6-Substituted Pyrrolo[2,3-*d*]pyrimidine Thienoyl Regioisomers as Targeted Antifolates for Folate Receptor α and the Proton-coupled Folate Transporter in Human Tumors

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Molecular modeling studies

Table 1S. Elemental Analysis

		Calculated, %				Found, %			
Cpd	**Formula**	**C**	**H**	**N**	**S**	**C**	**H**	**N**	**S**
5	$C_{19}H_{21}N_5O_6S \cdot 1.7\ H_2O$	47.73	5.14	14.65	6.71	47.75	4.95	14.38	6.59
7	$C_{19}H_{21}N_5O_6S \cdot 1.4\ H_2O$	48.28	5.08	14.82	6.78	48.23	4.90	14.74	6.73
6	$C_{19}H_{21}N_5O_6S \cdot 1.2\ HCO_2H$	48.26	4.69	13.93	6.39	48.33	4.83	13.55	6.82
8	$C_{19}H_{21}N_5O_6S \cdot 0.3\ CHCl_3$	47.97	4.44	14.49	6.64	48.36	4.58	14.25	6.45

Table 2S. High-Resolution Mass Spectra (HRMS) (ESI)

Compound	Formula	Calculated mass	Found mass
6	$C_{19}H_{21}N_5O_6S\ (M+H)^+$	448.1285	448.1280

Table 3S. Crystallographic data collection and refinement statistics for glycinamide ribonucleotide formyltransferase (GARFTase) in complex with β-GAR and 5, 7, or pemetrexed (PMX). Values in parentheses are for the highest resolution shell.

	GARFTase / Cpd 5	GARFTase / Cpd 7	GARFTase / PMX
PDB ID	4ZZ1	4ZZ2	4ZZ3
Data Collection			
Space group	$P3_221$	$P3_221$	$P3_221$
a=b, c (Å)	75.34, 100.74	75.58, 101.00	74.98,100.93
Molecule per a.s.u	1	1	1
Resolution	50-1.35 (1.37-1.35)	50-1.45 (1.48-1.45)	50-2.50 (2.54-2.50)
I/σI	27.8 (2.3)	31.3 (1.9)	28.2 (3.8)
Completeness (%)	98.9 (88.9)	99.6 (96.8)	99.6 (99.4)
Redundancy	9.7 (3.6)	10.3 (4.9)	8.0 (4.9)
R_{sym}	7.0 (43.0)	6.6 (52.1)	6.7 (35.6)
R_{pim}	2.3 (23.1)	2.1 (24.7)	2.5 (17.5)
CC* (high resolution shell)	0.957	0.951	0.975
Refinement			
Resolution (Å)	40-1.35	40-1.45	40-2.50
No. reflections			
Used in refinement	70157	57888	11574
Used in Rfree calculation	2004	2005	1158
Rfactor (%)	13.96	14.15	19.55
Rfree (%)	15.77	16.24	24.33
No. atoms			
Protein	1608	1580	1489
Ligand	49	49	49

Water molecules	328	300	27
B-factors (Å2, average)			
Overall	23.4	22.8	49.4
Ligand	21.5	21.1	62.0
Water molecules	37.9	37.7	35.6
r.m.s. deviations			
Bond lengths (Å)	0.006	0.006	0.002
Bond angles (º)	1.163	1.144	0.695
MolProbity clash score	0.90	0.61	3.90

Table 4S. Molecular contacts of GARFTase with antifolates based on crystallographic models. The interacting atoms and the contact distances between heavy atoms are shown with the distances greater than 3.2 Å in red. Atom labels are in accordance with those in the PDB coordinate files.

GARFTase / Cpd 5			GARFTase / Cpd 7			GARFTase / PMX		
Atom 1	Atom 2	Dist. (Å)	Atom 1	Atom 2	Dist. (Å)	Atom 1	Atom 2	Dist. (Å)
Asp 951 N	Cpd 5 O10	3.1	Asp 951 N	Cpd 7 O10	3.0	Ala 947 O	PMX N16	3.1
Ala 947 O	Cpd 5 N3	2.8	Ala 947 O	Cpd 7 N3	2.8	Glu 948 O	PMX N19	3.1
Glu 948 O	Cpd 5 N11	3.0	Glu 948 O	Cpd 7 N11	3.0	Leu 899 N	PMX N18	3
Leu 899 O	Cpd 5 N11	3.1	Leu 899 O	Cpd 7 N11	3.1	Arg 897 O	PMX N11	2.7
Leu 899 N	Cpd 5 N1	3.0	Leu 899 N	Cpd 7 N1	3.0	Met 896 O	PMX N22	2.8
Arg 897 O	Cpd 5 N5	2.9	Arg 897 O	Cpd 7 N5	2.9	Arg 897 NH1	PMX O30	4.2
Met 896 O	Cpd 5 N19	2.8	Met 896 O	Cpd 7 N19	2.9	Arg 897 NH1	PMX O31	3.8
Ile 898 N	Cpd 5 O28	2.9	Ile 898 N	Cpd 7 O28	2.9	Arg 897 NH2	PMX O31	2.5
Arg 871 NH1	Cpd 5 O28	2.8	Arg 871 NH1	Cpd 7 O28	2.8			
Arg 871 NH2	Cpd 5 O27	2.6	Arg 871 NH2	Cpd 7 O27	2.6			
Arg 897 NH2	Cpd 5 O27	3.7	Arg 897 NH2	Cpd 7 O27	3.7			
H_2O 110	Cpd 5 O25	2.3	H_2O 300	Cpd 7 O24	2.6			
Arg 897 NH1	H_2O 110	2.8	Arg 897 NH1	H_2O 300	2.8			
Lys 844 NZ	Cpd 5 O25	4.7	Lys 844 NZ	Cpd 7 O25	5.0			

Molecular modeling studies. The X-ray crystal structures of human folate receptor (FR) FRβ (PDB: 4KN0, 2.10 Å resolution)[1] and human glycinamide ribonucleotide formyltransferase (GARFTase) (PDB: 1NJS, 1.98 Å resolution)[2] are known. We docked our proposed 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs **5** and **7** into the known structures for FRβ and GARFTase using the software LeadIT 2.1.6[3] and previously described methods[4] to predict activities of the analogs for uptake by FRs and for GARFTase inhibition.

FRβ

FRα and FRβ share high sequence identity (82%) and sequence similarity (92%).[1] **Figure 4** in the manuscript shows the superimposition of the docked poses of **5** (red) and **7** (green) in the human FRα (PDB ID: 4LRH)[5] active site. The docked conformations of **5** and **7** in the X-ray crystal structure of FRβ bound to PMX (PDB: 4KN0; **Figure 1S**)[1] were very similar to those seen in the docking studies with FRα.

Figure 1S shows the superimposition of the docked poses of **5** (green) and **7** (red) in the human FRβ (PDB ID: 4KN0)[1] active site. The original crystal structure ligand, methotrexate (MTX), is not shown. The N1 nitrogens of **5** and **7** can interact with Arg119 while the 2-NH_2 moieties of both compounds interact via water molecules with Phe123. The pyrrolo[2,3-*d*]pyrimidine scaffolds can form hydrophobic interactions with Tyr76, Tyr101 and Trp187, similar to that seen with the bicyclic scaffold of MTX in the crystal structure. The thiophene rings of the two compounds can interact with Phe78, Trp118 and Trp156, analogous to the benzoyl moiety of MTX. The *L*-glutamate moieties of both compounds are similarly oriented and thus mimic the corresponding glutamate in MTX. The α-carboxylic acid of the glutamate side chain can form hydrogen bonds with the backbone NH of Gly153, Trp154 and Trp156. The γ-carboxylic acid can interact with the side chain NH group of Trp118. The docking scores of **5** and **7** were -47.40 kJ/mol and -42.44 kJ/mol, compared with -48.67 kJ/mol for folic acid.

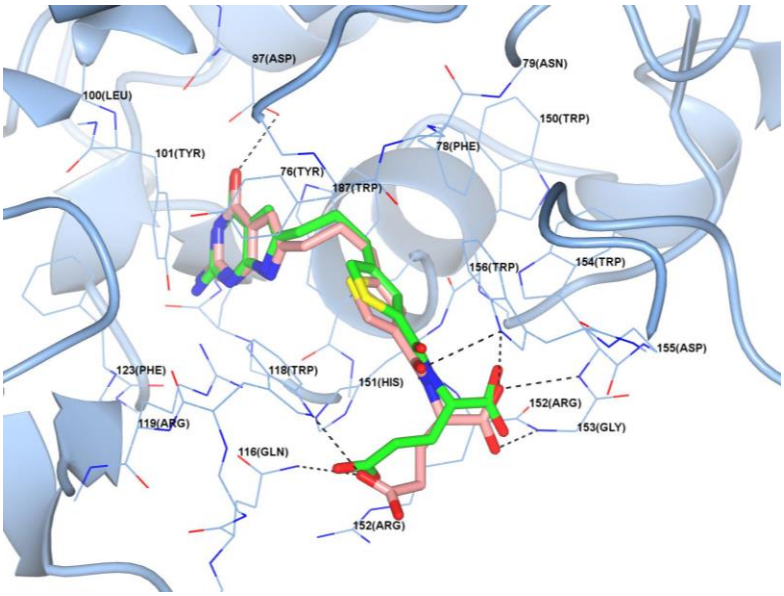

Figure 1S. Molecular modeling of 5 and 7 with FRβ. Superimposition of the docked poses of **5** (green) and **7** (pink) in the folate site of human FRα (PDB: 4KN0).

GARFTase

Docking studies were carried out using the X-ray crystal structure of human GARFTase bound to trifluoroacetyl-5,10-dideaza-acyclic-5,6,7,8-tetrahydrofolic acid (10-CF_3CO-DDACTHF, PDB: 1NJS).[2] **Figure 3S** shows the superimposition of the docked poses of **5** (green) and **7** (pink) in the GARFTase active site. As was seen with the docked conformations of these compounds in FRα, the docked conformations of both compounds are very similar. The 2-NH_2 and N1 moieties of **5** and **7** form hydrogen bonds with the backbone carbonyl and NH of Leu92, respectively. Additional hydrogen bonds are formed between 2-NH_2 of the two compounds with the backbone carbonyl of Asp142, 3-NH with the backbone of Ala140, and between the 4-oxo and backbone NH of Asp144. The pyrrolo[2,3-*d*]pyrimidine scaffolds of **5** and **7** bind in the region occupied by the diaminopyrimidine ring in 10-CF_3CO-DDACTHF (not shown) and form hydrophobic interactions with Leu85, Leu92, Val139. The pyrrole N7-nitrogens of the two compounds form hydrogen bonds with the backbone NH of Arg90. The pyrrolo[2,3-*d*]pyrimidine scaffolds of the two compounds form hydrophobic interactions with Leu85, Ile91, Ile91, Leu92, Val97 and

Val143. Subtle differences in the docked conformations of **5** and **7** occur in the thienoyl moieties, resulting from interaction of the thienoyl carbonyl of **5**, forming a hydrogen bond with the side chain of Ser116, and the corresponding carbonyl of **7** predicted to form a hydrogen bond with the backbone NH of Ile91. The glutamate chains of **5** and **7** are oriented similarly with the γ-COOH interacting with the side chain of Arg90 and the backbone NH of Leu91 and the α-COOH solvent exposed. The docking scores of **5** and **7** were -50.49 kJ/mol and -57.77 kJ/mol, respectively, compared with -53.90 kJ/mol for the crystal structure ligand 10-CF_3CO-DDACTHF. Molecular docking studies, described above, provided additional support for the synthesis and biological evaluation of **5** and **7** as FR-transportable analogs and as potent GARFTase inhibitors.

A comparison of the docked pose of **7** and its X-ray crystal structure pose in the 10-formyl tetrahydrofolate binding site of human GARFTase is shown in **Figure 6S**.

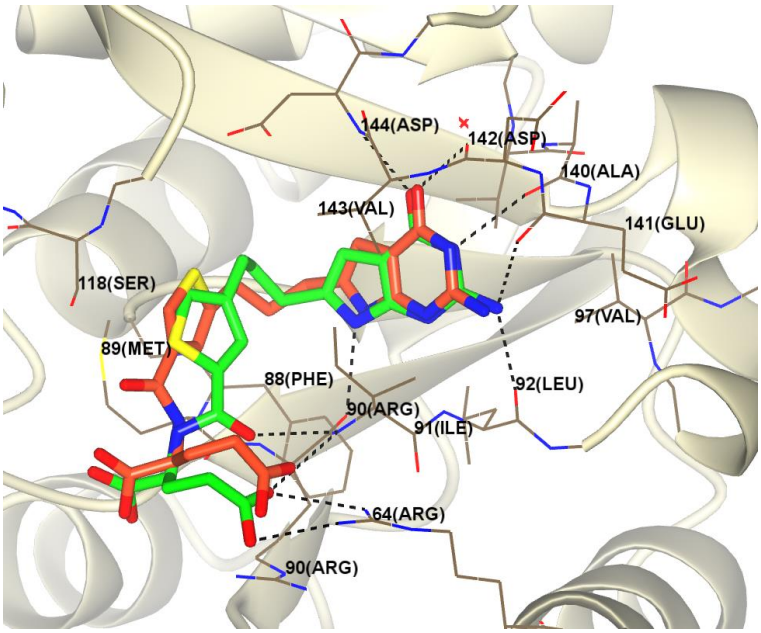

Figure 2S. Molecular modeling of 5 and 7 with human GARFTase. Superimposition of the docked poses of **5** (green) and **7** (red) in the folate site of human GARFTase (PDB: 1NJS).[2]

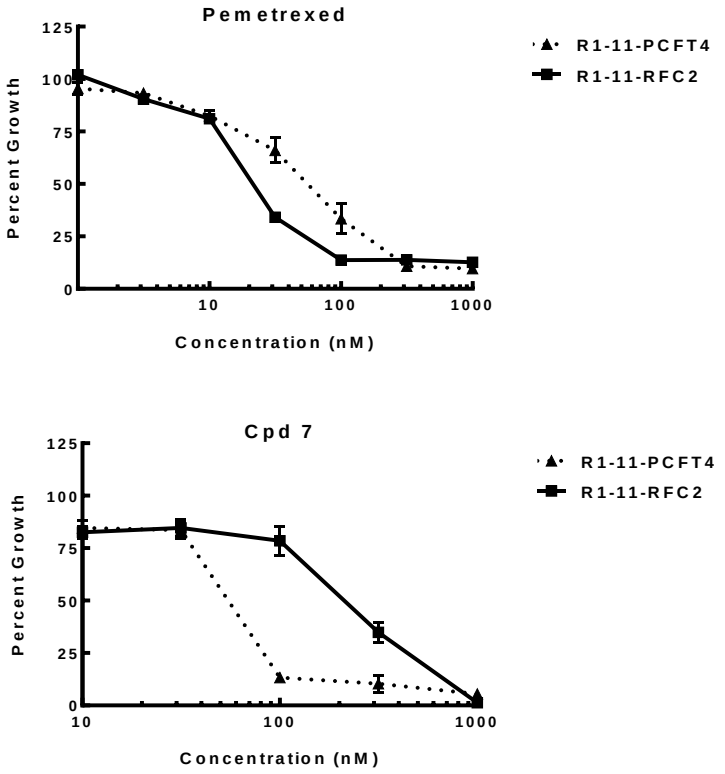

Figure 3S. Inhibition of R1-11-PCFT4 and R1-11-RFC2 cells by PMX and 7. Growth inhibition curves for folate-depleted R1-11-PCFT4 and –RFC2 cells treated with pemetrexed or **7** for 96 h are shown. Calculated IC_{50} values for R1-11-PCFT4 were 59 nM and 56 nM with pemetrexed and **7**, respectively. IC_{50} values for R1-11-RFC2 were 21 nM and 220 nM with pemetrexed and **7**, respectively. Abbreviation: Cpd, Compound.

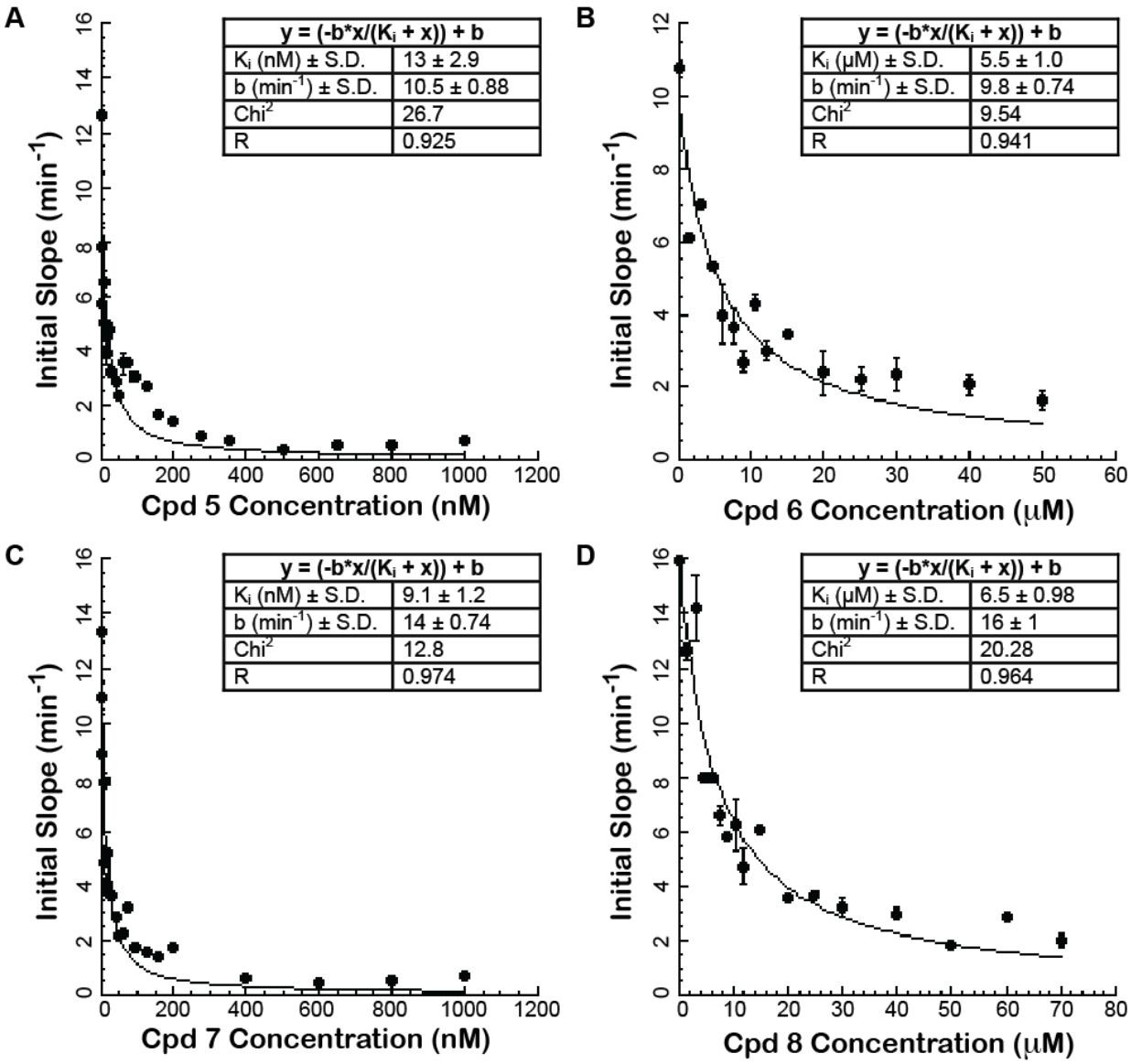

Figure 4S. (A-D) Graphs of antifolate concentration against initial slope of GARFTase for 5-8. Each graph was fit to a hyperbola to determine K_i. The hyperbola equation, determined K_i and y-intercept (b) values, as well as the Chi^2 and R-value for each curve is listed in the inset table.

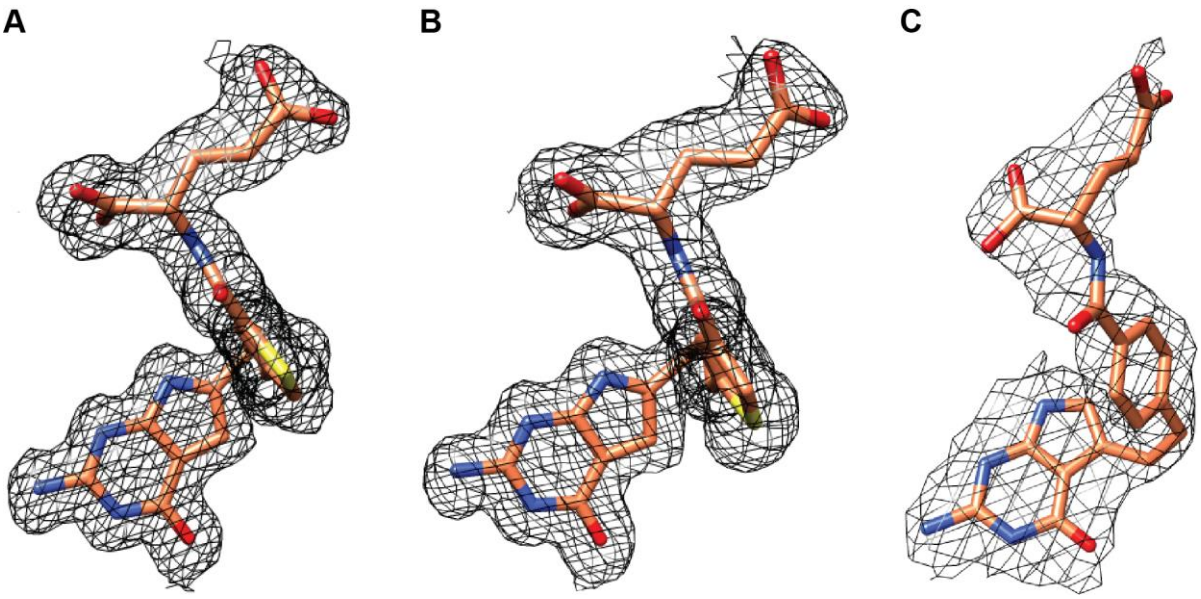

Figure 5S. Ligand structure and electron density. (A-C) The structures of **5**, **7**, and PMX, respectively, bound to GARFTase with the protein hidden and $2F_o - F_c$ map density contoured at 0.7 σ in mesh.

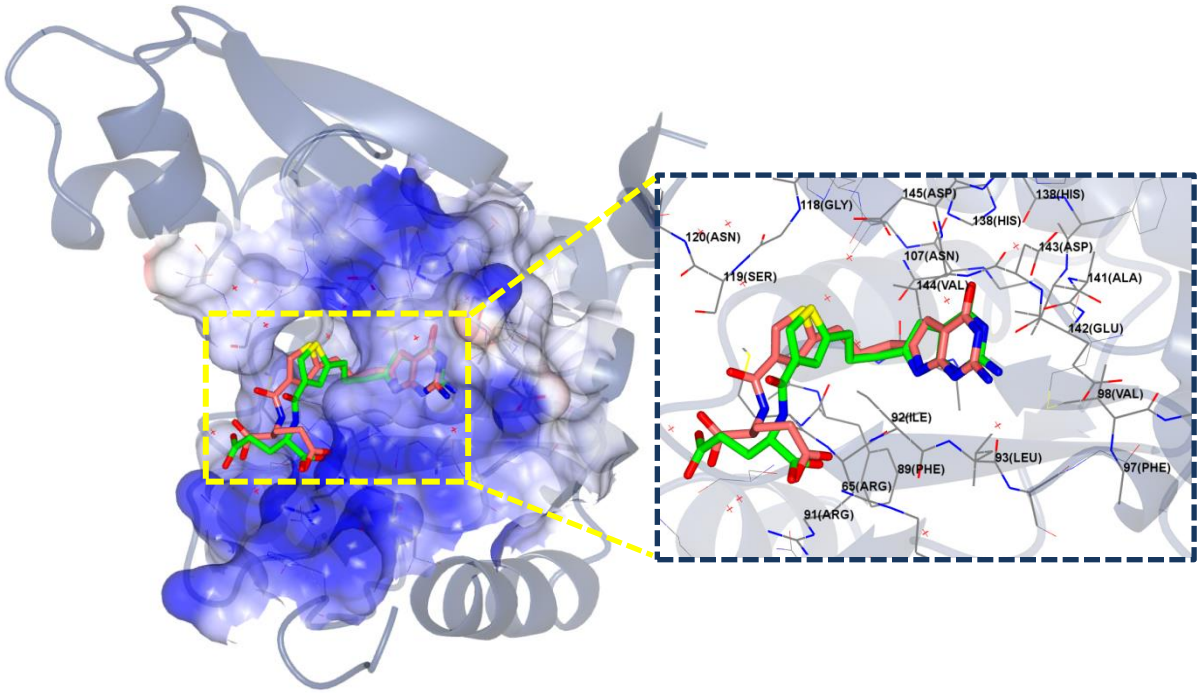

Figure 6S. Comparison of the docked pose of **7** (pink) and its X-ray crystal structure pose (green) in the 10-formyl tetrahydrofolate binding site of human GARFTase.

References

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