Supplementary Information for

An Enolase Inhibitor for the Targeted Treatment of *ENO1*-Deleted Cancers

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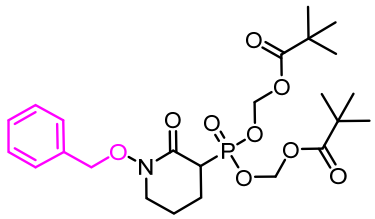
Supplementary Figure S1

Data Collection	Enolase 2:Hex (PDB 5IDZ)
Wavelength (Å)	1.116
Space group	$P2_1\ 2_1\ 2_1$
Cell dimensions	
a, b, c (Å)	68.1, 108.7. 116.5
α, β, γ (°)	90. 90, 90
N°. of **unique reflections**	26400
Resolution (Å)	79.5-2.63 (2.75-2.63)
Rmerge (all I^+ and I^-)	0.089 (0.314)
I/σI	15.6 (5.1)
Completeness (%)	99.9 (99.3)
Redundancy	6.9 (6.6)
Refinement	
Resolution (Å)	54.4 – 2.63
σF	1.34
N°. of reflections	26345
R_{work}/R_{free}	0.162/0.218
Wilson B	35.4
N°. of atoms	
Protein	6694
Ligands	44
Ions	2
Water	154
Average B-factors ($Å^2$)	
Protein	35.8
Ligands	39.0
Ions	28.0
Water	31.1
r.m.s.d.	
Bond lengths (Å)	0.004
Bond angles (°)	0.692

Supplementary Figure S1. X-ray diffraction data collection parameters and refinement statistics.

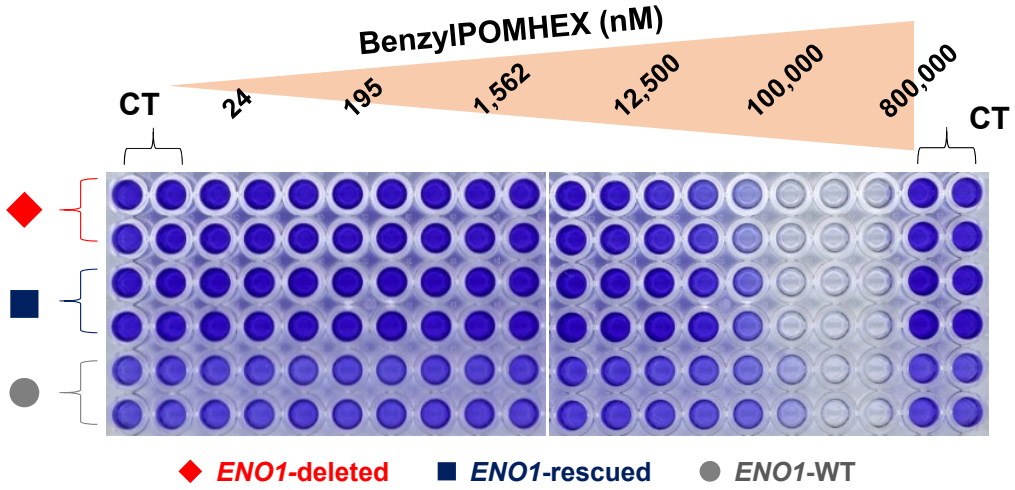
Supplementary Figure S2

a

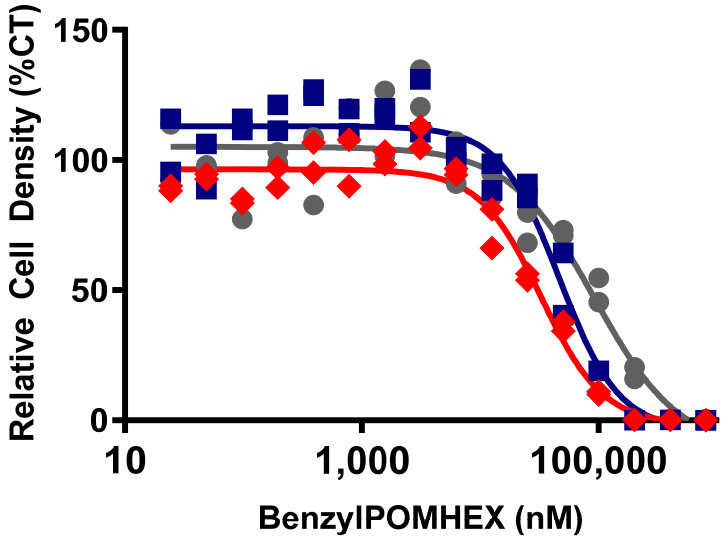


BenzylPOMHEX

b

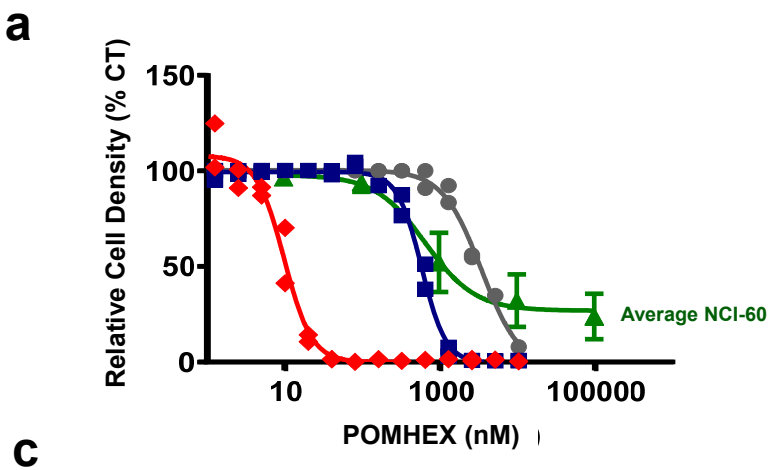


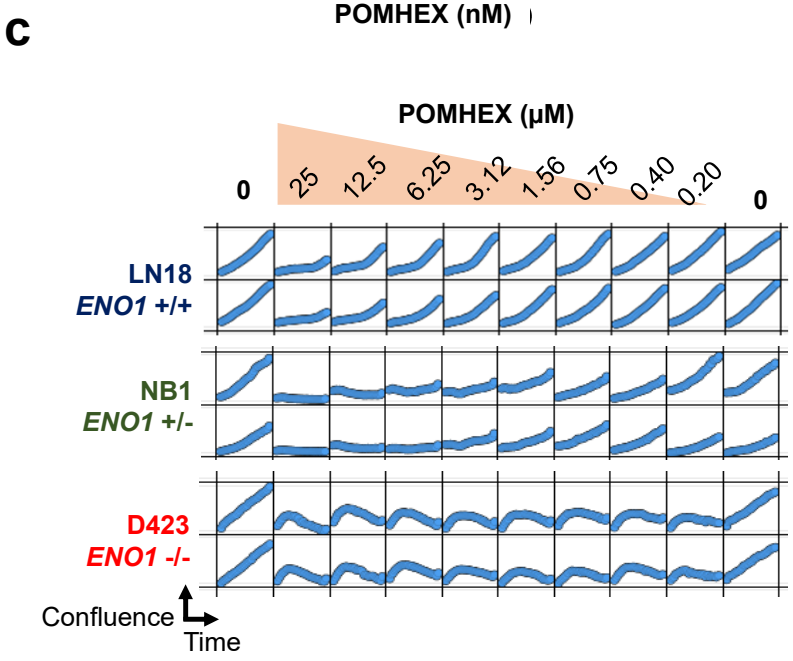
c

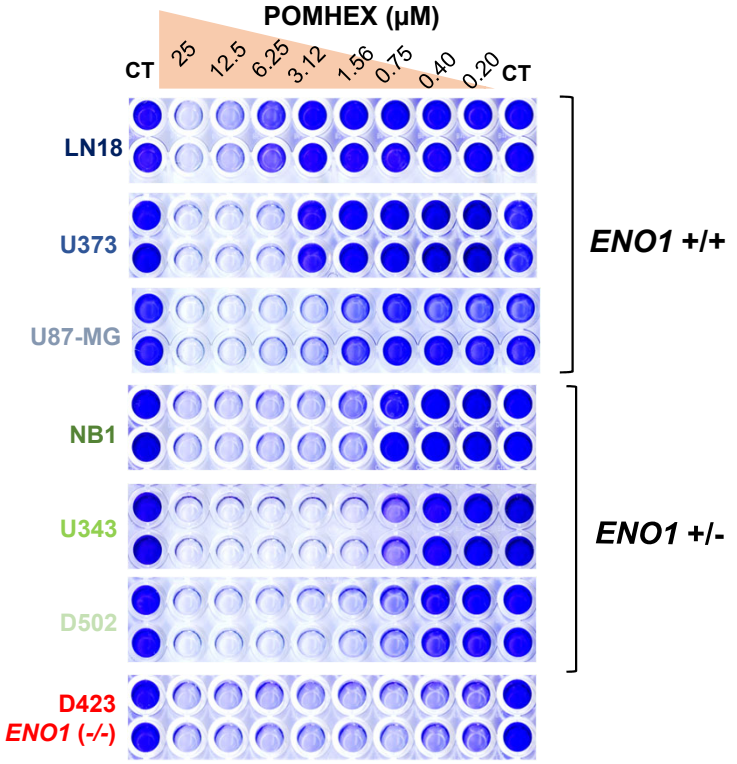


Supplementary Figure S2. BenzylPOMHEX does not display selective toxicity against *ENO1*-deleted glioma cells. **a.** Structure of the synthetic precursor to POMHEX, BenzylPOMHEX (Intermediate **3** in Supplementary Note); the benzyl moiety is indicated in pink. **b, c.** *ENO1*-deleted (D423, red), *ENO1*-isogenically rescued (D423 ENO1, blue), and ENO1-WT (LN319, grey) cells were treated for 7 days with BenzylPOMHEX in duplicate. Cell density was determined by crystal violet was expressed relative to a no-drug control. Toxicity against *ENO1*-deleted D423 cells is only evident at concentrations ~50,000 nM, or ~1,000-times higher than POMHEX with negligible selectivity against *ENO1*-deleted cells. This suggests that, in agreement with previous literature, the benzyl ether group is not labile in biological systems and the toxicity observed is due to effects unrelated to Enolase inhibition. This experiment was repeated once with similar results.

Supplementary Figure S3





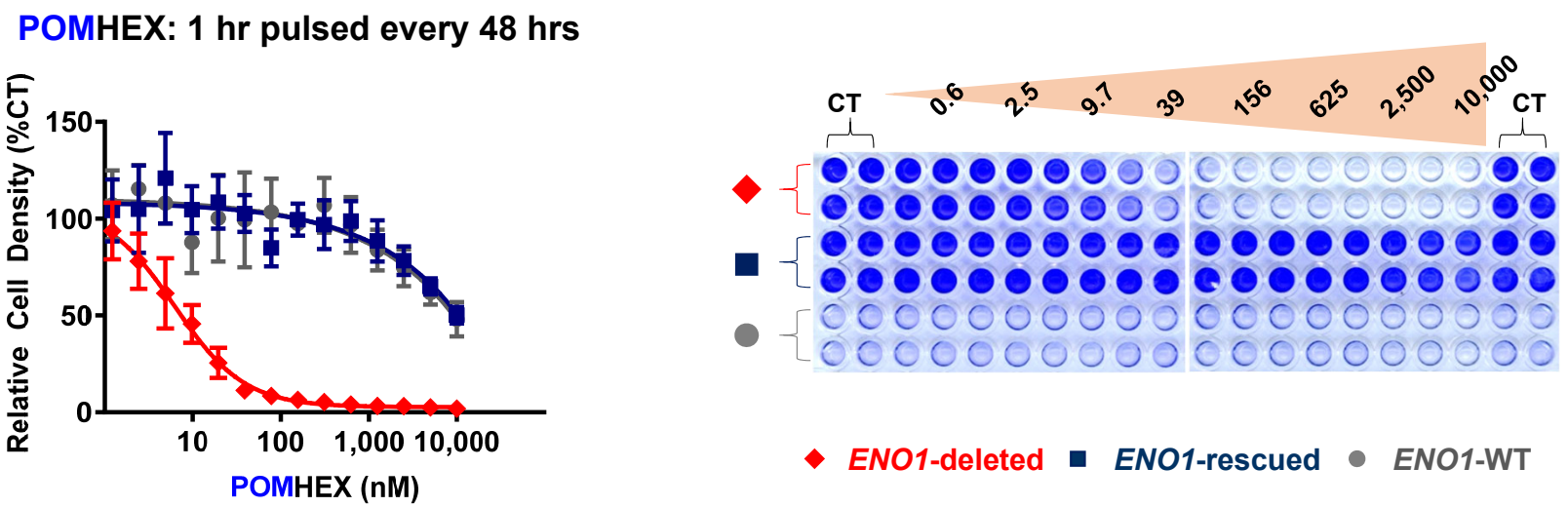


Cell Line	IC_{50} (nM)	ENO1 status
LN18	14,235 ± 1641	+/+
U373	2,802 ± 488	+/+
U87-MG	2,100 ± 339	+/+
A1207	1,355 ± 191	+/+
NB1	696 ± 38	+/-
U343	644 ± 143	+/-
D502	72 ± 4	+/-
D423	66 ± 8	-/-

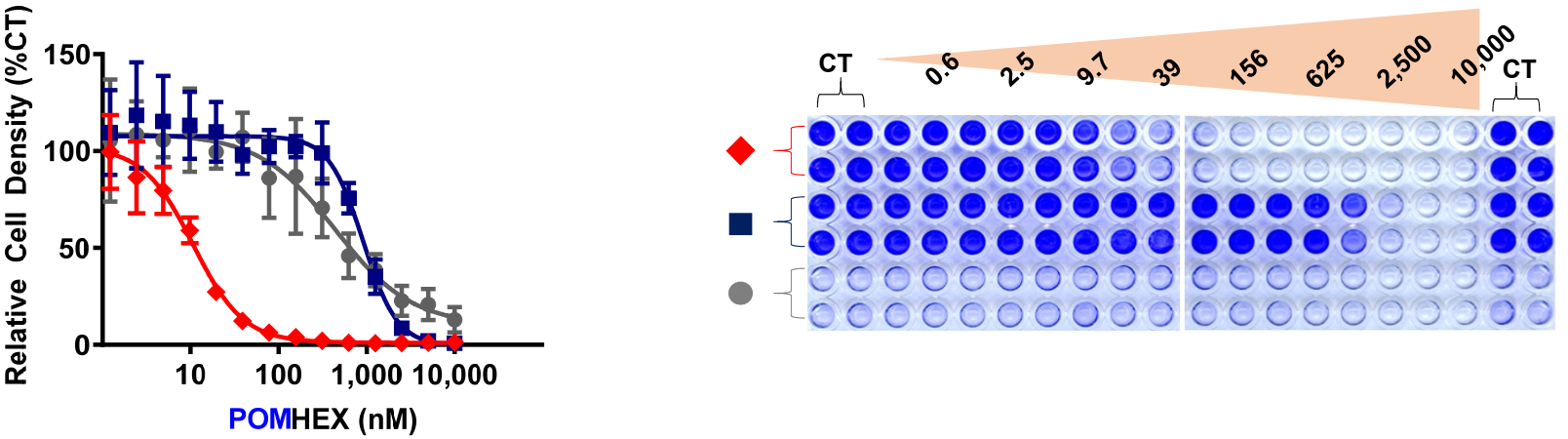
Supplementary Figure S3. *ENO1*-deletion status modulates sensitivity to POMHEX. a. *ENO1*-deleted (D423, red), *ENO1*-isogenically rescued (D423 ENO1, blue), and *ENO1*-WT (LN319, grey) cells were treated POMHEX in RPMI media under the same experimental conditions used for NCI-60 screening. Note that the sensitivity to POMHEX in RPMI is ~3-fold greater than in DMEM media. The relative terminal cell density of the mean +/- S.D. of 60 cells lines screened by the NCI-60 shown in green (data replotted from NSC784584; attached as **Supplementary Note 2**). **b.** Crystal violet stained plates of common glioma cell lines treated with a serial dilution of POMHEX (N = 2 biological replicates), with a summary of IC_{50} values. **c**. Live cell imaging incucyte confluency curves, (x-axis, time; y-axis, confluence: 0 to 100%) for representative *ENO1*-WT, *ENO1*-heterozygous deleted and *ENO1*-homozygous deleted glioma cell lines. Each box represents one biological replicate. Positive slopes indicate proliferating cells, flat slopes indicate cells in stasis, and negative slopes correspond to dying cells. Note the distinct negative slopes in *ENO1*-deleted D423 cells.

Supplementary Figure S4

a

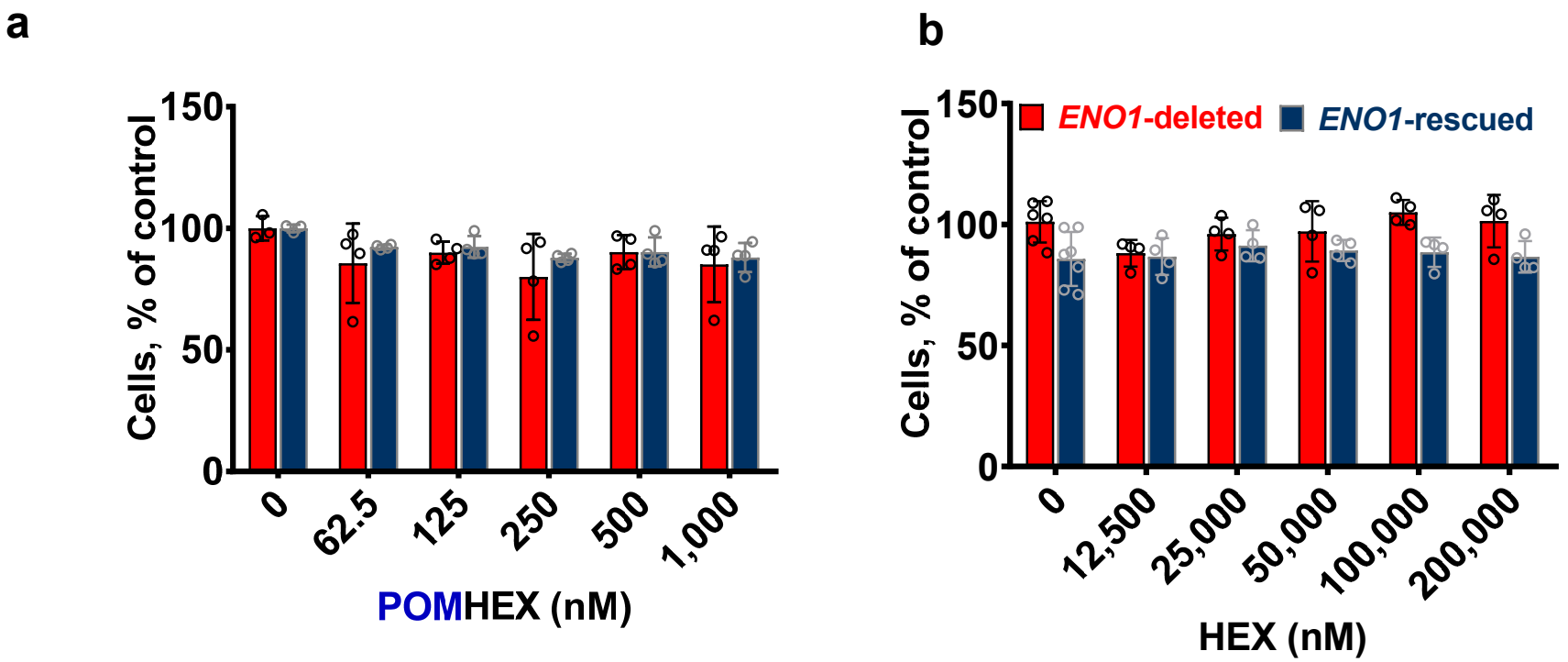


b



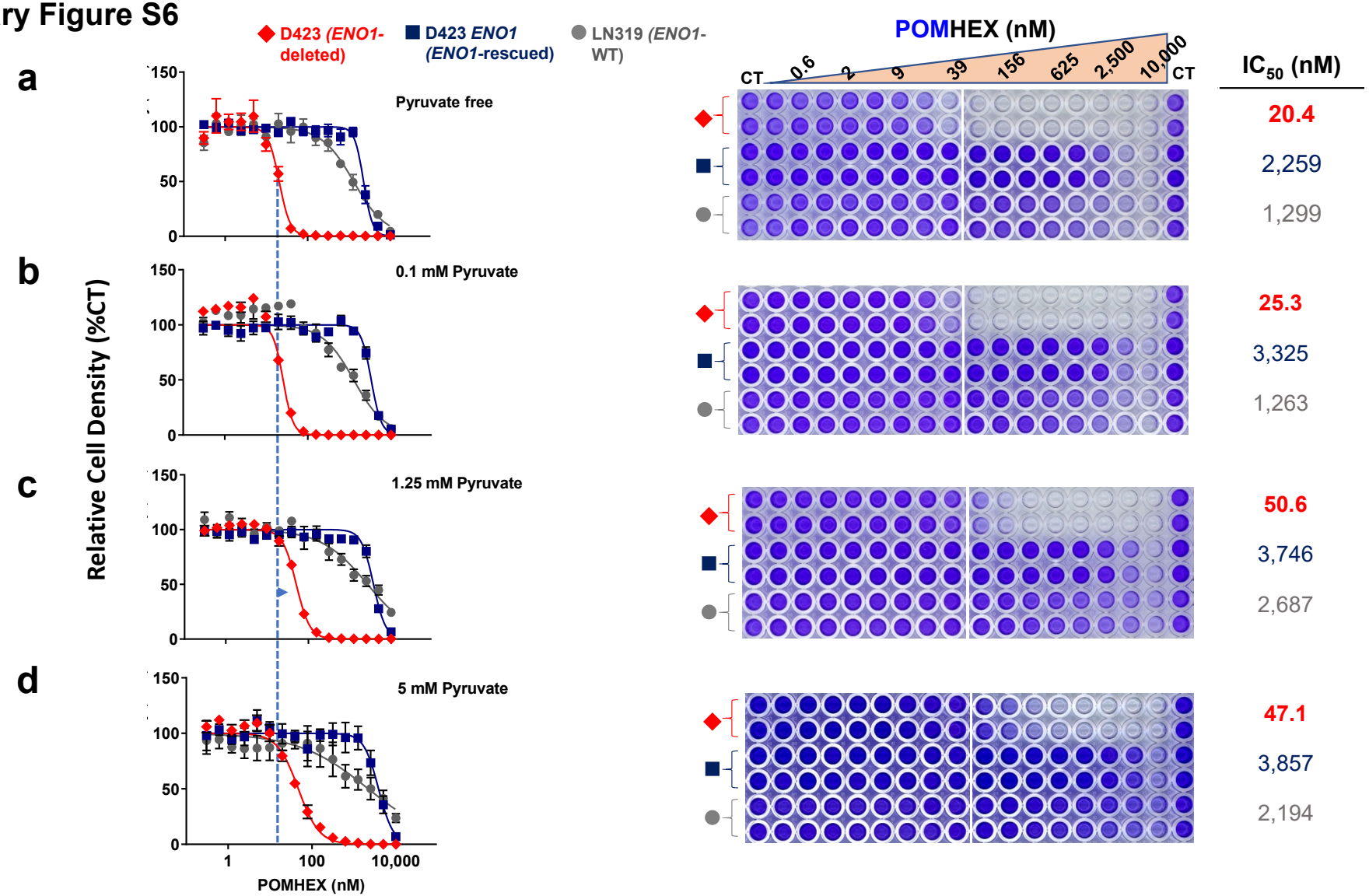
Supplementary Figure S4. Short pulse or continuous treatment with POMHEX results in similar levels of potency against *ENO1*-deleted glioma cells. a. Pulsed drug treatment. *ENO1*-deleted (D423, red), *ENO1*-isogenically rescued (D423 ENO1, blue), and *ENO1*-WT (LN319, grey) cells were treated POMHEX at the doses indicated (x-axis). Cells were exposed to media (DMEM, 10% FBS) containing POMHEX at the concentrations indicated for 1 hr. The drug-containing media was then removed and replaced with fresh, non-drug containing media. This was repeated every 48 hrs., until one week elapsed. Plates were then fixed and cell density quantified by crystal violet. **b.** For continuous POMHEX exposure experiments, experimental conditions were the same as in panel a, except that the drug-containing media was left on and only changed every 48 hrs. Cell density after 1-week total exposure was determined by crystal violet staining and expressed relative to non-drug contain controls. For both a and b, mean of n = 6 experiments ± S.D. is shown. The IC_{50} for continuous versus pulsed exposures for *ENO1*-deleted glioma cells were essentially the same (~10 nM). The non-target *ENO1*-rescued and *ENO1*-WT glioma cells were substantially less affected by pulsed versus continuous POMHEX (IC50 ~1,500 nM vs ~6,000 nM, and ~500 nM vs ~4,500 nM for D423 ENO1 rescued and LN319 ENO1-WT respectively.

Supplementary Figure S5



Supplementary Figure S5. ECAR inhibition is not due to decreased cell viability. Viability of cell populations at the end of the Seahorse experiment for HEX and POMHEX are unchanged. Thus, differences in ECAR and OCAR cannot be explained by cell number changes. Individual data points and the mean ± S.D. for POMHEX-treated [N=3(CT), 4(treatments)], and for HEX-treated [N=6 *ENO1*-deleted and 7 for *ENO1*-rescued (CT), 4 (treatments)]. Biological replicates are shown.

Supplementary Figure S6



Supplementary Figure S6. Exogenous pyruvate modestly attenuates sensitivity to POMHEX. *ENO1*-deleted (D423, red), *ENO1*-isogenically rescued (D423 *ENO1*, blue), and *ENO1*-WT (LN319, grey) cells were treated POMHEX at the doses indicated (x-axis) in media (DMEM) either free of pyruvate (**a**), with 0.1 mM (**b**), 1.25 mM (**c**) or 5 mM pyruvate (**d**). Pyruvate levels in human blood are around 0.05 mM. Cell density after 5 days exposure was determined by crystal violet staining and expressed relative to non-drug contain controls. Mean +/- S.E.M and n = 4 experiments are indicated. The experiment was independently replicated once. The IC_{50} for pyruvate-free media is indicated by a dashed line, for comparison. Exogenous pyruvate supplementation, even at supraphysiological levels, exerts a modest effect on sensitivity to Enolase inhibitors. Saturable transport through monocarboxylate transporters may limit the efficacy of this rescue (see Extended Figure 8).

Supplementary Figure S7

POMHEX 20 mpk IV

Time point	RBC ^6uL	HGB g/dL	**HCT %**	MCV fL	MCHC g/dL	MCH pg	RDW %	RET% %	RETI ^9/L	PLT ^3uL	WBC ^3uL	NEUT ^3uL	LYMP ^3uL	MONO ^3uL	EOSO ^3uL	BASO ^3uL	LUC ^3uL
Pre-dose	5.27	14.0	**44.2**	83.7	31.8	26.6	13.2	1.0	54.8	505	15.24	8.17	6.25	0.56	0.11	0.03	0.13
24hr Post	4.90	12.9	**41.1**	83.9	31.4	26.3	13.2	1.2	58.3	494	16.44	8.24	7.10	0.74	0.07	0.02	0.27

POMHEX 40 mpk IV

Time point	RBC ^6uL	HGB g/dL	**HCT %**	MCV fL	MCHC g/dL	MCH pg	RDW %	RET% %	RETI ^9/L	PLT ^3uL	WBC ^3uL	NEUT ^3uL	LYMP ^3uL	MONO ^3uL	EOSO ^3uL	BASO ^3uL	LUC ^3uL
Pre dose	5.45	12.0	**37.2**	68.2	32.2	22.0	13.7	3.0	164.7	418	9.52	5.32	3.86	0.20	0.08	0.01	0.05
24-Hour	5.69	12.4	**39.2**	68.8	31.8	21.9	13.6	2.8	156.8	470	20.42	18.86	1.11	0.39	0.01	0.01	0.05

HEX 100 mpk SC

Time point	RBC ^6uL	HGB g/dL	**HCT %**	MCV fL	MCHC g/dL	MCH pg	RDW %	RET% %	RETI ^9/L	PLT ^3uL	WBC ^3uL	NEUT ^3uL	LYMP ^3uL	MONO ^3uL	EOSO ^3uL	BASO ^3uL	LUC ^3uL
Pre-Dose	5.52	13.3	**41.6**	75.3	32.0	24.1	13.4	ND	ND	640	16.80	5.11	10.92	0.49	0.13	0.03	0.13
Post Dose	4.66	11.5	**35.7**	76.6	32.2	24.7	13.5	ND	ND	596	22.46	9.23	12.37	0.53	0.08	0.03	0.21

HEX 200 mpk SC

Time point	RBC ^6uL	HGB g/dL	**HCT %**	MCV fL	MCHC g/dL	MCH pg	RDW %	RET% %	RETI ^9/L	PLT ^3uL	WBC ^3uL	NEUT ^3uL	LYMP ^3uL	MONO ^3uL	EOSO ^3uL	BASO ^3uL	LUC ^3uL
Pre-dose	4.93	13.2	**42.0**	85.1	31.5	26.8	13.5	1.8	86.9	560	11.99	3.89	7.41	0.34	0.14	0.02	0.20
24 hour	4.48	11.9	**38.2**	85.4	31.1	26.6	13.4	2.1	93.1	504	15.76	9.98	4.94	0.62	0.03	0.01	0.18

BenzylHEX 200 mpk SC

Time point	RBC ^6uL	HGB g/dL	**HCT %**	MCV fL	MCHC g/dL	MCH pg	RDW %	RET% %	RETI ^9/L	PLT ^3uL	WBC ^3uL	NEUT ^3uL	LYMP ^3uL	MONO ^3uL	EOSO ^3uL	BASO ^3uL	LUC ^3uL
Pre-dose	5.58	13.4	**40.9**	73.3	32.9	24.1	15.1	1.2	68.7	409	11.51	7.12	3.76	0.44	0.10	0.01	0.08
24 Hour	5.15	12.3	**37.7**	73.3	32.7	24.0	15.3	1.0	49.2	351	14.52	6.86	6.86	0.49	0.15	0.02	0.15

Code	Name
WBC	White Blood Cell
# NEUT	Neutrophil Count
# LYM	Lymphocyte Count
# MONO	Monocyte Count
# EOS	Eosinophil Count
# BASO	Basophil Count
# LUC	Large Unstained Cells
# RETIC	Reticulocyte Count
RBC	Red Blood Cell
HGB	Hemoglobin Concentration
HCT	Hematocrit
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
PLT	Platelet Count
MPV	Mean Platelet Volume

Supplementary Figure S7. Nominal hematology parameters in NHP when dosed with Enolase inhibitor at concentrations higher-than-required for therapeutic efficacy. Male cynomolgus monkeys were fasted overnight and IV injected with either a single bolus dose of POMHEX or SC for HEX and BenzylHEX (inactive synthetic precursor; negative control). Blood was collected for PK measurements at time intervals (Figure 7), and a final blood draw, 24 h after dosing was used for veterinary panel hematology profiling. All experiments were performed at Charles River Laboratories on different individual animals. Minimal, non-dose-dependent decreases in hematocrit and RBC were observed in animals post-treatment with both POMHEX with HEX, which Charles River veterinary pathologists attribute to repeated blood draws for PK. In corroboration, similar decreases in hematocrit were observed post-treatment with inactive BenzylHEX. The data agree with the findings in mice (Supplementary Figure S2) and indicate minimal haemopoietic toxicity of HEX and POMHEX.

Supplementary Figure S8

Blood chemistry
HEX 100 mpk SC/day

	Accession : Hematology / Clinical Chemistry of Samples from CRL-Shrewsbury Study 20243814 Tested on 3-Mar-2020 :Chemistry																						
Time Point	Animal #	Analysis Sample ID	ALT U/L	ALB g/dL	A/G rato	ALP U/L	AST U/L	CA mg/dL	CL mEqL	CHOL mg/dL	CK U/L	CREA mg/dL	GGT U/L	GLOB g/dL	GLUC mg/dL	PHOS mg/dL	K mEqL	NA mEqL	TBIL mg/dL	TP g/dL	TRIG mg/dL	UREA mg/dL	SAQB	Weight Kg
Pre dose	1001	20243814-01	70	4.1	2.0	616	49	9.8	108	164	237	0.5	38	2.0	91	6.4	4.0	146	0.06	6.1	62	19	N	3.03
24 hr post	1001	20243814-02	92	4.2	2.3	619	55	9.6	102	168	618	0.5	40	1.8	90	7.1	4.3	139	0.11	6.0	31	14	N	N
48 hr post	1001	20243814-03	95	4.4	2.3	605	44	9.7	104	189	328	0.5	40	1.9	109	7.3	4.4	142	0.24	6.3	28	18	N	N
72 hr post	1001	20243814-04	86	4.4	1.9	570	32	10.2	103	184	315	0.5	38	2.3	100	6.2	4.6	142	0.25	6.7	24	16	N	2.96

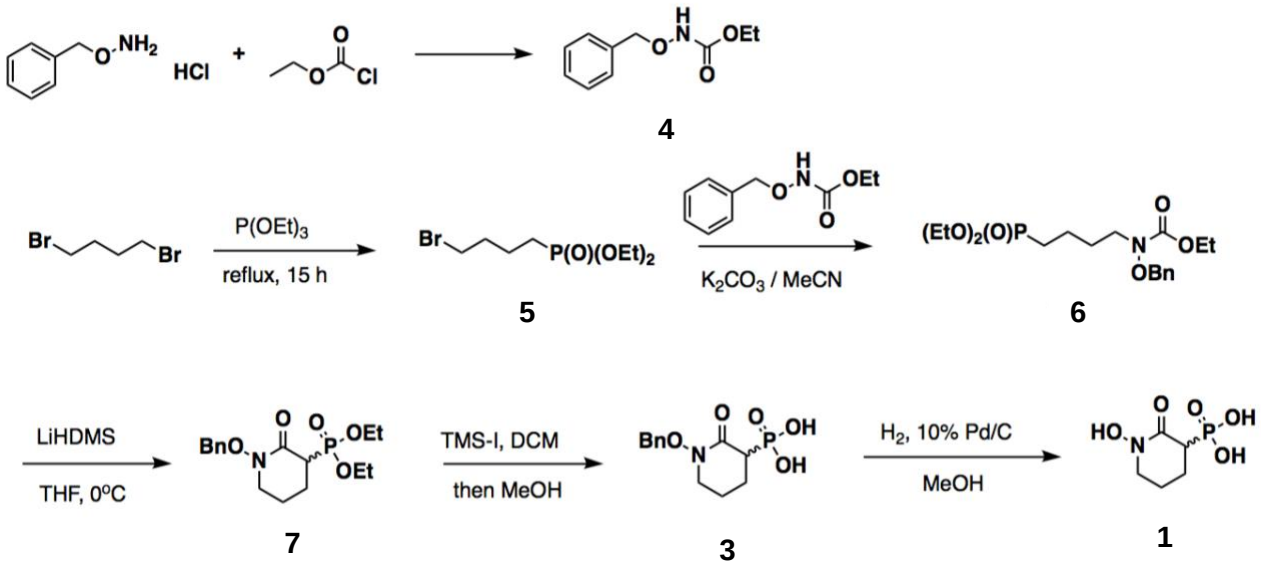
Hematology
HEX 100 mpk SC/day

Accession : Hematology / Clinical Chemistry of Samples from CRL-Shrewsbury Study 20243814 Tested on 3-Mar-2020 :Hematology																		
Time Point	Animal #	Analysis Sample ID	RBC ^6uL	HGB g/dL	**HCT %**	MCV fL	MCHC g/dL	MCH pg	RDW %	RETI ^9/L	PLT ^3uL	WBC ^3uL	NEUT ^3uL	LYMP ^3uL	MONO ^3uL	EOSO ^3uL	BASO ^3uL	LUC ^3uL
Pre dose	1001	20243814-01	5.64	13.6	**41.9**	74.3	32.6	24.2	14.7	71.3	464	8.69	1.36	7.06	0.16	0.06	0.01	0.04
24 hr post	1001	20243814-02	5.16	12.9	**38.8**	75.3	33.2	25.0	14.7	72.6	399	6.58	2.64	3.84	0.06	0.01	0.01	0.02
48 hr post	1001	20243814-03	5.21	13.0	**38.4**	73.8	33.7	24.9	14.6	66.0	431	7.55	2.25	5.05	0.18	0.02	0.00	0.04
72 hr post	1001	20243814-04	5.16	12.5	**38.3**	74.3	32.5	24.1	14.3	99.1	436	9.03	1.62	7.09	0.22	0.03	0.01	0.06

Supplementary Figure S8. No obvious systemic toxicities or anemia with repeated HEX treatment in NHP. Blood chemistry and hematology veterinary panels were performed at Charles River Laboratories on the same animal profiled for HEX levels in plasma in Figure 7, with daily 100 mg/kg SC injections. Times indicated are in reference to the first injection. The values for hematocrit (**bold**) were plotted in Figure 7. No obvious increases in blood chemistry parameters indicative of hepatotoxicity (ALT, AST), nephrotoxicity (CREA, Urea), myotoxicity (CK, ALP). Hematological parameters were also normal, except for the initial decrease in hematocrit, which is attributed to multiple blood draws for pharmacology.

Supplementary Note 1 . Synthesis of (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid, (HEX)

Synthesis of (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid HEX (1) CAS: 2004714-32-1



Step 1: Synthesis of ethyl benzyloxycarbamate (4). A mixture of O-benzylhydroxylamine hydrochloride (1.6 g, 10 mmol) and pyridine (5 mL) was stirred at RT for 2 h under N_2. This was then cooled to 0℃. Next, ethyl carbonochloridate (1.1 g, 10 mmol) was added and the mixture was stirred at RT for 2 h. The reaction mixture was then diluted with EtOAc (60 mL), washed with 2N HCl (30 mL x 3) and aq $NaHCO_3$ (30 mL x 2), dried over sodium sulfate, filtered and concentrated to yield ethyl benzyloxycarbamate **3** as a yellow oil (1.7 g, 87%). MS (ES+) C10H13NO3 requires: 195, found 196 $[M+H]_+$.

Step 2: Synthesis of diethyl 4-bromobutylphosphonate (5). Triethyl phosphite (30.0 g, 181 mmol) was slowly added to 1, 4-dibromobutane (117 g, 542 mmol) at 90℃.Then the mixture was stirred at 90℃ overnight. The mixture was purified by silica gel column using gradient elution (DCM/MeOH: 0-8%) to yield the diethyl 4-bromobutylphosphonate **5** as a light-yellow oil (30 g, 61%). MS (ES+) C8H18BrO3P requires: 272, found 273 $[M+H]_+$.

Step 3: Synthesis of ethyl benzyloxy(4-(diethoxyphosphoryl)butyl)carbamate (6). A mixture of diethyl 4-bromobutylphosphonate (1.7 g, 6.2 mmol), ethyl benzyloxycarbamate (1.1 g, 5.6 mmol, **3**) and potassium carbonate (3.9 g, 28 mmol) in MeCN (20 mL) was stirred at 90 °C overnight. The solvent was removed under reduced pressure. The residue was diluted with water (60 mL), extracted with DCM (2 x 50 mL). The combined organic extracts were washed with brine (100 mL), dried over sodium sulfate, filtered and concentrated to give a yellow oil. The oil was purified by silica gel column using gradient elution (DCM/MeOH: 0-5%, 5-8%) to afford ethyl benzyloxy(4-(diethoxyphosphoryl)butyl)carbamate **6** as a yellow oil (1.8 g, 83%). MS (ES+) $C_{18}H_{30}NO_6P$ requires: 387, found 388 $[M+H]^+$.

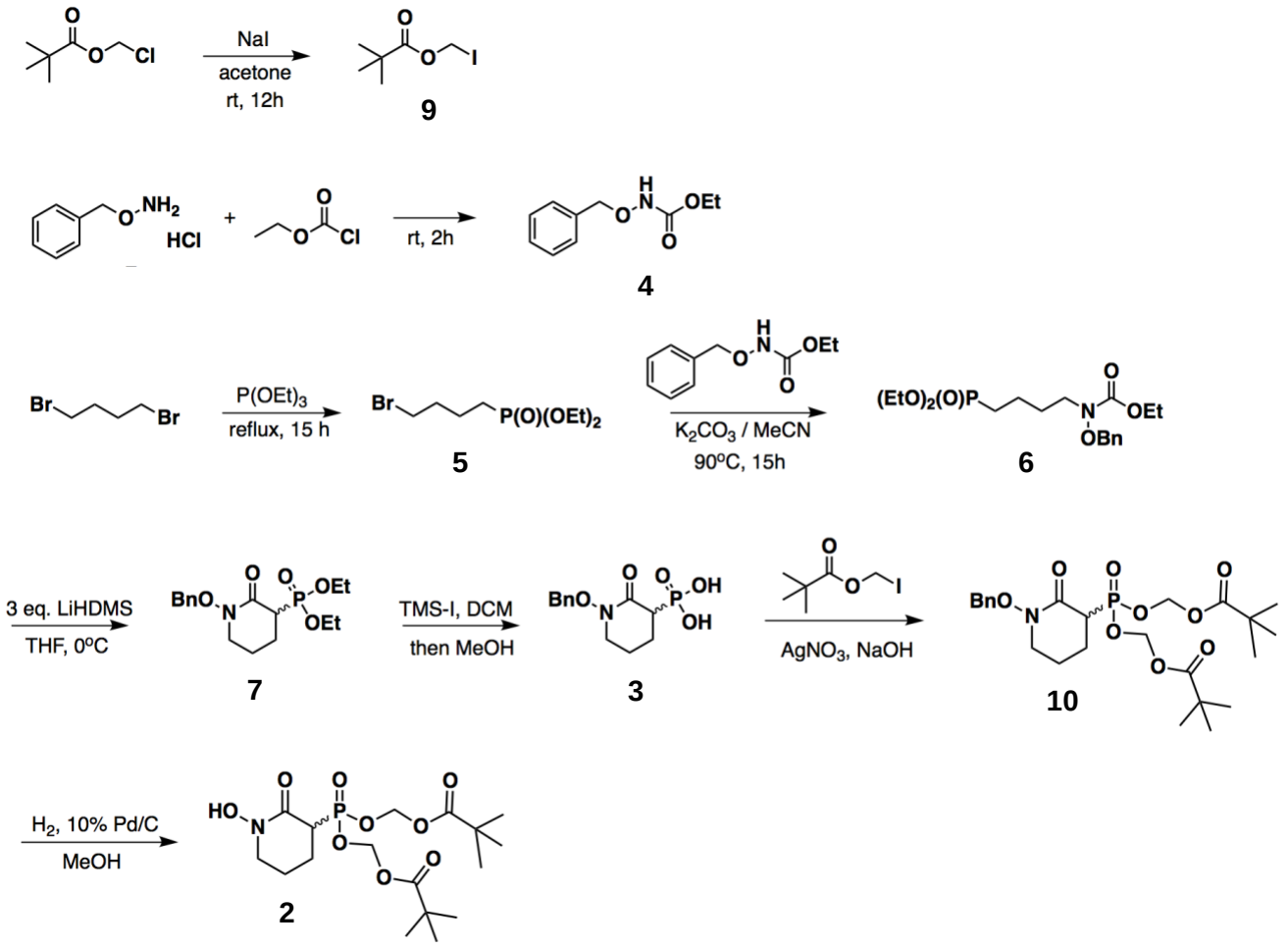
Step 4: Synthesis of diethyl 1-(benzyloxy)-2-oxopiperidin-3-ylphosphonate (7). To a solution of ethyl benzyloxy(4-(diethoxyphosphoryl)butyl)carbamate (1.16 g, 3 mmol, **6**) in THF (10 mL) at 0℃, LiHMDS (1M solution in THF, 9 mL, 9 mmol) was slowly added. The mixture was stirred at 0℃ for 3 h under N_2. Then, the reaction was quenched at 0℃with 10% aq. AcOH (10mL) and solvent was removed under reduced pressure. The residue was dissolved in EtOAc (100mL), washed with brine (50 mL), dried over sodium sulfate, filtered and concentrated to give a yellow oil. The oil was purified by silica gel column using gradient elution (DCM/MeOH = 0-8%) to afford diethyl 1-(benzyloxy)-2-oxopiperidin-3-ylphosphonate **7** as a yellow oil (850 mg, 83%). MS (ES+) $C_{16}H_{24}NO_5P$ requires: 341, found 342 $[M+H]^+$. **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.46-7.31 (m, 5H), 4.91 (q, *J*=10.81, 10.81, 15.36 Hz, 2H), 4.14 (m, 4H), 3.29 (m, 2H), 2.98 (dt, *J*=25.80 Hz, 1H), 2.08 (m, 2H), 1.90 (m, 1H), 1.68 (m, 1H), 1.33 (t, *J*=7.0, 7.0 Hz, 6H).**^{13}C NMR** (75 MHz, $CDCl_3$) δ 163.09 (d, *J*=5.41 Hz, 1C), 135.85, 130.19 (s, 2C), 129.18, 128.93 (s, 2C), 76.38, 63.55 (d, *J*=6.84 Hz, 1C), 62.70 (d, *J*=6.84 Hz, 1C), 51.39, 42.71 (d, *J*=136.97, 1C), 23.24 (d, *J*=4.76, 1C), 22.32 (d, *J*=7.48, 1C), 16.97 (d, *J*=6.12, 1C), 16.84 (d, *J*=6.12, 1C). **^{31}P NMR** (121 MHz, $CDCl_3$) δ 23.83.

Step 5: Synthesis of 1-(benzyloxy)-2-oxopiperidin-3-ylphosphonic acid (3). To a solution of diethyl 1-(benzyloxy)-2-oxopiperidin-3-ylphosphonate (3.41 g, 10 mmol, **7**) in DCM (30 mL) at 0°C, iodotrimethylsilane (6.0 g, 30 mmol) was slowly added. The mixture

was stirred at RT for 4 h. Next, MeOH (40 mL) was added and the solvent was removed. The aforementioned step was repeated twice. Then, the residue was purified by preparative HPLC to yield 1-(benzyloxy)-2-oxopiperidin-3-ylphosphonic acid **3** as a yellow solid (1.7 g, 60%). MS (ES+) C12H16NO5P requires: 285, found 286 $[M+H]^+$. **^{1}H NMR** (300 MHz, D_2O) δ 7.56-7.48 (m, 5H), 4.97 (q, *J*=6.48, 10.32, 10.43 Hz, 2H), 3.54 (t, *J*=6.22, 6.22 Hz, 2H), 2.86 (dt, *J*=24.08 Hz, 1H), 2.10 (m, *J*=4.80, 6.10, 6.47, 6.79 Hz, 1H), 1.97 (m, *J*=5.92, 6.24, 6.31, 6.40, 8.25 Hz, 1H, 1.79 (m, *J*=5.76, 5.92, 6.24, 6.56, 6.31 Hz, 1H). **^{13}C NMR** (75 MHz, D_2O) δ 166.24 (d, *J*=5.49 Hz, 1C), 134.38, 130.00 (s, 2C), 129.22, 128.84 (s, 2C), 75.76, 50.23, 42.18 (d, *J*=129.09, 1C), 22.12 (d, *J*=6.44, 1C), 21.24 (d, *J*=7.81, 1C). **^{31}P NMR** (121 MHz, D_2O) δ 17.02 (m, *J*=12.10, 12.10, 17.84, 17.84 Hz).

<u>Step 6</u>: Synthesis of (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid—HEX (1). To a solution of 1-(benzyloxy)-2-oxopiperidin-3-ylphosphonic acid (0.8 g, 2.80 mmol, **8**) dissolved in MeOH (10 mL), palladium on carbon was added (10%, 80 mg). Next, the resulting mixture was hydrogenated at 5 psi at RT for 1 h in a Parr apparatus. Then, the catalyst was removed by filtering through Celite ®. The filtrate was concentrated to yield (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid **HEX** (**1**) as a light-yellow oil (0.54 g, 98%). MS (ES^+) $C_5H_{10}NO_5P$ requires: 195, found 196 $[M+H]^+$. MS (ES^-) $C_5H_{10}NO_5P$ requires: 195, found 194 $[M-H]^-$. **^{1}H NMR** (300 MHz, D_2O) δ 3.52 (m, 2H), 2.72 (dt, *J*=24.24, 1H), 2.09 (m, 1H), 1.92 (m, 2H), 1.77 (m, *J*=7.21, 6.32, 6.25, 6.09, 5.93, 5.38 Hz, 1H). **^{1}H (^{31}P decoupled)** NMR (300 MHz, D_2O) δ 3.61 (m, 2H), 3.02 (t, J=6.35, 6.49 Hz, 1H), 2.15-1.86 (m, 4H). **^{13}C NMR** (75 MHz, D_2O) δ 165.07 (d, *J*=6.10 Hz, 1C), 51.55, 41.48 (d, *J*=130.59 Hz, 1C), 22.05 (d, *J*=4.05 Hz, 1C), 21.05 (d, *J*=8.17 Hz, 1C). **^{31}P NMR** (121 MHz, D_2O) δ 20.53 (m, *J*=11.59, 13.04, 14.18, 14.49 Hz, 1P). **^{31}P (^{1}H decoupled)** NMR (121 MHz, D_2O) δ 20.77 (s, 1P).

Synthesis of (((1-hydroxy-2-oxopiperidin-3-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) POMHEX (2) CAS: 2004714-34-3



Step 1: Synthesis of iodomethyl pivalate (9). A mixture of chloromethyl pivalate (30 g, 199 mmol, **7**) and sodium iodide (60 g, 400 mmol) in acetone (250 mL) was stirred vigorously in a foil covered flask for 12 h at RT. The mixture was filtered, and the salts were rinsed with acetone (50 mL) and concentrated. Then, the residue was dissolved in ether (250 mL) and transferred to a separatory funnel. The organic phase was then washed with 10 % aqueous sodium hydrogen sulfite (3 x 50 mL) followed by brine (1 x 50 mL), dried (Na_2SO_4), filtered, and concentrated affording iodomethyl pivalate **9** as a light-yellow liquid (43.8 g, 91%). **^{1}H NMR** (300 MHz, $CDCl_3$) δ 1.2 (s, 9H), 5.9 (s, 2H).

The synthetic route (steps 2-6) towards POMHEX follows the synthesis of HEX up to intermediate (3).

Step 7: Synthesis of (((1-(benzyloxy)-2-oxopiperidin-3-yl)phosphoryl)bis(oxy))bis (methylene) bis(2,2-dimethylpropanoate) (10). To a solution of (1-(benzyloxy)-2-oxopiperidin-3-yl)phosphonic acid (1 g, 3.51 mmol **3**) in water (17.53 ml), sodium hydroxide (0.280 g, 7.01 mmol) was added. The mixture was stirred at 25°C for 1 h. Once the solution reached an alkaline pH (~9), a solution of silver nitrate (1.787 g, 10.52 mmol) in water (4 mL) was added and the resulting mixture was stirred at 25°C for 2 h. Then, the solid was collected by vacuum filtration and rinsed with cold water (50 mL) and ether (25 mL) and dried under vacuum. The resulting solid was added to a solution of iodomethyl pivalate **9** (1.867 g, 7.71 mmol) in toluene (17.53 ml). The mixture was stirred at 25°C for 6 h. After filtration, the filtrate was concentrated and purified via silica gel chromatography using gradient elution (EtOAc/hexanes: 20-100%), which yielded (((1-(benzyloxy)-2-oxopiperidin-3-yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2- dimethyl propanoate) **10** as a light-yellow oil (1.08 g, 60%). MS (ES+) $C_{24}H_{36}NO_9P$ requires: 513, found 514 $[M+H]^+$. ^{1}**H NMR** (300 MHz, $CDCl_3$) δ 7.43-7.5 (m, 2H), 7.33-7.41 (m, 3H), 5.7-5.9 (m, 4H), 4.96 (s, 2H), 3.27-3.4 (m, 2H), 3.15 (dt, *J*=26.13, 7.0 Hz, 1H), 1.96-2.1 (m, 3H), 1.71 (m, 1H), 1.25 (s, 9H), 1.24 (s, 9H). ^{13}**C NMR** (75 MHz, $CDCl_3$) δ 177.01 (s, 2C), 162.42 (d, *J*=5.21 Hz, 1C), 135.74, 130.34 (s, 2C), 129.34, 129.10 (s, 2C), 81.34 (d, *J*=5.74 Hz, 1C), 76.58, 51.34, 42.85 (d, *J*=142.65 Hz, 1C), 39.36 (s, 2C), 27.47 (s, 3C), 27.45 (s, 3C), 22.98 (d, *J*=4.71 Hz, 1C), 22.75 (d, *J*=10.98 Hz, 1C). ^{31}**P NMR** (121 MHz, $CDCl_3$) δ 23.30.

Step 8: Synthesis of (((1-hydroxy-2-oxopiperidin-3-yl)phosphoryl)bis(oxy))bis (methylene) bis(2,2-dimethylpropanoate)—POMHEX (2). To a solution of (((1-(benzyloxy)-2-oxopiperidin-3- yl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethyl propanoate) **10** (0.8 g, 1.55 mmol) dissolved in MeOH (10 mL), palladium on carbon was added (10%, 80 mg). Next, the resulting mixture was hydrogenated at 5 psi at RT for 1 h in a Parr apparatus. Then, the catalyst was removed by filtering through Celite. The filtrate was concentrated to yield (((1-hydroxy-2-oxopiperidin-3-yl)phosphoryl)bis(oxy))bis

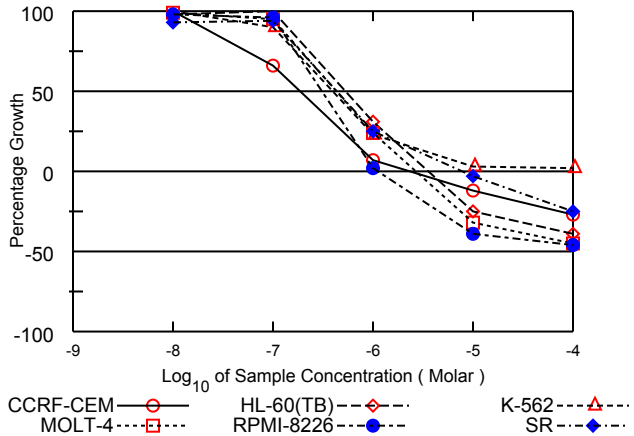
(methylene) bis(2,2-dimethylpropanoate) **POMHEX (2)** as a light-yellow oil (0.65 g, 98%). MS (ES+) C17H30NO9P requires: 423, found 424 [M+H]+. **$_{1}$H NMR** (300 MHz, $CDCl_3$) δ 5.76 (dd, J=12.76 Hz, 1H), 5.72-5.55 (m, 3H), 3.59 (m, 2H), 3.10 (dt, J=26.39 Hz, 1H), 2.15 (m, 1H), 2.05 (m, 2H), 1.84 (m, 1H), 1.21 (s, 9H), 1.20 (s, 9H). **$_{1}$H ($_{31}$P decoupled) NMR** (300 MHz, $CDCl_3$) δ 5.77 (d, J=5.17 Hz, 1H), 5.71-5.66 (m, 3H), 3.59 (m, 2H), 3.14 (t, J=7.48, 7.17 Hz, 1H), 2.15 (m, 1H), 2.09 (m, 2H), 1.88 (m, 1H), 1.21 (s, 9H), 1.20 (s, 9H). **$_{13}$C NMR** (75 MHz, $CDCl_3$) δ 177.70, 177.69, 159.84 (s, J=5.39 Hz, 1C), 83.27 (d, J=5.67 Hz, 1C), 82.43 (d, J=6.49 Hz, 1C) 49.42, 41.13 (d, J=143.71 Hz, 1C), 23.06 (d, J=4.40 Hz), 22.16 (d, J=10.59 Hz, 1C), 22.61 (s, 3C), 22.61 (s, 3C). **$_{31}$P NMR** (121 MHz, C_6D_6) δ 22.75 (m, 1P). **$_{31}$P ($_{1}$H decoupled) NMR** (121 MHz, $CDCl_3$) δ 22.83 (s, 1P).

Supplementary Note 2. POMHEX NCI-60

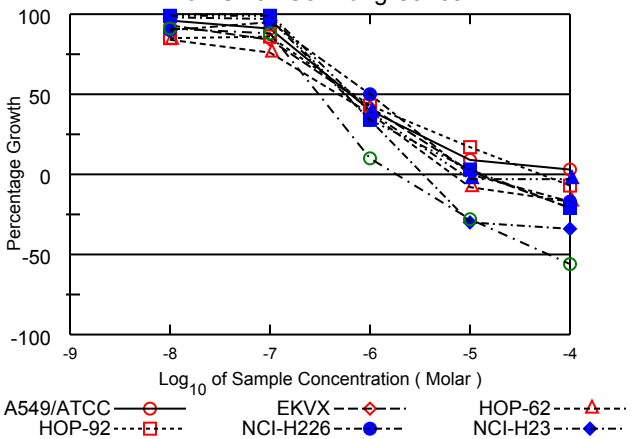
National Cancer Institute Developmental Therapeutics Program
Dose Response Curves

NSC: D - 784584 / 1	SSPL: 0ZOT	EXP. ID: 1507NS15
Report Date: January 19, 2017	Test Date: July 06, 2015	

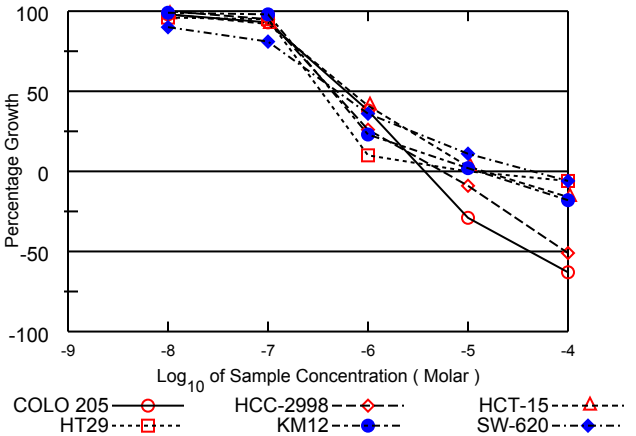
Leukemia



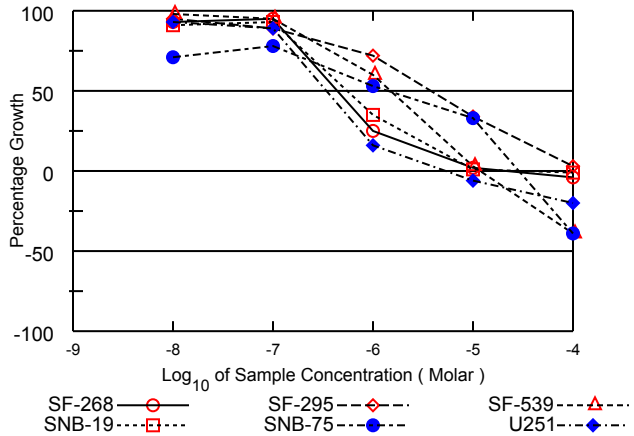
Non-Small Cell Lung Cancer



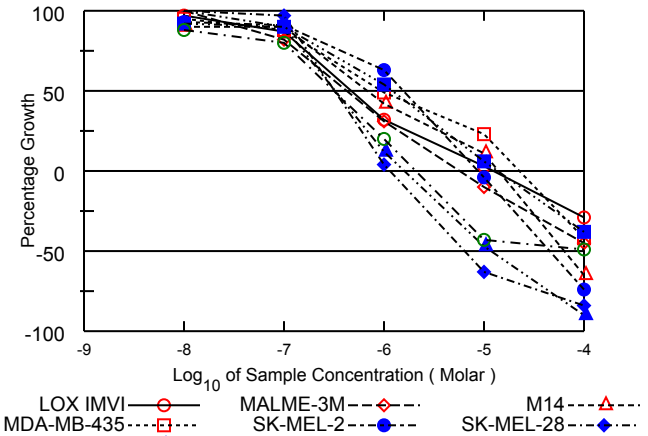
Colon Cancer



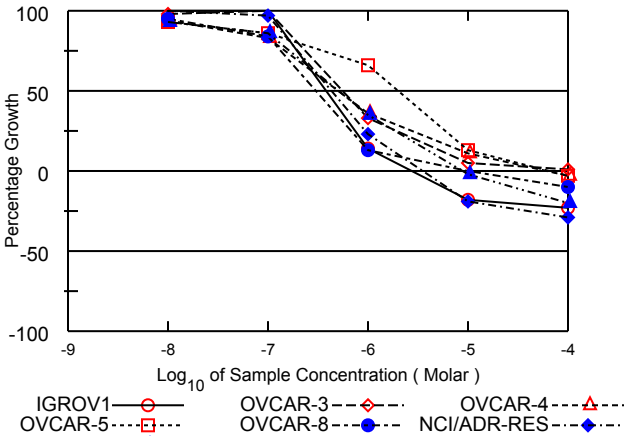
CNS Cancer



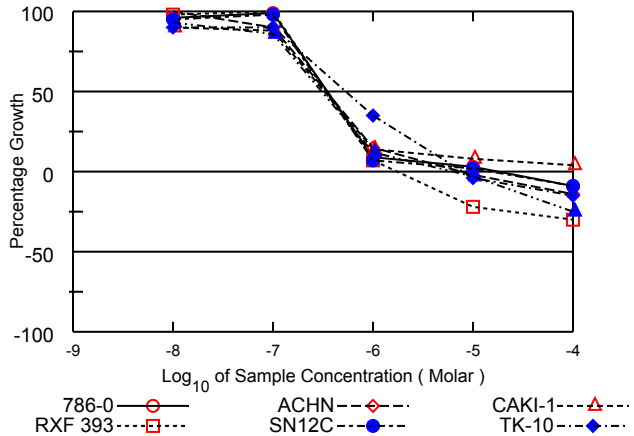
Melanoma



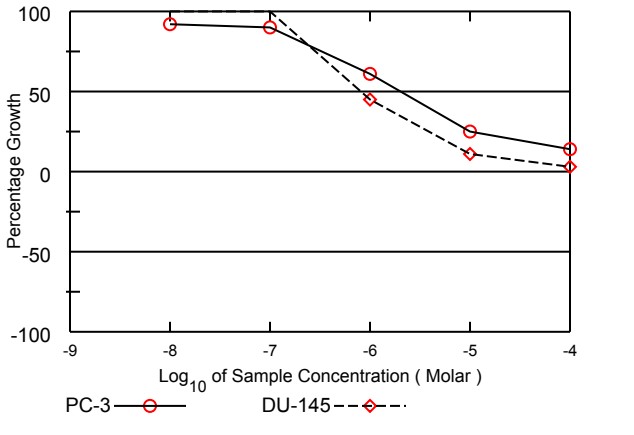
Ovarian Cancer



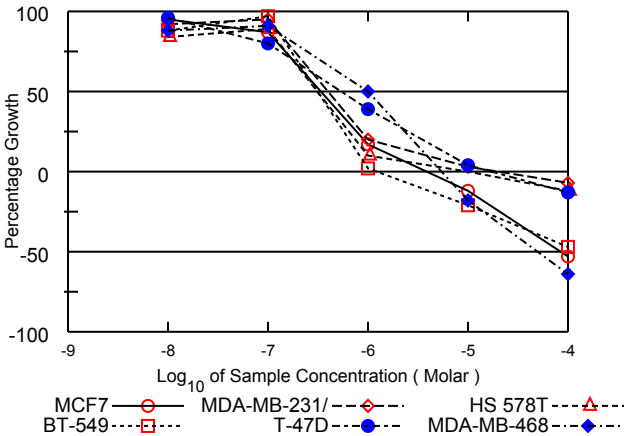
Renal Cancer



Prostate Cancer



Breast Cancer



National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC : D - 784584 / 1	Experiment ID : 1507NS15	Test Type : 08	Units : Molar
Report Date : January 19, 2017	Test Date : July 06, 2015	QNS :	MC :
COMI : POMHEX	Stain Reagent : SRB Dual-Pass Related	SSPL : 0ZOT	

			Log10 Concentration												
	Time		Mean Optical Densities					Percent Growth							
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.692	2.635	2.633	1.970	0.829	0.607	0.508	100	66	7	-12	-27	1.85E-7	2.30E-6	> 1.00E-4
HL-60(TB)	0.841	2.702	2.657	2.754	1.410	0.629	0.517	98	103	31	-25	-39	5.38E-7	3.53E-6	> 1.00E-4
K-562	0.295	2.251	2.244	2.052	0.741	0.357	0.336	100	90	23	3	2	3.93E-7	> 1.00E-4	> 1.00E-4
MOLT-4	0.637	2.604	2.581	2.506	1.109	0.434	0.352	99	95	24	-32	-45	4.31E-7	2.69E-6	> 1.00E-4
RPMI-8226	0.825	2.421	2.394	2.354	0.858	0.501	0.449	98	96	2	-39	-46	3.08E-7	1.12E-6	> 1.00E-4
SR	0.433	1.955	1.843	1.866	0.811	0.421	0.325	93	94	25	-3	-25	4.33E-7	7.87E-6	> 1.00E-4
Non-Small Cell Lung Cancer															
A549/ATCC	0.461	2.330	2.260	2.167	1.208	0.628	0.510	96	91	40	9	3	6.37E-7	> 1.00E-4	> 1.00E-4
EKVX	0.686	2.323	2.205	2.063	1.380	0.723	0.543	93	84	42	2	-21	6.57E-7	1.25E-5	> 1.00E-4
HOP-62	0.802	1.652	1.517	1.447	1.127	0.736	0.667	84	76	38	-8	-17	4.87E-7	6.63E-6	> 1.00E-4
HOP-92	1.384	2.280	2.149	2.157	1.771	1.532	1.292	85	86	43	17	-7	6.95E-7	5.15E-5	> 1.00E-4
NCI-H226	0.817	2.021	1.904	1.956	1.418	0.810	0.675	90	95	50	.	-17	9.96E-7	9.62E-6	> 1.00E-4
NCI-H23	0.712	2.210	2.179	2.164	1.228	0.496	0.468	98	97	34	-30	-34	5.64E-7	3.40E-6	> 1.00E-4
NCI-H322M	0.574	1.616	1.631	1.641	0.982	0.557	0.558	101	102	39	-3	-3	6.73E-7	8.50E-6	> 1.00E-4
NCI-H460	0.322	3.214	3.192	3.184	1.294	0.399	0.254	99	99	34	3	-21	5.61E-7	1.29E-5	> 1.00E-4
NCI-H522	0.945	2.777	2.604	2.559	1.129	0.680	0.420	91	88	10	-28	-56	3.08E-7	1.83E-6	6.28E-5
Colon Cancer															
COLO 205	0.441	1.712	1.689	1.625	0.920	0.311	0.163	98	93	38	-29	-63	6.00E-7	3.64E-6	4.09E-5
HCC-2998	0.472	1.803	1.855	1.736	0.815	0.431	0.231	104	95	26	-9	-51	4.46E-7	5.59E-6	9.39E-5
HCT-15	0.529	2.984	2.936	2.789	1.544	0.610	0.444	98	92	41	3	-16	6.75E-7	1.48E-5	> 1.00E-4
HT29	0.349	2.188	2.124	2.090	0.527	0.347	0.327	96	95	10	.	-6	3.35E-7	8.53E-6	> 1.00E-4
KM12	0.710	3.376	3.350	3.336	1.335	0.776	0.585	99	98	23	2	-18	4.43E-7	1.33E-5	> 1.00E-4
SW-620	0.265	1.955	1.779	1.641	0.875	0.444	0.249	90	81	36	11	-6	4.93E-7	4.26E-5	> 1.00E-4
CNS Cancer															
SF-268	0.682	2.237	2.124	2.166	1.070	0.709	0.655	93	95	25	2	-4	4.41E-7	2.02E-5	> 1.00E-4
SF-295	0.986	2.876	2.786	2.671	2.355	1.637	1.048	95	89	72	34	3	3.90E-6	> 1.00E-4	> 1.00E-4
SF-539	1.258	2.985	2.958	2.903	2.301	1.312	0.762	98	95	60	3	-39	1.52E-6	1.18E-5	> 1.00E-4
SNB-19	0.660	2.134	2.007	2.032	1.176	0.677	0.651	91	93	35	1	-1	5.51E-7	2.87E-5	> 1.00E-4
SNB-75	0.955	2.064	1.738	1.821	1.544	1.321	0.579	71	78	53	33	-39	1.43E-6	2.86E-5	> 1.00E-4
U251	0.407	1.924	1.824	1.751	0.654	0.381	0.327	93	89	16	-6	-20	3.41E-7	5.22E-6	> 1.00E-4
Melanoma															
LOX IMVI	0.258	1.921	1.876	1.703	0.784	0.307	0.183	97	87	32	3	-29	4.65E-7	1.23E-5	> 1.00E-4
MALME-3M	0.719	1.192	1.199	1.106	0.867	0.650	0.398	101	82	31	-10	-45	4.25E-7	5.81E-6	> 1.00E-4
M14	0.435	1.857	1.712	1.713	1.039	0.599	0.151	90	90	42	11	-65	6.93E-7	1.41E-5	6.32E-5
MDA-MB-435	0.448	2.645	2.532	2.379	1.530	0.953	0.259	95	88	49	23	-42	9.55E-7	2.25E-5	> 1.00E-4
SK-MEL-2	1.098	2.635	2.524	2.492	2.060	1.056	0.289	93	91	63	-4	-74	1.55E-6	8.76E-6	4.58E-5
SK-MEL-28	0.467	1.344	1.343	1.321	0.500	0.172	0.077	100	97	4	-63	-84	3.21E-7	1.14E-6	6.34E-6
SK-MEL-5	0.550	2.619	2.707	2.416	0.789	0.294	0.055	104	90	12	-47	-90	3.24E-7	1.58E-6	1.20E-5
UACC-257	1.075	2.440	2.329	2.310	1.808	1.152	0.668	92	90	54	6	-38	1.19E-6	1.35E-5	> 1.00E-4
UACC-62	0.748	2.455	2.255	2.112	1.097	0.427	0.379	88	80	20	-43	-49	3.18E-7	2.10E-6	> 1.00E-4
Ovarian Cancer															
IGROV1	0.790	2.590	2.652	2.636	1.044	0.646	0.606	103	103	14	-18	-23	3.93E-7	2.73E-6	> 1.00E-4
OVCAR-3	0.501	1.888	1.857	1.907	0.954	0.569	0.513	98	101	33	5	1	5.59E-7	> 1.00E-4	> 1.00E-4
OVCAR-4	0.595	1.395	1.344	1.259	0.885	0.681	0.577	94	83	36	11	-3	5.08E-7	5.97E-5	> 1.00E-4
OVCAR-5	0.953	1.849	1.785	1.720	1.546	1.066	0.923	93	86	66	13	-3	2.01E-6	6.27E-5	> 1.00E-4
OVCAR-8	0.501	2.267	2.182	1.992	0.735	0.498	0.449	95	84	13	.	-10	3.05E-7	8.91E-6	> 1.00E-4
NCI/ADR-RES	0.536	1.784	1.804	1.746	0.827	0.434	0.383	102	97	23	-19	-29	4.34E-7	3.55E-6	> 1.00E-4
SK-OV-3	0.857	2.126	2.039	1.944	1.303	0.840	0.688	93	86	35	-2	-20	5.08E-7	8.81E-6	> 1.00E-4
Renal Cancer															
786-0	1.179	2.979	2.906	2.961	1.342	1.237	1.070	96	99	9	3	-9	3.50E-7	1.81E-5	> 1.00E-4
ACHN	0.564	2.412	2.412	2.231	0.833	0.555	0.487	100	90	15	-2	-14	3.40E-7	7.97E-6	> 1.00E-4
CAKI-1	0.621	3.069	2.813	2.777	0.953	0.820	0.709	90	88	14	8	4	3.24E-7	> 1.00E-4	> 1.00E-4
RXF 393	0.947	1.697	1.681	1.725	1.000	0.737	0.659	98	104	7	-22	-30	3.60E-7	1.74E-6	> 1.00E-4
SN12C	0.653	2.242	2.161	2.206	0.759	0.677	0.597	95	98	7	2	-9	3.34E-7	1.41E-5	> 1.00E-4
TK-10	0.816	1.816	1.719	1.713	1.169	0.786	0.690	90	90	35	-4	-15	5.37E-7	8.05E-6	> 1.00E-4
UO-31	0.783	2.212	2.117	2.009	0.955	0.761	0.588	93	86	12	-3	-25	3.06E-7	6.41E-6	> 1.00E-4
Prostate Cancer															
PC-3	0.474	1.466	1.392	1.363	1.079	0.724	0.616	92	90	61	25	14	2.02E-6	> 1.00E-4	> 1.00E-4
DU-145	0.429	1.945	1.944	1.997	1.116	0.598	0.475	100	103	45	11	3	8.31E-7	> 1.00E-4	> 1.00E-4
Breast Cancer															
MCF7	0.571	2.887	2.768	2.594	0.960	0.504	0.267	95	87	17	-12	-53	3.38E-7	3.86E-6	8.35E-5
MDA-MB-231/ATCC	0.572	1.467	1.395	1.426	0.748	0.598	0.534	92	95	20	3	-7	3.97E-7	2.00E-5	> 1.00E-4
HS 578T	0.984	2.098	1.918	1.980	1.098	0.975	0.863	84	89	10	.	-12	3.14E-7	8.27E-6	> 1.00E-4
BT-549	0.701	1.570	1.469	1.544	0.721	0.556	0.369	88	97	2	-21	-47	3.13E-7	1.26E-6	> 1.00E-4
T-47D	0.892	1.819	1.783	1.634	1.255	0.933	0.775	96	80	39	4	-13	5.43E-7	1.77E-5	> 1.00E-4
MDA-MB-468	0.579	1.181	1.109	1.125	0.878	0.478	0.207	88	91	50	-18	-64	9.77E-7	5.48E-6	4.95E-5

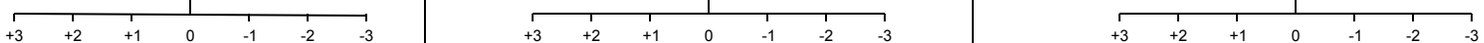
National Cancer Institute Developmental Therapeutics Program

Mean Graphs

NSC : D - 784584/1 | Units :Molar | SSPL :0ZOT | EXP. ID :1507NS15

Report Date :January 19, 2017 | Test Date :July 06, 2015

Panel/Cell Line	Log_{10}GI50	GI50	Log_{10}TGI	TGI	Log_{10}LC50	LC50
Leukemia						
CCRF-CEM	-6.73		-5.64		> -4.00	
HL-60(TB)	-6.27		-5.45		> -4.00	
K-562	-6.41		> -4.00		> -4.00	
MOLT-4	-6.37		-5.57		> -4.00	
RPMI-8226	-6.51		-5.95		> -4.00	
SR	-6.36		-5.10		> -4.00	
Non-Small Cell Lung Cancer						
A549/ATCC	-6.20		> -4.00		> -4.00	
EKVX	-6.18		-4.90		> -4.00	
HOP-62	-6.31		-5.18		> -4.00	
HOP-92	-6.16		-4.29		> -4.00	
NCI-H226	-6.00		-5.02		> -4.00	
NCI-H23	-6.25		-5.47		> -4.00	
NCI-H322M	-6.17		-5.07		> -4.00	
NCI-H460	-6.25		-4.89		> -4.00	
NCI-H522	-6.51		-5.74		-4.20	
Colon Cancer						
COLO 205	-6.22		-5.44		-4.39	
HCC-2998	-6.35		-5.25		-4.03	
HCT-15	-6.17		-4.83		> -4.00	
HT29	-6.47		-5.07		> -4.00	
KM12	-6.35		-4.88		> -4.00	
SW-620	-6.31		-4.37		> -4.00	
CNS Cancer						
SF-268	-6.36		-4.70		> -4.00	
SF-295	-5.41		> -4.00		> -4.00	
SF-539	-5.82		-4.93		> -4.00	
SNB-19	-6.26		-4.54		> -4.00	
SNB-75	-5.85		-4.54		> -4.00	
U251	-6.47		-5.28		> -4.00	
Melanoma						
LOX IMVI	-6.33		-4.91		> -4.00	
MALME-3M	-6.37		-5.24		> -4.00	
M14	-6.16		-4.85		-4.20	
MDA-MB-435	-6.02		-4.65		> -4.00	
SK-MEL-2	-5.81		-5.06		-4.34	
SK-MEL-28	-6.49		-5.94		-5.20	
SK-MEL-5	-6.49		-5.80		-4.92	
UACC-257	-5.92		-4.87		> -4.00	
UACC-62	-6.50		-5.68		> -4.00	
Ovarian Cancer						
IGROV1	-6.41		-5.56		> -4.00	
OVCAR-3	-6.25		> -4.00		> -4.00	
OVCAR-4	-6.29		-4.22		> -4.00	
OVCAR-5	-5.70		-4.20		> -4.00	
OVCAR-8	-6.52		-5.05		> -4.00	
NCI/ADR-RES	-6.36		-5.45		> -4.00	
SK-OV-3	-6.29		-5.05		> -4.00	
Renal Cancer						
786-0	-6.46		-4.74		> -4.00	
ACHN	-6.47		-5.10		> -4.00	
CAKI-1	-6.49		> -4.00		> -4.00	
RXF 393	-6.44		-5.76		> -4.00	
SN12C	-6.48		-4.85		> -4.00	
TK-10	-6.27		-5.09		> -4.00	
UO-31	-6.51		-5.19		> -4.00	
Prostate Cancer						
PC-3	-5.69		> -4.00		> -4.00	
DU-145	-6.08		> -4.00		> -4.00	
Breast Cancer						
MCF7	-6.47		-5.41		-4.08	
MDA-MB-231/ATCC	-6.40		-4.70		> -4.00	
HS 578T	-6.50		-5.08		> -4.00	
BT-549	-6.50		-5.90		> -4.00	
T-47D	-6.26		-4.75		> -4.00	
MDA-MB-468	-6.01		-5.26		-4.30	
_MID	-6.27		-4.97		-4.06	
Delta	0.46		0.98		1.14	
Range	1.32		1.95		1.2	



National Cancer Institute Developmental Therapeutics Program Dose Response Curves	NSC : D - 784584/1	SSPL:0ZOT	EXP. ID:1507NS15
	Report Date:January 19, 2017	Test Date:July 06, 2015