

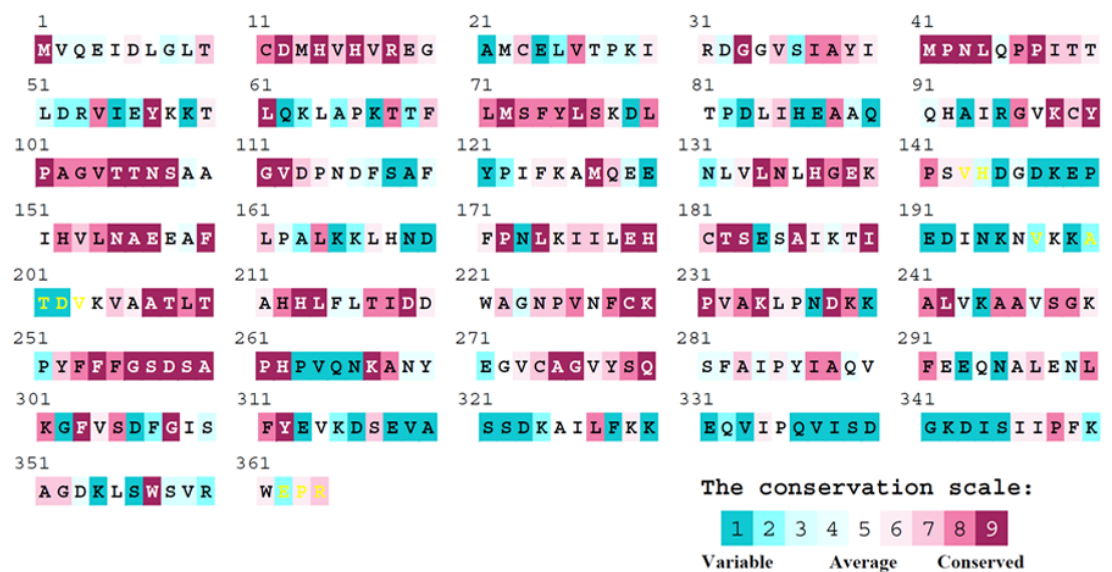
SUPPLEMENTARY INFORMATION

**Plumbagin, a natural product with potent anticancer activities, binds to and inhibits dihydroorotase, a key enzyme in pyrimidine biosynthesis**

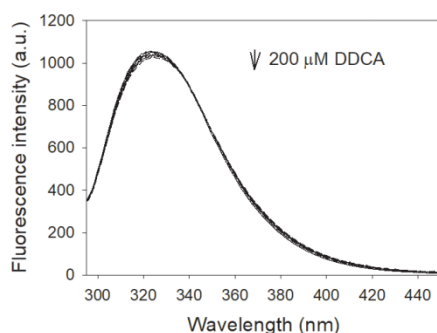
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**Supplementary information includes:**

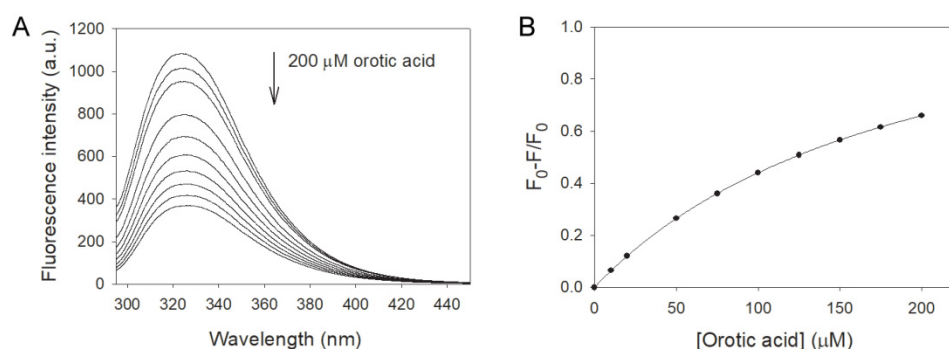
Figure S1: An alignment consensus of sequenced DHOase homologs by ConSurf. Figure S2: Binding of DDCA to ScDHOase. Figure S3: Binding of orotic acid to ScDHOase. Table S1: The formation of hydrogen bonds in the complexed structure of ScDHOase with PLU. Table S2: Primers used for construction of plasmids. Table S3: Anticancer activity of PLU and 5-FU against 4T1 cells.



**Supplementary Figure S1. An alignment consensus of sequenced DHOase homologs by ConSurf.** To show the degree of variability at each position along the sequence, the alignment consensus of 1694 unique sequenced DHOase homologs was performed by ConSurf. Amino acid residues that are highly variable are colored teal, while highly conserved amino acid residues are colored burgundy. A consensus sequence was established by determining which amino acid residue is most commonly found at each position relative to the primary sequence of ScDHOase. The binding sites mentioned above are highly conserved among varying organisms.



**Supplementary Figure S2. Binding of DDCA to ScDHOase.** The fluorescence emission spectra of ScDHOase with DDCA of different concentrations (0–200  $\mu\text{M}$ ). The decrease in intrinsic fluorescence of protein was measured at 324 nm upon excitation at 280 nm with a spectrofluorimeter. When DDCA (200  $\mu\text{M}$ ) was added into the ScDHOase solution, the intrinsic fluorescence was only quenched by 3.0%, suggesting low interactions.



**Supplementary Figure S3. Binding of orotic acid to ScDHOase.** (A) The fluorescence emission spectra of ScDHOase with orotic acid of different concentrations (0–200  $\mu\text{M}$ ; 0, 10, 20, 50, 75, 100, 125, 150, 175, and 200  $\mu\text{M}$ ). The decrease in intrinsic fluorescence of protein was measured at 324 nm upon excitation at 280 nm with a spectrofluorimeter. The fluorescence intensity emission spectra of ScDHOase significantly quenched with orotic acid. (B) An aliquot amount of orotic acid was added to the enzyme solution for determining the  $K_d$ . The  $K_d$  was obtained by the equation:  $\Delta F = \Delta F_{\text{max}} - K_d(\Delta F/[\text{orotic acid}])$ . Data points are an average of 2–3 determinations within 10% error. The  $K_d$  value of orotic acid for ScDHOase was  $198.3 \pm 1.2 \mu\text{M}$  as determined through this curve.

**Supplementary Table S1.** The formation of hydrogen bonds in the complexed structure of ScDHOase with PLU.

Subunit	Ligand	Distance (Å)	Residue
B	PLU [O2]	2.62	H16 [ND1]
B	PLU [O2]	3.11	R18 [NH1]
B	PLU [O2]	2.99	N43 [ND2]
B	PLU [O1]	2.01	K230 [O]
B	PLU [O3]	3.39	K230 [N]
D	PLU [O2]	2.97	T105 [OG1]
D	PLU [O1]	3.59	D258 [O]
D	PLU [O1]	2.54	D258 [OD2]
A	Malate [O1B]	2.83	R18 [NH2]
A	Malate [O2]	3.66	N43 [ND2]
A	Malate [O1A]	2.35	H262 [NE2]
C	Malate [O4A]	2.24	R18 [NH1]
C	Malate [O4A]	3.75	N43 [ND2]
C	Malate [O2]	2.34	T105 [OG1]
C	Malate [O2]	3.44	T106 [N]
C	Malate [O2]	3.77	T106 [O]
C	Malate [O4B]	3.21	T106 [OG1]

The formation of hydrogen bonds was analyzed by PISA, an automatic analytical tool for macromolecular assemblies in the crystalline state.

**Supplementary Table S2.** Primers used for construction of plasmids.

Oligonucleotide	Primer
ScDHOase-wt-NdeI-N	GGG <u>CATAT</u> GGTACAAGAAATCGATTTA
ScDHOase-wt-XhoI-C	GGG <u>CTCGAG</u> ACGAGGTTCCCATCTCAC
ScDHOase H14A-N	TTGACATGTGATATGG <u>CTGT</u> TCATGTAAGA
ScDHOase H14A-C	CTCTCTTACATGAACAG <u>CC</u> CATATCACATGT
ScDHOase H16A-N	ACATGTGATATGCATGTTG <u>CTGT</u> AAGAGAG
ScDHOase H16A-C	CGCACCCCTCTCTTACAG <u>CA</u> ACATGCATATC
ScDHOase R18A-N	GCATGTTTCATGTAG <u>CA</u> GAGGGTGCGATGTG
ScDHOase R18A-C	TCGCACCCTCTG <u>CT</u> TACATGAACATGCATAT
ScDHOase N43A-N	TACATCATGCCAG <u>CT</u> TTACAACCTCCAATT
ScDHOase N43A-C	TGGAGGTTGTAAAG <u>CT</u> TGGCATGATGTAAGC
ScDHOase K98A-N	TGCCATTTCGTGGAGTAG <u>CG</u> TGTTATCCAGC
ScDHOase K98A-C	TCCCGCTGGATAACACG <u>CT</u> ACTCCACGAAT

ScDHOase T105A-N	CCAGCGGGAGTAG <u>CA</u> ACAAATTCGGCTGCT
ScDHOase T105A-C	CCGAATTTGTTG <u>CT</u> ACTCCCGCTGGATAAC
ScDHOase T106A-N	CCAGCGGGAGTAACAG <u>CA</u> AAATTCGGCTGCT
ScDHOase T106A-C	CCGAATTTG <u>CT</u> TGTTACTCCCGCTGGATAAC
ScDHOase H137A-N	ACCTGGTATTAAATTTGG <u>CT</u> TGGGGAAAAAC
ScDHOase H137A-C	CAGAAGGTTTTTCCCCAG <u>CC</u> AAATTTAATA
ScDHOase H180A-N	TAATTCTGGAAG <u>CC</u> TGCACTAGCGAGTCGG
ScDHOase H180A-C	CGCTAGTGCAG <u>GCT</u> TCCAGAATTATTTTCA
ScDHOase D258A-N	CTTTGGATCTG <u>CT</u> TCAGCACCTCATCCTG
ScDHOase D258A-C	CAGGATGAGGTGCTGAAG <u>C</u> AGATCCAAAG
ScDHOase D258E-N	TCTTTGGATCTGA <u>AT</u> CAGCACCTCATCCTG
ScDHOase D258E-C	CAGGATGAGGTGCTGA <u>TT</u> CAGATCCAAAGA
ScDHOase H262A-N	ATTCAGCACCTG <u>CT</u> TCCTGTACAAAATAAGG
ScDHOase H262A-C	TTGTACAGGAG <u>C</u> AGGTGCTGAATCAGATC
huDHOase1456-1846-NdeI-N	GGG <u>CATATG</u> ACCTCCCAAAGCTTGTG
huDHOase1456-1846-XhoI-C	GGG <u>CTCGAG</u> ATCAGGAAGCCCTGGGATGCC
CAD(aa1612 TAT to TCT)-N	GCTGAGTGCACAT <u>CT</u> GTTCACGTGGCAC
CAD(aa1612 TAT to TCT)-C	GTGCCACGTGACAG <u>AT</u> GTGCACTCAGC
StDHOase-wt-NdeI-N	AAGAT <u>CATATG</u> ACTGCACCATCCCAGG
StDHOase-wt-XhoI-C	ACTGG <u>CTCGAG</u> TTTTTTTACTGACCAG

These plasmids were verified by DNA sequencing. Underlined nucleotides indicate the designated site for mutation or the restriction site.

**Supplementary Table S3. Anticancer activity of PLU and 5-FU against 4T1 cells.**

Cytotoxicity	None	PLU (2 $\mu$ M)	5-FU (5 $\mu$ M)	PLU (2 $\mu$ M) and 5-FU (5 $\mu$ M)
Death rate (%)	0	8	27	59
Migration rate (%)	99	84	65	37
Apoptotic rate (%)	1	8	26	58
Colony count (%)	100	73	54	17

Value is an average of 3 determinations within 10% error.