SUPPLEMENTAL DATA

Targeting non-squamous non-small cell lung cancer via the proton-coupled folate transporter with 6-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl antifolates

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Table S1. Immunohistochemistry (IHC) results are shown for 61 non-squamous non-small cell lung cancer (NS-NSCLC) and 10 normal lung tissues from a tissue microarray (TMA) (US Biomax, Inc). The TMA was incubated with affinity-purified PCFT-specific antibody or rabbit IgG, the slides developed, counterstained and mounted, as described in Materials and Methods. Representative images are shown in Figure 3 (panel C) for IgG and for 3 NS-NSCLC specimens with low, intermediate and high level staining. The slides were manually scored by two independent pathologists with the staining intensities scored as negative (0), weak (1+), moderate (2+), or strong (3+), then scanned by Aperio Image Scanner (Aperio Technologies, Inc.) for microarray image scanning. The total positive cell numbers and intensity of antibody staining of each tissue core were computed and are plotted in Figure 3 (panel B). Sex, age, pathology, grade, stage, and staining intensity for each patient are summarized in the table. Abbreviations: F, female; M, male.

No.	Sex	Age	Pathology	Grade	Stage	Staining intensity	Relative level
1	M	46	Normal lung tissue	-	-	0	1.30
2	F	14	Normal lung tissue	-	-	0	1.78
3	M	47	Normal lung tissue	-	-	0	0.18
4	M	42	Normal lung tissue	-	-	0	1.20
5	M	25	Normal lung tissue	-	-	0	0.63
6	M	35	Normal lung tissue	-	-	0	0.81
7	F	15	Normal lung tissue	-	-	0	1.19
8	M	50	Normal lung tissue	-	-	0	0.20
9	M	47	Normal lung tissue	-	-	0	1.73
10	M	24	Normal lung tissue	-	-	0	0.42
11	M	50	Mucinous adenocarcinoma	1	II	1	4.32
12	M	70	Papillary adenocarcinoma	1	I	1	2.13
13	M	42	Mucinoius adenocarcinoma	1	I	0	0.32
14	F	48	Mucinoius adenocarcinoma	1	I	1	6.75
15	F	64	Mucinoius adenocarcinoma	1	II	0	2.87
16	F	62	Adenocarcinoma	1	II	1	3.58
17	F	44	Adenocarcinoma	1	IIIa	1	0.06
18	F	43	Adenocarcinoma	1	IIIa	0	0.78
19	M	63	Adenocarcinoma	2	IIIa	1	2.37
20	M	51	Adenocarcinoma (sparse)	-	IIIa	1	2.49
21	F	63	Adenocarcinoma	2	II	1	0.83
22	M	50	Adenocarcinoma	2	IIIa	2	1.16
23	F	44	Adenocarcinoma	2	II	2	6.46
24	M	56	Adenocarcinoma	2	IIIa	1	2.09
25	F	71	Adenocarcinoma	2	II	2	5.44
26	F	56	Adenocarcinoma	1	II	2	7.85
27	F	50	Papillary adenocarcinoma	1	I	2	5.29
28	F	56	Adenocarcinoma	2	I	2	5.12
29	M	67	Adenocarcinoma (lung tissue)	-	II	0	0.31
30	M	59	Adenocarcinoma	2	IIIa	1	5.40
31	M	61	Adenocarcinoma	2	I	0	2.60
32	M	50	Adenocarcinoma	2	I	1	2.03
33	F	61	Adenocarcinoma	2	II	0	4.00
34	F	60	Adenocarcinoma	2	I	0	5.00
35	M	66	Adenocarcinoma	2	II	1	4.40
36	F	68	Adenocarcinoma with necrosis	2	IIIa	2	5.12
37	F	55	Adenocarcinoma	2	I	1	3.58
38	F	50	Adenocarcinoma	2	IIIa	0	2.26
39	M	57	Adenocarcinoma	2	II	1	0.51
40	M	50	Adenocarcinoma	2-3	II	1	6.10
41	M	68	Adenocarcinoma (sparse)	-	IV	1	6.17
42	M	60	Adenocarcinoma	2-3	II	1	4.17
43	F	51	Adenocarcinoma (lung tissue)	-	I	0	2.92
44	M	68	Adenocarcinoma (sparse)	-	II	0	3.70

45	F	64	Adenocarcinoma	2	I	1	2.98
46	F	59	Adenocarcinoma	3	IIIa	2	3.02
47	M	53	Adenocarcinoma	3	I	2	3.07
48	F	57	Adenocarcinoma	2	I	1	1.02
49	F	48	Adenocarcinoma	2	I	2	3.99
50	M	60	Adenocarcinoma	3	IIIa	1	2.01
51	M	54	Adenocarcinoma	3	I	1	6.07
52	M	76	Adenocarcinoma	3	II	2	8.83
53	M	67	Adenocarcinoma	3	IIIa	2	4.84
54	F	35	Adenocarcinoma	3	I	1	4.56
55	F	68	Adenocarcinoma	3	IIIa	1	5.88
56	M	57	Adenocarcinoma (lung tissue)	-	I	0	1.27
57	M	42	Adenocarcinoma (sparse)	-	IIIa	1	3.25
58	M	59	Adenocarcinoma	2	II	0	3.51
59	F	51	Bronchioloalveolar carcinoma	-	I	1	1.11
60	M	72	Bronchioloalveolar carcinoma	-	I	1	1.20
61	M	60	Bronchioloalveolar carcinoma (sparse carcinoma with necrosis)	-	II	1	6.01
62	M	53	Bronchioloalveolar carcinoma	-	II	0	0.17
63	M	53	Large cell carcinoma	-	IIIb	2	6.93
64	F	54	Large cell carcinoma	-	II	2	4.80
65	M	54	Large cell carcinoma	-	I	1	2.10
66	M	65	Large cell carcinoma	-	I	2	5.90
67	M	60	Atypical carcinoid	-	I	1	3.18
68	M	57	Atypical carcinoid	-	I	1	4.77
69	F	36	Atypical carcinoid	-	I	2	3.39
70	M	49	Atypical carcinoid	-	IIIb	2	4.47
71	M	43	Carcinoid	-	I	2	4.74

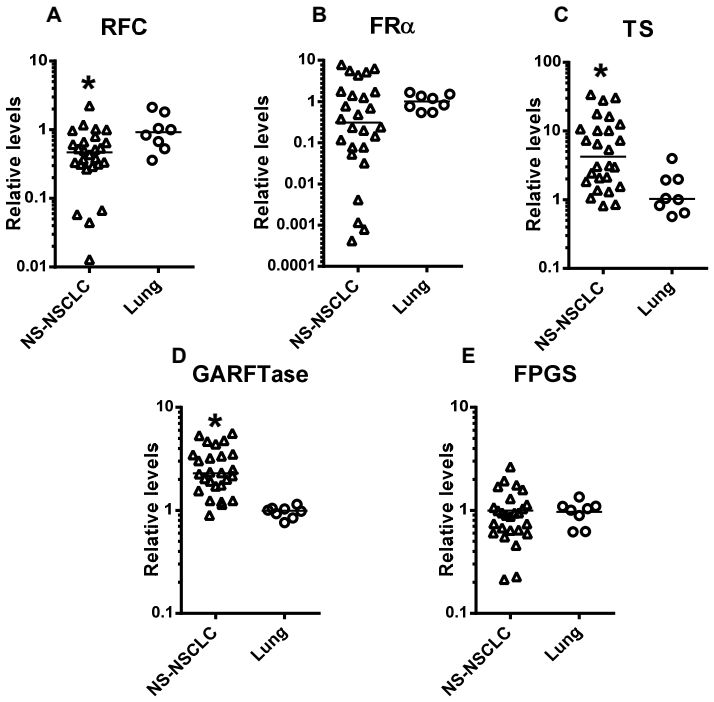

Figure S1. Expression of folate uptake and metabolism genes in primary non-squamous non-small cell lung cancer (NS-NSCLC) and normal lung tissues. A-E: Results for quantitative real-time RT-PCR are shown for 26 NS-NSCLC and 9 normal lung specimens (Origene) for various genes including reduced folate carrier (RFC), folate receptor (FR) α, thymidylate synthase (TS), glycinamide ribonucleotide formyltransferase (GARFTase), and folylpolyglutamate synthetase (FPGS). Transcript levels of the target genes were normalized to transcript levels for β-actin. The median values for the normal lung specimens were assigned a value of 1. Statistical significance between the groups was analyzed by a student's t-test. An asterisk indicates a statistically significant difference between the median NS-NSCLC value and the median value for the normal lung specimens, $p < 0.05$. Experimental details are described in Materials and Methods.

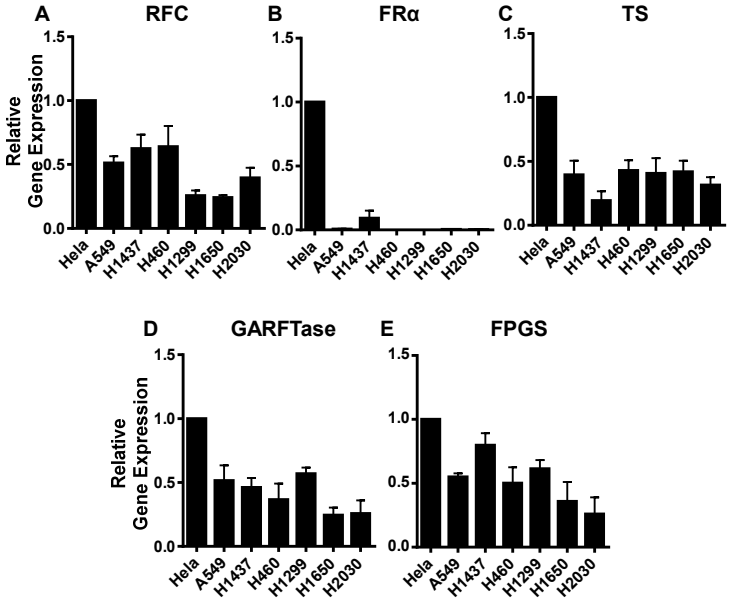

Figure S2. Expression of folate uptake and metabolism genes in NS-NSCLC cell lines. A-E) To measure transcript levels of folate transporters and folate metabolism enzymes in NS-NSCLC cell lines (A549, H1437, H460, H1299, H1650, and H2030), including RFC, FRα, TS, GARFTase, and FPGS, quantitative real-time RT-PCR was performed. Transcript levels for the target genes were normalized to transcript levels for β-actin. The mean transcript levels for the HeLa cell line were assigned a value of 1. Results are shown as mean values ± standard errors, representative of triplicate experiments. Experimental details are described in Materials and Methods.

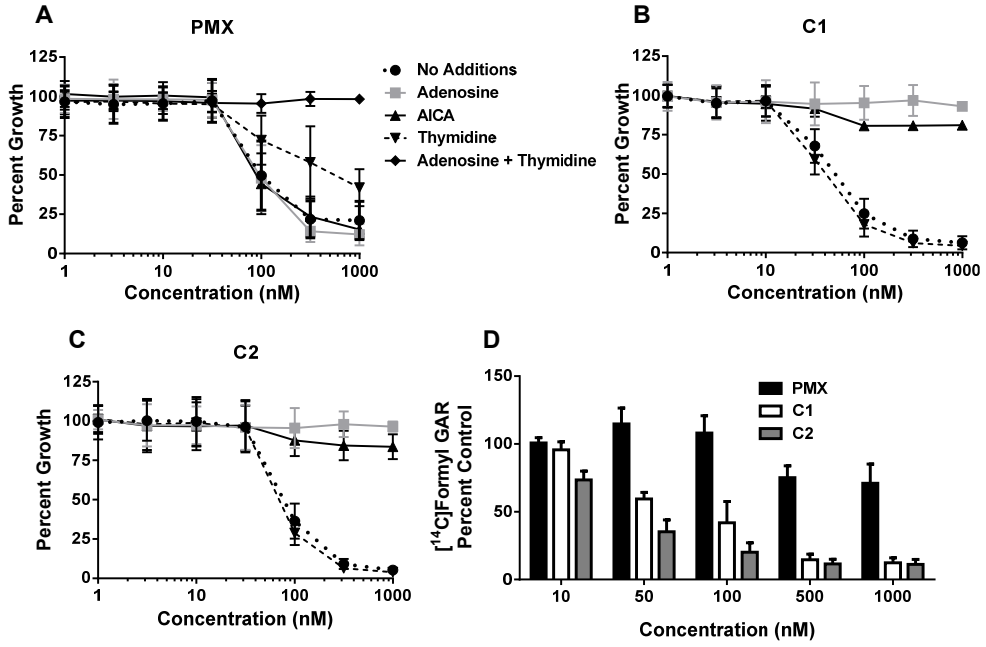

Figure S3. Determination of inhibition of *de novo* purine biosynthesis and GARFTase by C1 and C2. A-C) H460 cells were seeded in the presence of 0-1000 nM pemetrexed (PMX), **C1** or **C2**, with or without additions of 60 μM adenosine, 320 μM 5-aminoimidazole-4-carboxamide (AICA), 10 μM thymidine, or adenosine plus thymidine. Cell viabilities were determined with a fluorescence-based assay (Cell Titer Blue™). D) Incorporation of [^{14}C(U)]glycine into [^{14}C]formyl glycinamide ribonucleotide (GAR) as an *in situ* measure of endogenous GARFTase was determined. For the GARFTase assays, IC_{50} values were 78.9 nM and 27.1 nM for **C1** and **C2**, respectively. For PMX, the IC_{50} value was >1000 nM. Experimental details are described in Materials and Methods. For A-D, the plots show mean values ± standard errors and represent triplicate experiments.