Supporting Information

Crystallographic Investigation and Selective Inhibition of Mutant Isocitrate Dehydrogenase

Baisong Zheng,[†,§] Yuan Yao,[†,§] Zhen Liu,[†,§] Lisheng Deng,[†] Justin L. Anglin,[†] Hong Jiang,[†] B. V. Venkataram Prasad,[#] Yongcheng Song[,†]*

[†]Department of Pharmacology, [#]Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas 77030

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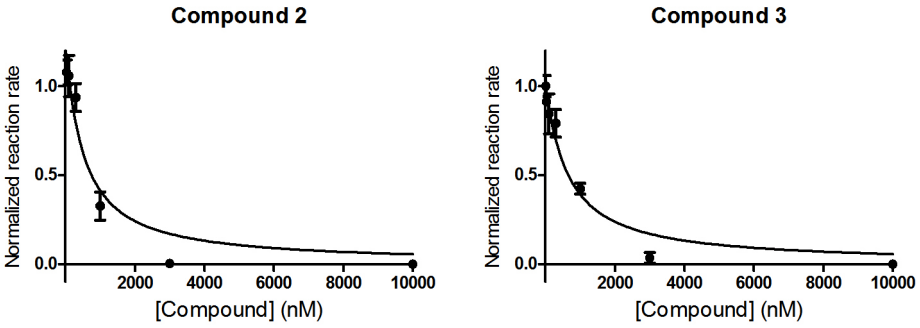
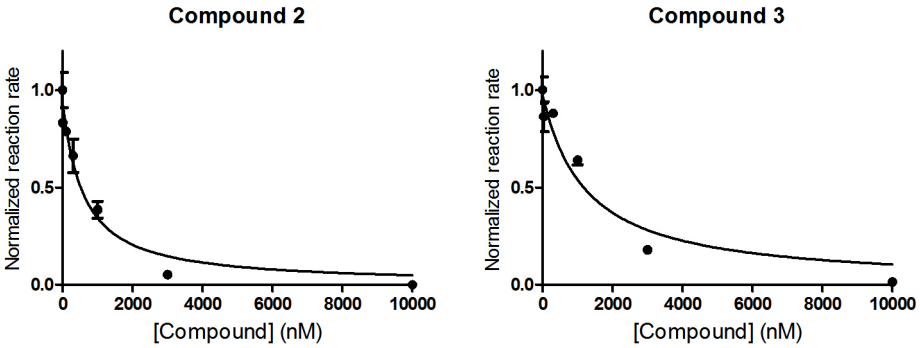


(**A**) Morrison tight binding fitting curves against IDH1(R132H)



(**B**) Morrison tight binding fitting curves against IDH1(R132C).

Figure S1. Enzyme activities of compounds **2** and **3** against (**A**) IDH1(R132H) and (**B**) IDH1(R132C), from which the K_i values were obtained.

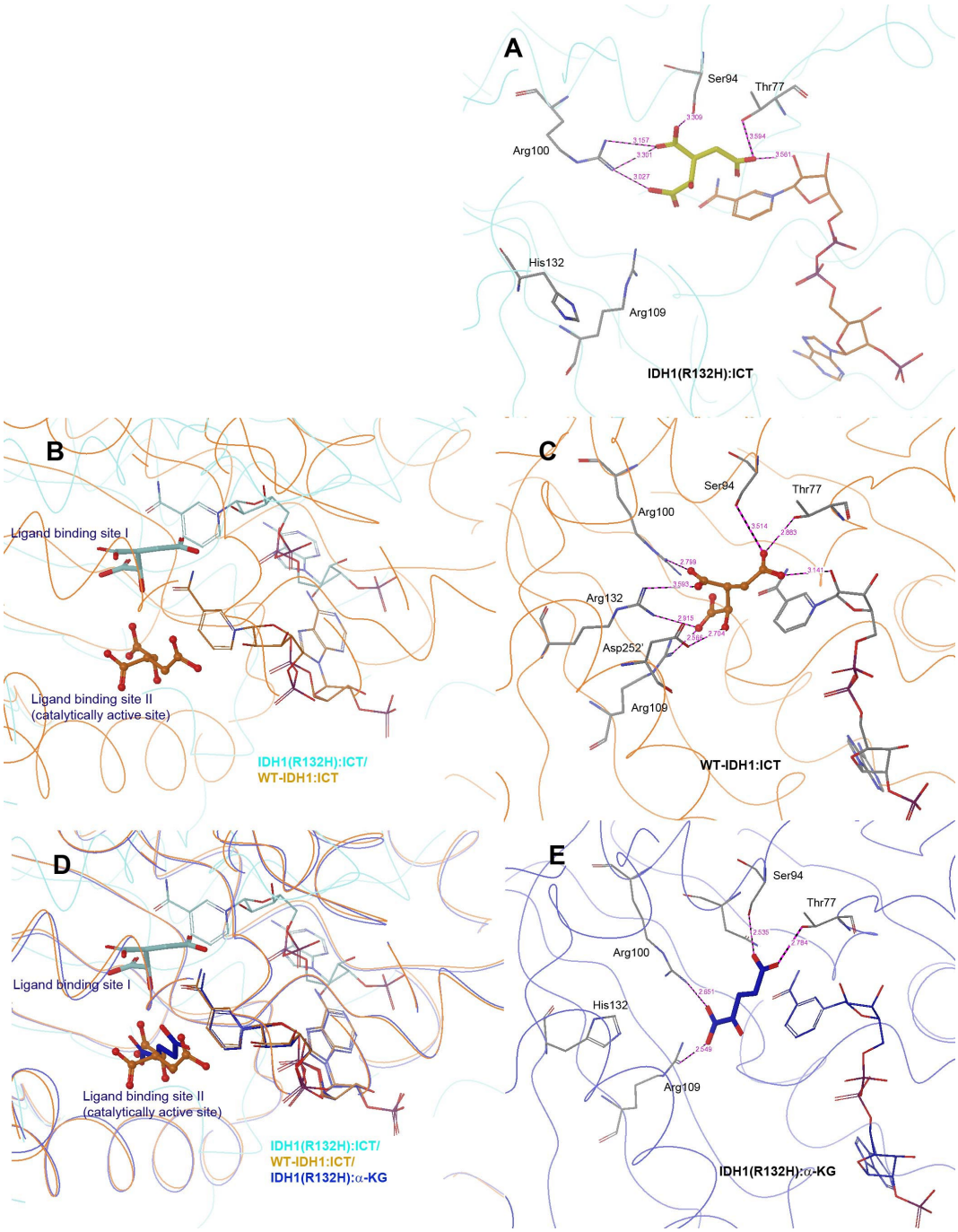


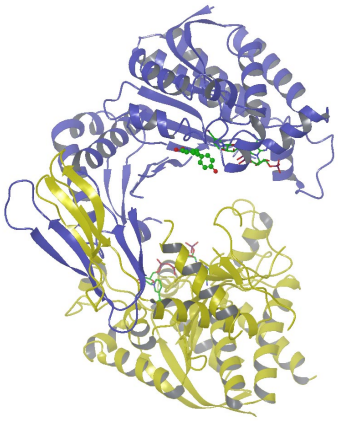
Figure S2. (**A**) Active site of the IDH1(R132H):ICT structure (PDB: 3MAP); (**B**) Alignment of WT-IDH1:ICT (in orange, PDB: 1T0L) with IDH1(R132H):ICT (in cyan), showing ligand binding sites I and II; (**C**) Active site of the WT-IDH1:ICT structure; (**D**) Alignment of IDH1(R132H):α-KG (in blue, PDB: 3INM) with IDH1(R132H):ICT (in cyan) and WT-IDH1:ICT (in orange); (**E**) Active site of the IDH1(R132H):α-KG structure.

Table S1. Data processing and refinement statistics

	IDH1(R132H):**2**	IDH1(R132H):**3**
A. Data processing		
Wavelength (Å)	1.542	1.542
Space group	$P4_32_12$	$P4_32_12$
Unit cell dimensions		
a, b, c (Å)	80.85, 80.85, 304.84	82.86, 82.86, 304.04
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	50.0-3.3(3.36-3.30[b])	49.0-3.3(3.36-3.31)
Unique reflections	15797(773)	15759(782)
Completeness (%)	95.7(85.2)	88.4(69.0)
Redundancy	15.5(16.4)	5.8(7.0)
R_{merge} (%)	12.5(62.8)	19.0(61.4)
$I/\sigma(I)$[b]	15.5(4.2)	8.3(2.3)
B. Refinement		
Resolution (Å)	45.7-3.3(3.36-3.3)	49.02-3.31(3.56-3.31)
Number of reflections used in working set	14773(849)	14792(2140)
Number of reflections for R_{free} calculation	774(52)	750(109)
R_{work} (%)	22.4(27.5)	20.1(20.6)
R_{free} (%)[a]	29.3(39.0)	27.7(29.4)
Mean B-factor from Wilson plot (Å^2)	49.8	17.8
RMSD of bond length (Å)	0.01	0.01
RMSD of bond angle (°)	1.45	1.37
Ramachandran plot[c]		
Residues in most favored regions	91.9%	93.9%
Residues in additional allowed regions	8.1%	6.1%
Residues in generously allowed regions	0.0%	0.0%
Residues in disallowed regions	0.0%	0.0%

[a]A subset of the data (5%) was excluded from the refinement and used to calculate R_{free}.
[b]Numbers in the parenthesis refer to highest resolution bin
[c]Ramachandran plot was generated using Procheck[1].

A.



B.

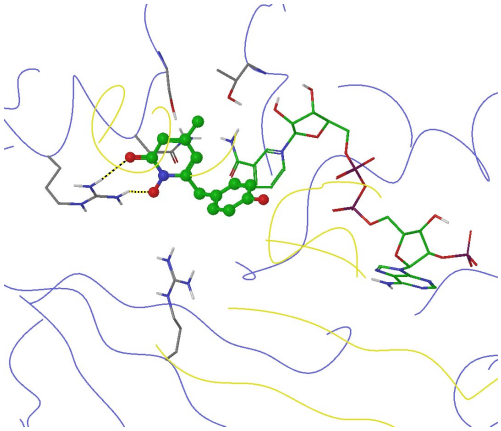
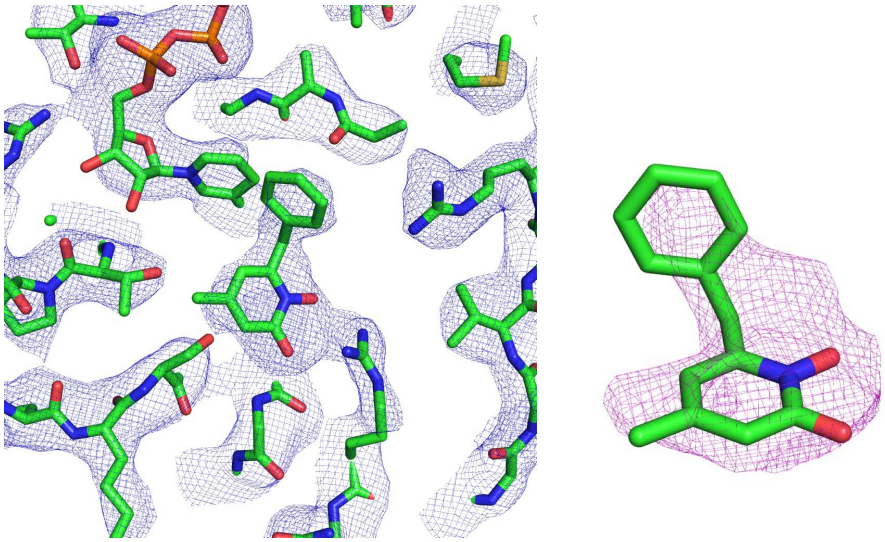
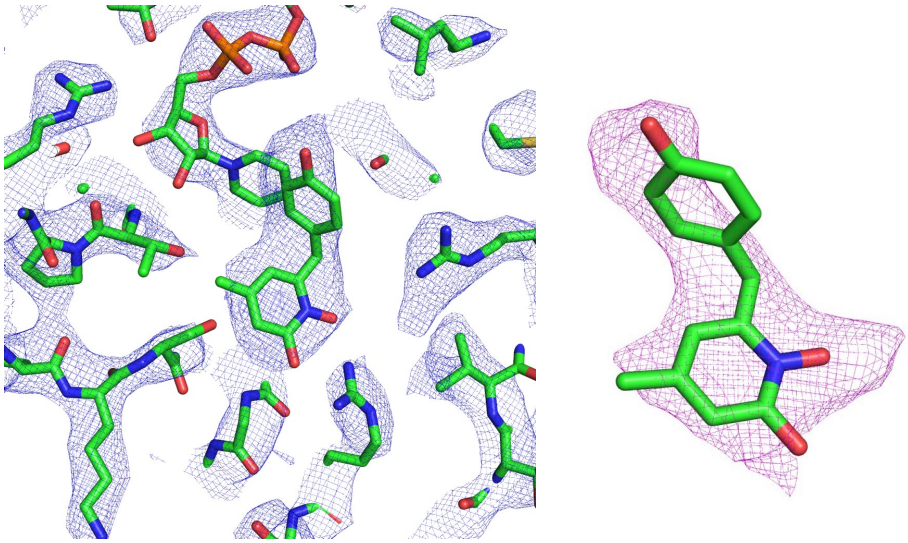


Figure S3. (**A**) The overall structure of IDH1(R132H):**3** (ball and stick model); (**B**) Close-up view of the active site of IDH1(R132H):**3**.



(Left) The $2F_o$-F_c electron density map of **2**, contoured at 1σ; (Right) The F_o-F_c omit map at 2σ.



(Left) The $2F_o$-F_c electron density map of **3**, contoured at 1σ; (Right) The F_o-F_c omit map at 2σ.

Figure S4.

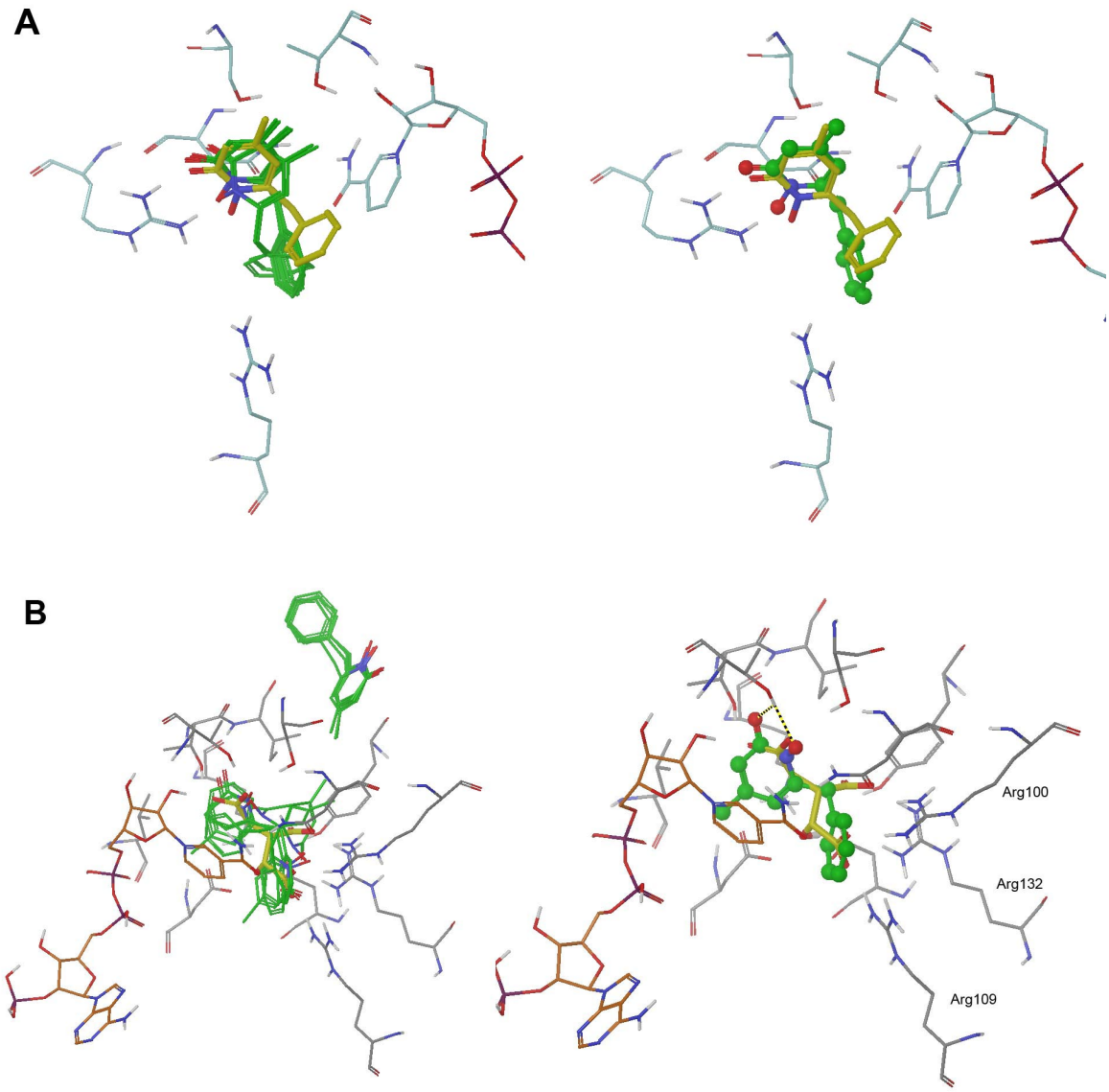


Figure S5. (**A**) Left: 10 lowest energy docking structures of compound **2** (C atoms in green) in the IDH1(R132H):**2** structure, as compared with the crystal structure of **2** (in yellow); Right: The lowest energy docking structure of **2** (in ball & stick model), mimicking the binding conformation of the crystal structure of **2**. (**B**) Left: 10 lowest energy docking structures of **2** (C atoms in green) in the WT-IDH1:ICT structure (PDB: 1T0L), showing 4 poses are not located in the ITC (in yellow) binding site; Right: The lowest energy docking structure of **2**, in which the phenyl ring, rather than more favorable 1-hydroxypyridin-2-one ring, is located in the arginine-rich pocket (including Arg100, 109 and 132). The docking results suggest binding of **2** to WT-IDH1 is not favored.

Experimental Section

All reagents were purchased from Alfa Aesar (Ward Hill, MA) or Aldrich (Milwaukee, WI). Compounds were characterized by ^{1}H NMR on a Varian (Palo Alto, CA) 400-MR spectrometer and the purities monitored by a Shimadzu Prominence HPLC with a Phenomenex C18 column (4.6 x 250 mm, Methonal:H_2O 60:40, monitored at 254 and 280 nm). The purities of all compounds were found to be >95%. Identities of biologically active compounds **2** and **3** were confirmed with high resolution mass spectra (HRMS) using a ThermoFisher LTQ-Orbitrap mass spectrometer. Detection and quantitation of D2HG was performed using a Shimadzu LCMS-2020 HPLC-MS system.

General method A: To a solution of 5-Bromo-2-methoxypyridine or 6-Bromo-2-methoxypyridine (1.0 mmol, 188.0 mg), Pd(dppf)Cl_2 (0.05 mmol, 40.8 mg) in anhydrous THF (5 mL) was added benzylmagnesium chloride (1.5 mmol, 1.5 mL of 1 M solution in THF). The reaction mixture was refluxed for 12 h and the solvent was removed under reduced pressure. The residue was purified with a flash column chromatography (silica gel, ethyl acetate:hexane 1:3, v/v), affording a benzyl substituted 2-methoxypyridine (57 – 63% yield). To the product (0.5 mmol, 99.6 mg) in $CHCl_3$ (5 mL) was added 3-chloroperoxybenzoic acid (258.9 mg, 1.5 mmol) and the reaction mixture was heated to 50 ^oC for 12 h. Upon removal of the solvent, a flash column chromatography (silica gel, ethyl acetate/MeOH 1:0 – 2:1, v/v) to give a pyridine N-oxide (73 – 80% yield), which (0.3 mmol, 64.6 mg) was then refluxed with AcCl (5.0 mL) for 10 h. Upon removal of the solvent under reduced pressure, the residue was dissolved in MeOH (5.0 mL). After 12 h, the solvent was removed under reduced pressure and the powder washed with ethyl acetate to give compound **1** or **4** as an off-white powder (82 – 85% yield).

5-Benzyl-1-hydroxypyridin-2(1H)-one (1). It was prepared from 5-Bromo-2-methoxypyridine (564 mg, 3 mmol) using general method A as an off-white powder (206 mg, 34% overall yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.81 (2H, s), 6.74-6.81 (1H, m), 7.16-7.17 (2H, m), 7.25-7.35 (4H, m), 7.59-7.60 (1H, m).

6-Benzyl-1-hydroxypyridin-2(1H)-one (4). It was prepared from 6-Bromo-2-methoxypyridine (564 mg, 3 mmol) using general method A as an off-white powder (260 mg, 43% overall yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.14 (2H, s), 5.97 (1H, d, J = 7.6 Hz), 6.61 (1H, d, J = 8.8 Hz), 7.26-7.31 (4H, m), 7.34-7.37 (2H, m).

General method B: To a solution of a carboxylic acid (10 mmol) in CH_2Cl_2 (10 mL) was added oxalyl chloride (30 mmol, 8.6 mL) followed by 0.05 mL of DMF. After 3 h, the solvents were removed under reduced pressure and the acid chloride thus obtained was dissolved in CH_2Cl_2 (10 mL), followed by addition of ethyl 3-methyl-but-2-enate (10 mmol, 1.3 g) and $AlCl_3$ (35 mmol, 4.7 g). The reaction mixture was refluxed for 5 h and the reaction quenched by HCl (aq.) and ice. The product was extracted with CH_2Cl_2 (50 mL x3) and the combined organic phases were washed with saturated $NaHCO_3$, dried over sodium sulfate, and evaporated to dryness to give crude **7**. It was dissolved in CH_3COOH (10 mL) followed by adding concentrated H_2SO_4 (3 mL). The reaction mixture was heated to 100 ^{o}C for 5 h before carefully quenched with $NaHCO_3$ (aq.) at 0 ^{o}C. The product was extracted with EtOAc (50 mL x3) and the organic layers were dried over sodium sulfate, evaporated, and purified with a flash column chromatography (silica gel, EtOAc/Hexane 3/1) to give **8** as a white solid. To a solution of **8** (2 mmol) in C_6H_6 (10 mL) was added P_4S_{10} (5 mmol, 2.2 g) and the reaction mixture was stirred at 80 ^{o}C for 5 h.[1] Upon removal of the solvent, the residue was subjected to a flash column chromatography (silica gel, EtOAc/hexane 10/1, v/v) to give **9**, which (1 mmol) in pyridine (10 mL) was heated to 115 ^{o}C with hydroxyamine hydrochloride (5 mmol, 347.4 mg) for 5 h. The resulting mixture was filtered and the filtrate was evaporated to dryness and washed with EtOAc to give compounds **2**, methyl protected **3** or **6**.

6-Benzyl-4-methyl-1-hydroxypyridin-2(1H)-one (2). It was prepared from phenylacetic acid (408 mg, 3 mmol) using general method B as an off-white powder (168 mg, 26% overall yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.18 (3H, s), 4.08 (2H, s), 5.78 (1H, s), 6.40 (1H, s), 7.26-7.31 (3H, m), 7.34-7.37 (2H, m); HRMS (ESI) $[M+H]^+$ Calcd for $C_{13}H_{14}NO_2^+$: 216.1019, Found: 216.1017.

6-(4-hydroxybenzyl)-4-methyl-1-hydroxypyridin-2(1H)-one (3). Methyl protected **3** was prepared from 4-methoxyphenylacetic acid (498 mg, 3 mmol) using general method B, which in CH_2Cl_2 (5 mL) was stirred with BBr_3 (1.5 mmol, 0.15 mL) at room temperature for 12 h before quenched with H_2O. The product was extracted with CH_2Cl_2 (50 mL x3) and the organic layers were washed with HCl and water, dried over sodium sulfate, and evaporated to give **3** as an off-white powder (173 mg, 25% overall yield). ^1H NMR ($CDCl_3$, 400 MHz): δ 2.16 (3H, s), 4.01 (2H, s), 5.79 (1H, s), 6.40 (1H, s), 6.82-6.84 (2H, m), 7.11-7.12 (2H, m); HRMS (ESI) $[M+H]^+$ Calcd for $C_{13}H_{14}NO_3^+$: 232.0968, Found: 232.0975.

6-Phenyl-4-methyl-1-hydroxypyridin-2(1H)-one (6). It was prepared from benzoic acid (366 mg, 3 mmol) using general method B as an off-white powder (121 mg, 20% overall yield). ^1H NMR ($CDCl_3$, 400 MHz): 2.21 (3H, s), 6.05 (1H, s), 6.53 (1H, s), 7.42-7.45 (3H, m), 7.81-7.83 (2H, m).

6-Benzyl-4-isopropyl-1-hydroxypyridin-2(1H)-one (5). 2,6-Dihydroxypyridine-4-carboxylic acid (30 mmol, 4.6 g) and $POBr_3$ (150 mmol, 43 g) in a dried flask were heated at 130 ^oC for 8 h. After cooling, MeOH (50 mL) was added at 0 ^oC and the reaction mixture was stirred at room temperature for 12 h. Upon removal of the solvents, crude compound **10** thus obtained was dissolved in THF (30 mL) and CH_3MgBr (43 mmol, 43 mL of 1 M solution in THF) added at -78 ^oC. The reaction mixture was warmed to room temperature and stirred overnight before quenched with H_2O. The product was extracted with CH_2Cl_2 (50 mL x3) and the organic layers were removed under reduced pressure. The remaining oil was purified by a flash column chromatography (silica gel, ethyl acetate/hexane 1/3, v/v) to give a tertiary alcohol as a colorless oil (5.2 g, 61%). To a solution of the product (17 mmol, 5.0 g) and Et_3N (51 mmol, 7.1 mL) in CH_2Cl_2 (20 mL) was added methanesulfonyl chloride (43 mmol, 3.3 mL) at 0 ^oC. The reaction mixture was stirred at room temperature for 12 h. Upon removal of the solvent under reduced pressure, the residue was purified with a flash column chromatography (silica gel, ethyl acetate/hexane 1/10, v/v) to give compound **11** as a white solid (2.7 g, 57%). **11** (9 mmol, 2.5 g) and NaOMe (13.5 mmol) in MeOH (10 mL) were heated to 65 ^oC and stirred for 12 h. The solvent was removed under reduced pressure and the remaining oil was purified with a flash column chromatography (silica gel,

ethyl acetate/hexane 1/5, v/v) to afford 2-methoxy substituted product as a white solid (1.3 g, 61%), to which (5 mmol, 1.1 g) and tetramethylethylene diamine (7.5 mmol, 1.1 mL) in THF (10 mL) was added *n*-BuLi (7.5 mmol, 7.5 mL of 1 M solution in THF) at -78 ^{o}C. After 1 h, benzaldehyde (10 mmol, 1.0 mL) was added and the reaction mixture warmed to room temperature. After stirring for 12 h, the reaction was quenched with H_2O and the product was extracted with of CH_2Cl_2 (50 mL x3). The organic phases were evaporated and the residue was purified with a flash column chromatography (silica gel, ethyl acetate/hexane 1/3, v/v) to give compound **12** as a colorless oil (1.0 g, 79%). **12** (3.5 mmol, 0.89 g) was hydrogenated using $Pd(OH)_2$ (300 mg of 20% on activated charcoal) in ethyl acetate (5 mL) at room temperature for 12 h. The mixture was filtered and evaporated to dryness and the residue oil was dissolved in dichloroethane (5 mL), followed by adding trifluoroacetic acid (9.3 mmol, 0.71 mL) and Et_3SiH (6.2 mmol, 2.5 mL).[2] The reaction mixture was heated to 50 ^{o}C for 12 h. Upon removal of the solvents, the residue was purified with a flash column chromatography (silica gel, ethyl acetate/hexane 1/5, v/v) to give compound **13** as a white solid (396 mg, 53%). Following the last two steps in general method A (oxidation with 3-chloroperoxybenzoic acid and AcCl-mediated deprotection), compound **5** can be obtained as a light-yellow solid (208 mg, 52%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.12 (6H, d, J = 6.8 Hz), 2.63-2.67 (1H, m), 4.12 (2H, s), 5.86 (1H, s), 6.46 (1H, s), 7.24-7.29 (3H, m), 7.32-7.35 (2H, m).

Expression and purification of human WT and R132H mutant IDH1. The wild-type IDH1 gene was cloned using 5'-GATCCGAATTCGATGTCCAAAAAAATCAGTG-3' and 5'-TGGTGCTCGAGTAAGTTTGGCCTGAGCTAG-3' as forward and reverse primers, respectively, and inserted into pET-24b vector. Correctness of the insert was verified by sequencing. *E. coli* BL21-CodonPlus strain (Agilent) was transformed with the plasmid and cultured at 37 °C in LB medium containing kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL). Upon reaching an optical density of ~0.6 at 600 nm, IDH1 expression was induced by adding 0.1 mM isopropylthiogalactoside at 18 °C for 20 hours. Cells were harvested, lysed, centrifuged at 20,000 rpm for 20 min and the supernatant was

collected and purified using a Ni-affinity (HisTrap HP, GE Healthcare) followed by Superdex 75 (GE Healthcare) column chromatography. WT-IDH1 was obtained with ~90% purity (SDS-PAGE).

R132H and R132C mutant IDH1 genes were generated from the wild-type IDH1 plasmid, using QuikChange™ Site-Directed Mutagenesis Kit (Agilent) following the manufacturer's protocol. Correctness of the gene sequences was verified. The mutant genes were then transferred to pGEX-KG vector for a better expression. Expression of mutant IDH1 enzymes were performed similarly as the wild-type protein. Cells were harvested, lysed, centrifuged at 20,000 rpm for 20 min and the supernatant was collected and the recombinant protein trapped in glutathione sepharose resin (GE Healthcare). The GST-IDH1 fusion protein was eluted with 10 mM of glutathione solution and the GST tag was removed by thrombin digestion overnight at 4 °C. IDH1(R132H) and IDH1(R132C) were obtained in ~90% purity (SDS-PAGE) using a glutathione sepharose column followed by Superdex 75 gel filtration column chromatography.

Enzyme inhibition assays. Determination of the activity and inhibition of IDH1(R132H) and IDH1(R132C) is based on the initial linear consumption of NADPH of the reaction. The enzyme activity assay was performed in a 96-well microplate using the purified IDH1 mutant (100 nM), 4 mM $MgCl_2$, 1 mM α-KG, 100 μM NADPH (>>K_m for NADPH) in 50 mM HEPES buffer (pH = 7.5) containing 0.1 mg/mL bovine serum albumin. For inhibition assay, triplicate samples of compounds were incubated with the protein for 5 min, before adding α-KG to initiate the reaction. The optical absorbance of each well was monitored every 30s at 340 nm, where NADPH has the maximum absorption, using a Beckman DTX-880 microplate reader. The data were imported into Prism (version 5.0, GraphPad) and the IC_{50} values were calculated by using a standard dose response curve fitting. For compounds with IC_{50}s >> [enzyme], K_i values were calculated using the Cheng-Prusoff equation $K_i = IC_{50}/(1+[S]/K_m)$, where [S] is the concentration of α-KG (1 mM) and K_m is the literature value of 0.965 mM for R132H and 0.295 mM for R132C.[3] For compounds with IC_{50}s < 1 μM, K_i values were calculated using the Morrison tight inhibition modeling in Prism.

Determination of the activity/inhibition of WT-IDH1 is based on the initial linear production of NADPH. In brief, the enzyme activity assay was performed in a 96-well microplate using the purified IDH1 (15 nM), 4 mM $MgCl_2$, 50 μM sodium (*D*)-isocitrate (K_m: 65 μM),[3] 1 mM $NADP^+$ (>>K_m for NADP) in 50 mM HEPES buffer (pH = 7.5). The reaction can be readily monitored by an increase in optical absorbance at 340 nm. Activity and compound inhibition can be determined similarly as described for mutant IDH1 using Prism 5.0.

Cell growth inhibition. The cytotoxicity assay was done using our previous method.[4] In brief, $1x10^5$ WI-38 (fibroblast) cells were inoculated into each well of a 96-well plate and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere with 100% humidity overnight for cell attachment. After addition of compounds (from 1 – 300 μM), plates were incubated for 48h after which cell viability was assessed by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, using a commercially available kit (Promega). IC_{50}s of each compound was calculated from dose response curves by Prism 5.0.

D2HG production inhibition in HT1080 cells. HT1080 fibrosarcoma cell line, which harbors an IDH1(R132C) mutation, was obtained from ATCC (Manassas, VA). D2HG production inhibition assay followed a published protocol.[3] In brief, 1 x 10^5 cells/well were seeded into wells of a 6-well plate and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% dialyzed fetal bovine serum at 37 °C in a 5% CO_2 atmosphere with 100% humidity overnight for cell attachment. Cells were treated with an increasing concentration of compound **2** in 2 mL of culture medium for 48h. Medium was collected and diluted with 8 mL of MeOH. After shaking, the mixture was centrifuged to remove any precipitation and the supernatant was subjected to HPLC-MS to separate and quantitate the amount of D2HG. HPLC was run using a Phenomenex C18 column (250 x 4.6 mm, 5 μm) with 50% TBA Buffer: 50% MeOH as an eluent at a flow rate of 0.5 mL/min (TBA Buffer: 10 mM tributylamine, 15 mM acetic acid, 3% MeOH in water). A single ion monitoring (SIM) for 147 Da was used to detect

and quantitate the amount of D2HG (parameters: Interface Voltage, -4.2kV; Detector Voltage, 1.3kV; Nebulizing Gas, 1.5 L/min; Drying Gas, 15 L/min; Desolvation Line Temperature, 250 °C; Heat Block Temperature, 200°C; Pirani Gauge Vacuum, 102 Pa; Ion Gauge Vacuum: 5e-4 Pa). Authentic D2HG purchased from Sigma-Aldrich (St Louis, MO) was used to validate and calibrate the HPLC-MS assay conditions, before measuring the D2HG concentrations secreted by the HT1080 cells untreated or treated with **2**. EC_{50} was calculated from the dose response curve by Prism 5.0.

Crystallization and structure determination. The crystallization of IDH1(R132H) was performed using a method similar to that described in the literature.[5] IDH1(R132H) (10 mg/mL) was crystallized together with 2 mM of compounds **2** or **3**, 10 mM NADPH and 10 mM $CaCl_2$ in a solution of 1.75 M $(NH_4)_2SO_4$ and 0.1 M NaOAc (pH 5.6). Catalytically inactive Ca^{2+} was used to mimic Mg^{2+}. Prism-like single crystals appeared in ~5 days. Diffraction data were collected using a Rigaku FR-E+ SuperBright X-ray source at Baylor College of Medicine and processed with the program the program HKL2000.[6] The initial structure was obtained by the program Phaser[7] using the coordinates of 3MAP (PDB code) as a target and the refinement carried out using the program Refmac[8] or PHENIX[9]. The final refinement statistics were summarized in Table S1 and the coordinates were deposited into Protein Data Bank as entries 4I3L and 4I3K. Figures 2, S2, S3 and S5 were generated using Maestro in Schrödinger Suite (version 2010).[10]

Molecular modeling. Docking studies were performed using the program Glide[11] in Schrödinger.[10] The crystal structure of the WT-IDH1:ICT complex (PDB: 1T0L) was prepared using the module "protein preparation wizard" in Maestro with default protein parameters: water molecules were removed, hydrogen atoms added, ligands (except for NADP) extracted and NADP remained in the protein structure for docking. H-bonds were then optimized and the protein was energy-minimized using OPLS2005 force field. A receptor grid, which is large enough to contain the whole active site, was generated using Glide without any constraints. Compound **2** was constructed, minimized using

OPLS2005 force field and then docked into the prepared protein structure using Glide (docking parameters: standard-precision and dock flexibly).

References for Supporting Information

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