclass was expected. In fact, it is surprising that any members of this class are capable of enzyme inactivation. Finally, the diacid inhibitors **29**–**40** were surprisingly less active inhibitors than the equivalent di-*t*-butyl ester inhibitors examined (**17**–**28**). In general, the activity of the diacid inhibitors closely follows the pattern seen in the corresponding di-*t*-butyl ester group of inhibitors.

The most consistently active series examined against GAR Tfase (**2**, **12**, **22** and **35**) all contain the 2,4-dinitro-5-fluoro substitution pattern in the fluoronitrophenyl group. All four agents showed inactivation of GAR Tfase within 6 h. The most active member of this series (**12**) also completely inhibited GAR Tfase in the presence of GAR following 6 h of incubation. Importantly, the activity of **12**, which lacks the glutamate side chain, indicates the potential of simplified antifolate inhibitors which do not incorporate this side chain.

Five of the compounds (**2**, **12**, **14**, **17** and **22**) that showed the most significant inhibition at 250 μM were further tested against GAR Tfase at a 10× lower concentration (25 μM). As expected, these agents still exhibited inhibition albeit at a lower level.

Table 1. Fluoronitrophenyl structural analogues synthesized and evaluated



1- 40

Compound	*n*	R^1	R^2	R^3	R^4	R^5
1	1	NO_2	H	F	F	H
2	2	NO_2	H	NO_2	F	H
3	2	F	F	NO_2	H	H
4	2	NO_2	H	F	F	H
5	2	NO_2	F	H	F	H
6	2	NO_2	H	H	F	H
7	1	NO_2	H	NO_2	F	CH_2-*para*-Ph-COOMe
8	1	F	F	NO_2	H	CH_2-*para*-Ph-COOMe
9	1	NO_2	F	H	F	CH_2-*para*-Ph-COOMe
10	1	NO_2	H	H	F	CH_2-*para*-Ph-COOMe
11	1	NO_2	H	F	F	CH_2-*para*-Ph-COOMe
12	2	NO_2	H	NO_2	F	CH_2-*para*-Ph-COOMe
13	2	F	F	NO_2	H	CH_2-*para*-Ph-COOMe
14	2	NO_2	F	H	F	CH_2-*para*-Ph-COOMe
15	2	NO_2	H	H	F	CH_2-*para*-Ph-COOMe
16	2	NO_2	H	F	F	CH_2-*para*-Ph-COOMe
17	1	NO_2	H	NO_2	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
18	1	F	F	NO_2	H	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
19	1	NO_2	F	H	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
20	1	NO_2	H	H	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
21	1	NO_2	H	F	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
22	2	NO_2	H	NO_2	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
23	2	F	F	NO_2	H	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
24	2	NO_2	F	H	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
25	2	NO_2	H	H	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
26	2	NO_2	H	F	F	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
27	1	NO_2	F	NO_2	H	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
28	2	NO_2	F	NO_2	H	CH_2-*para*-Ph-Glu(O-*t*-Bu)$_2$
29	1	NO_2	H	NO_2	F	CH_2-*para*-Ph-Glu-OH
30	1	F	F	NO_2	H	CH_2-*para*-Ph-Glu-OH
31	1	NO_2	F	H	F	CH_2-*para*-Ph-Glu-OH
32	1	NO_2	H	H	F	CH_2-*para*-Ph-Glu-OH
33	1	NO_2	H	F	F	CH_2-*para*-Ph-Glu-OH
34	1	NO_2	F	NO_2	H	CH_2-*para*-Ph-Glu-OH
35	2	NO_2	H	NO_2	F	CH_2-*para*-Ph-Glu-OH
36	2	F	F	NO_2	H	CH_2-*para*-Ph-Glu-OH
37	2	NO_2	F	H	F	CH_2-*para*-Ph-Glu-OH
38	2	NO_2	H	H	F	CH_2-*para*-Ph-Glu-OH
39	2	NO_2	H	F	F	CH_2-*para*-Ph-Glu-OH
40	2	NO_2	F	NO_2	H	CH_2-*para*-Ph-Glu-OH

The activity of these agents against AICAR Tfase after 12 h pre-incubation was also examined (Table 2). In general, the agents were less potent AICAR Tfase inhibitors. Only four of the agents examined (**29**, **34**, **35** and **40**) strongly inhibited AICAR Tfase after the 12 h pre-incubation. These all contain the free glutamic acid side chain and are the same four agents of this subclass that significantly inhibited GAR Tfase. In contrast, the simpler agents (**2**, **7**, **9**, **11**, **12** and **14**–**16**) that were inhibitors of GAR Tfase exhibited only slight or no inhibition of AICAR Tfase. Given the general reactivity of fluoronitrophenyl compounds, it is interesting to note the selectivity seen between the related GAR Tfase and AICAR Tfase.

Agents **1**–**40** as well as Lometrexol were examined for cytotoxic activity both in the presence (+) and absence (−) of added hypoxanthine and thymidine against CCRF-CEM cells (Table 2). The cytotoxic activity of most of the agents was relatively nonpotent and uniform against the CCRF-CEM cell line regardless of whether media purines (hypoxanthine) or pyrimidines (thymidine) were present or absent. This indicates a lack of activity due to specific inhibition of the purine or pyrimidine biosynthetic pathways. The one exception to this absence of selectivity was **32**, which in addition to being one of the most cytotoxic compounds in the series examined, also showed a significant increase in potency in the absence of media hypoxanthine indicating its selective cytotoxicity may be derived from inhibition of either GAR or AICAR transformylase.

In preliminary studies to elucidate the nature of the activity of this class of inhibitors, **12** (500 μM) was incubated with GAR Tfase (6.4 μM). Electrospray mass

Table 2. In vitro biological activity against GAR Tfase, AICAR Tfase and CCRF-CEM cells[12]

	Pre-incubation (time)										
	Gar Tfase + GAR[a]			GAR Tfase[a]			AICAR Tfase[a]	CCRF-CEM IC_{50} (μM)[b]			
Agent	3 min	30 min	(h)	3 min	30 min	(h)	12 h	(+) T, (+) H[c]	(−) T, (+) H	(+) T, (−) H	(−) T, (−) H
1	NA[d]	NA	NA	K_i = 35 μM			NA	58	37	43	55
2	100	75	2 (6)	76	73	39 (6)	64	1.2	1.0	1.1	1.00
2 (25 μM)	NA	NA	56 (6)	76	71	58 (6)	n.t.[d]				
3	NA	NA	88 (6)	80	77	59 (6)	NA	73	76	68	76
4	NA	NA	NA	87	82	63 (6)	98	97	97	>100	97
5	NA	NA	NA	80	70	53 (6)	NA	94	97	97	97
6	NA	NA	NA	86	69	64 (7)	NA	>100	>100	>100	>100
7	66	54	27 (6)	47	24	2 (6)	75	1.1	1.7	1.9	1.3
8	79	85	67 (6)	68	60	30 (6)	73	67	36	61	48
9	32	29	30 (6)	16	9	0 (6)	NA	>100	>100	91	>100
10	81	62	47 (6)	78	43	12 (6)	94	72	72	66	72
11	35	31	22 (6)	17	3	2 (6)	NA	11	11	13	12
12	64	22	0 (6)	46	9	0 (6)	77	3.5	4.3	3.9	2.5
12 (25 μM)	NA	83	2 (6)	83	77	65 (6)	n.t.				
13	82	78	75 (6)	61	57	41 (6)	96	45	55	45	41
14	25	12	6 (6)	6	5	0 (6)	NA	>100	>100	>100	>100
14 (25 μM)	NA	NA	84 (6)	78	76	39 (6)	n.t.				
15	75	44	8 (6)	70	41	0 (6)	NA	30	55	43	45
16	68	54	14 (6)	44	33	0 (6)	94	53	66	55	59
17	25	12	7 (6)	19	11	0 (6)	87	15	12	4.3	13
17 (25 μM)	81	98	79 (6)	75	68	39 (6)	n.t.				
18	42	47	47 (6)	52	47	52 (3)	99	46	40	46	46
19	40	48	49 (6)	82	57	40 (6)	98	44	47	50	47
20	66	57	58 (6)	65	52	48 (6)	NA	50	56	69	51
21	42	40	37 (6)	57	53	35 (6)	NA	20	27	27	23
22	76	65	40 (6)	38	29	4 (6)	90	1.8	1.5	2.6	3.1
22 (25 μM)	NA	NA	76 (6)	79	74	69 (6)	n.t.				
23	97	94	88 (6)	71	61	47 (6)	NA	20	20	36	24
24	39	42	51 (8)	26	24	20 (8)	99	7.7	2.4	17	13
25	98	93	85 (8)	78	56	27 (8)	NA	14	12	3.4	8.7
26	72	80	66 (6)	53	55	27 (6)	NA	3.1	1.7	3.4	4.8
27	68	59	42 (8)	32	22	3 (8)	81	8.9	7.5	4.8	7.1
28	96	93	63 (8)	71	58	44 (8)	84	4.0	3.9	3.5	3.2
29	91	nt	68 (6)	77	nt	31 (6)	7	52	52	36	56
30	NA	NA	NA	93	96	91 (7)	NA	>100	>100	>100	>100
31	89	92	89 (7)	90	84	73 (7)	NA	>100	>100	>100	>100
32	78	81	85 (8)	80	78	81 (8)	NA	>100	>100	15	2.1
33	NA	NA	NA	87	94	98 (8)	NA	>100	>100	>100	>100
34	NA	90	66 (6)	91	67	32 (6)	6	54	50	37	49
35	NA	nt	43 (6)	85	nt	13 (6)	11	43	51	44	40
36	NA	98	92 (8)	87	NA	88 (8)	NA	>100	>100	>100	>100
37	79	85	79 (8)	78	80	87 (8)	NA	>100	>100	>100	>100
38	91	82	79 (8)	96	78	74 (8)	NA	>100	>100	>100	>100
39	66	59	81 (8)	66	62	83 (8)	NA	>100	>100	>100	>100
40	96	97	45 (6)	89	71	13 (6)	11	36	46	48	36
Lometrexol								>100	>100	0.07	0.13

[a]GAR and AICAR Tfase inhibition following preincubation (%control activity remaining (normalized)). Agents were tested at a concentration of 250 μM.
[b]The cytotoxic assays were conducted in media free of purines or pyrimidines in the presence of (+) or absence of (−) thymidine or hypoxanthine.
[c]T = Thymidine, H = Hypoxanthine.
[d]NA = not active, nt = not tested.

spectrometry indicated the covalent attachment of 1 molar equivalent of **12** to GAR Tfase. Interestingly, when **12** (250 μM) was incubated with GAR Tfase (6.4 μM) in the presence of β-GAR (1.25 mM), electrospray mass spectrometry did not indicate covalent attachment of **12** to the enzyme, although **12** is still an effective time-dependent inhibitor of GAR Tfase in the presence of β-GAR. This suggests that direct active site alkylation occurs in the absence of β-GAR, but that β-GAR alkylation occurs within the active site when it is included in the pre-incubation.

In conclusion, a novel class of folate based inhibitors has been reported that incorporate the fluoronitrophenyl group. Members of this new class of agents have been shown to inhibit GAR Tfase in a time dependent fashion. In the one case (**12**) examined to date, this occurs via adduct formation with nucleophilic residues in the GAR Tfase active site (−β-GAR) or by β-GAR alkylation (+ β-GAR). Further studies with the most active series of compounds (**2**, **12**, **22** and **35** as well as **14** and **32**) are in progress and the results will be disclosed in due course.

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11. ^{1}H NMR characterization of representative compounds is as follows: **2**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 9.29 (bs, 1H), 8.87 (d, J = 8.0 Hz, 1H), 7.16 (d, J = 15.0 Hz, 1H), 6.12 (bs, 2H), 5.94 (bs, 2H), 3.18–3.10 (m, 2H), 2.26 (t, J = 6.8 Hz, 2H), 1.65–1.54 (m, 2H); **12**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.57 (d, J = 7.9 Hz, 1H), 7.92 (d, J = 8.2 Hz, 2H), 7.41 (d, J = 7.9 Hz, 2H), 7.31 (d, J = 14.9 Hz, 1H), 6.08 (bs, 2H), 5.84 (bs, 2H), 4.73 (s, 2H), 3.83 (s, 3H), 3.21 (t, J = 7.0 Hz, 2H), 2.09 (t, J = 7.9 Hz, 2H), 1.66–1.59 (m, 2H); **22**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.57 (d, J = 7.7 Hz, 1H), 7.83 (d, J = 8.0 Hz, 2H), 7.37 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 15.0 Hz, 1H), 4.73 (s, 2H), 4.36–4.25 (m, 1H), 3.26–3.15 (m, 2H), 2.33 (t, J = 6.6 Hz, 2H), 2.20–2.11 (m, 2H), 2.10–1.85 (m, 2H), 1.74–1.60 (m, 2H), 1.40 (s, 9H), 1.37 (s, 9H); **35**: ^{1}H NMR (DMSO-d_6, 250 MHz) δ 8.56 (d, J = 8.4 Hz, 1H), 7.84 (d, J = 9.1 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 15.0 Hz, 1H), 4.73 (s, 2H), 4.46–4.32 (m, 1H), 3.29–3.15 (m, 2H), 2.34 (t, J = 8.0 Hz, 2H), 2.20–2.05 (m, 2H), 2.03–1.88 (m, 2H), 1.74–1.60 (m, 2H).
12. The CCRF-CEM cytotoxicity assay, the AICAR Tfase inhibition studies and the time-dependent GAR Tfase inhibition studies were performed as described in Boger, D. L.; Haynes, N.-E.; Kitos, P. A.; Warren, M. S.; Ramcharan, J.; Marolewski, A. E.; Benkovic, S. J. *Bioorg. Med. Chem.* **1997**, *5*, 1817 with the following changes: In the GAR Tfase assay, solutions were made containing 50 nM purN GAR Tfase, 750 nM BSA, 1.25 mM GAR (if GAR was present during the preincubation) and 250 μM inhibitor. These solutions were incubated at room temperature. Aliquots of these stock solutions were taken, diluted 25-fold in assay buffer, and thermostated to 26 °C on a Cary 1 UV-Visible spectrophotometer. Assays were initiated by the addition of 20 μM fDDF at the indicated time points. In the AICAR Tfase assay, the assay solutions were incubated at room temperature for 12 h before the reaction was initiated by addition of AICAR.