A unique small molecule inhibitor of enolase clarifies its role in fundamental biological processes

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SUPPLEMENTARY METHODS SECTION

Reagents and antibodies

3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin were purchased from Sigma-Aldrich. Rosiglitazine was purchased from Cayman Chemicals. Antibodies for enolase (A-5), GAPDH (I-19) PECK (P-16) and α-tubulin (10DB) were purchased from Santa Cruz Biotechnology. The antibody for Akt (#9272) was purchased from R&D Systems. The antibody for Bcl-xL (#2762) was purchased from Cell Signaling. Emodin was a gift from Professor Won Keun Oh, Chosun University, Republic of Korea.

Cell culture conditions

The human colon carcinoma cell line HCT116 was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; purchased from Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (PenStrep; purchased from Gibco BRL). Human embryonic kidney cells (HEK) were cultured in DMEM supplemented with 10% FBS and PenStrep. 3T3-L1 murine fibroblasts were maintained in proliferation media, consisting of DMEM supplemented with 10% calf serum and 1% PenStrep. Huh7 human hepatocytes were cultured in DMEM

supplemented with 10% FBS and 1% PenStrep. THP-1 human monocytes were cultured in RPMI-1640 medium supplemented with 0.05 nM 2-mercaptoethanol, 10% FBS and PenStrep. 3T3-L1 murine pre-adipocyte cells were cultured in DMEM supplemented with 10% newborn calf serum CS and PenStrep. HEK, Huh7, THP-1 and 3T3-L1 cells were gifts from Professor Hyun Chul Lee, Yonsei University College of Medicine, Republic of Korea.

Construction of the tagged triazine library. The tagged triazine library was prepared according to the procedure reported previously (supplementary scheme 1).(*1*) The purity of synthesized compounds was determined by analytical HPLC. Please note that compounds in this library have previously been shown to be cell permeable.(*2*)

Screening for apoptosis inducers that maintain effectiveness under hypoxia. HCT116 colon carcinoma cells were seeded in 2 sets of 96 well plates at a density of 5 x 10^3 cells per well. 'Low glucose' DMEM (containing 5 mM glucose) was used for screening, because glycolysis inhibitors, such as 2-Deoxy-D-glucose (2-DG), are effective by competing with glucose. 24 h later, the hypoxic condition was induced in one set of 96 well plates by treatment with 150 μM of 0.22 μm filtered $CoCl_2$ and the culture media volume was reduced by 50% (to 100 μL/well), as previously described.(*3*) 4 h later, test compounds from a tagged triazine library(*1*) were screened at a concentration of 5 μM in duplicate wells. Cytotoxicity was determined by MTT assay 24 h after adding drug. 'Hits' for further analysis were classified as compounds that 25% or higher cytotoxicity in the hypoxia condition compared to the normoxia condition (as measured by MTT assay absorbance). 50 μM 2-DG, an inhibitor of glycolysis that selectively kills cancer cells in hypoxic conditions(*4*), was used as a positive control.

To confirm that $CoCl_2$ treatment induced hypoxia, enolase expression was measured. Enolase expression is known to increase in response to hypoxia.(*5*) Treatment of HCT116 carcinoma cells with 150 μM $CoCl_2$ for 4 h induced enolase expression (supplementary Figure 9).

Enolase activity assay. Enolase purified from rabbit muscle was purchased from Sigma-Aldrich. A single unit of enolase is defined as the amount of enzyme that produces 1 μmol of phosphoenol pyruvate from phospho-D-glycerate/min in standard assay.(*6*) Enolase activity assay was measured at 37°C by incubating pure enolase (3–9 U) in a buffer containing 50 mM imidazole-HCl (pH 6.8), 2.0 mM MgSO4 and 400 mM KCl in the absence or presence of ENOblock or NaF. The reaction was initiated by adding 1 μmol of 2-phospho-D-glycerate, and the OD was measured after 10 min of reaction time with a spectrophometer at 240 nm.

Cell death assay. 3 x 10^5 HCT116 cells were seeded in a 6 well plate. 24 h later, cells were treated with compound of interest (with or without prior induction of hypoxia for 4 h) for 24 h using triplicate wells. Cells were then trypsinized and resuspended in 2.5 mL PBS. A 100 μL aliquot was taken for staining with 0.2% trypan blue solution (Sigma) and counted using a hemocytometer. 150 cells were counted and dead cells were classified as those that could not exclude trypan blue.

Zebrafish tumor cell xenograft model. Zebrafish embryos were obtained using standard mating conditions and staged for cell xenoplantation at 48 h post fertilization. After staining of cancer cells (described below), embryos were de-chorionized using micro-forceps and anesthetized with 0.0016% tricaine and positioned on their right side on a wet 1.0% agarose pad. Tumor cells were detached from culture dishes using 0.05% trypsin-EDTA and washed twice with PBS at room

temperature. Cells were stained with 2 μg/ml DiI diluted in PBS and washed four times: once with FBS, twice with PBS and then once with 10% FBS diluted in PBS. Cells were kept on ice before injection. Cancer cells were counted by microscopy, suspended in 10% FBS and 100 cells were injected into the center of the yolk sac using an injector equipped with borosilicate glass capillaries (PV820 pneumatic picopump, World Precision Instruments). Injected embryos were transferred to a 96-well plate (one embryo/well) containing drug of interest diluted in 200 mL E3 media (without methylene blue) and maintained at the pre-selected incubation temperature. At 4 days post injection, the number of embryos exhibiting cancer cell dissemination from the injection site was counted and photographed using upright microscopy (Leica DM2500 microscope). The number of migrated cells was counted and embryos that exhibited more than 5 fluorescent microfoci distant from the yolk sac were scored for cell dissemination.

Measurement of glucose uptake in zebrafish. At 72 hpf, larvae were placed into a 96-well plate (6 eggs/well in 200 μL E3 water supplemented with 0.2 mM 2-phenylthiourea). Drug of interest was added for 1 h. The solution was then replaced with E3 water supplemented with 600 μM 2-NBDG and incubated for 3 h. The larva were washed with E3 water and anesthetized with 0.02 % tricaine-supplemented E3 water. One larva was then placed on a chamber slide, containing 3% methylcellulose in E3 water, for fluorescent microscopy (Leica DM2500 microscope equipped with a DFC425 C digital camera). Images were captured at 50X magnification. The remaining 5 larvae where lysed with 120 μL of CelLytic M solution (Sigma-Aldrich) and sonication (4℃, 10’’/5’’ pulse, 10 min). Lysed larvae were centrifuged at 10000 rpm for 10 min. 100 μL of the supernatant was transferred to a 96-well plate and fluorescence

was measured with a fluorescent microplate reader (SpectraMAX Gemini XS, Molecular Devices; λ_{ex}=466 nm λ_{ex}=540 nm).

Statistics. The student's t test was used for comparison between experimental groups (Microsoft Excel, reference number 14.0.6023.1000). *P* values of less than 0.05 were considered to be significant. Unless otherwise stated, all presented results are representative of three independent experiments, which were carried out at different days.

Cell proliferation assay. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay, as previously described.(*7*)

Small molecule target identification using affinity chromatography. Proteins were extracted from 3T3-L1 pre-adipocytes by incubation with extraction buffer (1 mM $CaCl_2$; 150 mM NaCl; 10 mM Tris, pH 7.4; 1 % Triton X-100; 1 mM PMSF plus one tablet of protease inhibitor cocktail (Roche) per 10 mL buffer) for 5 min on ice. Crude lysate was centrifuged at 10,000 rpm for 10 min. The protein concentration of the supernatant was measured by Bradford assay (Bio-Rad) and adjusted to a final concentration of 1 µg/µL prior to affinity chromatography. 50 µL of agarose affinity matrix conjugated compound was washed with 1 mL bead buffer (10 mM Tris, pH 7.4; 5 mM NaF; 250 mM NaCl; 5 mM EDTA; 5 mM EGTA; 0.1 % Triton X-100 plus one tablet of protease inhibitor cocktail per 10 ml buffer). Matrices were incubated with 300 µL of 300 µg protein extract plus and identical volume of bead buffer at 4 ºC for 2 h, using a tube rotator set at 10 rpm. For studies of competition drug binding to a cellular target, the competitor was added to the mixture of protein extract/bead buffer and incubated at room temperature for

30~60 min prior to incubation with the matrix. The supernatant containing unbound proteins was removed by centrifugation and the matrices were washed seven times with 1 mL bead buffer. Proteins bound to the matrices were eluted by incubation with 80 μL 2X Laemmli buffer (Bio-Rad) at 95 °C for 3 min. Eluted proteins were separated by 10 % SDS-PAGE and visualized with a silver staining kit (GE Healthcare). Prominent protein bands specific to active matrices were excised from each gel and identified by MALDI-TOF mass spectrometry (Korea Research Institute of Standards and Science). The database used for target identification was IPI_human373 20100716 (89652 sequences; 35854403 residues), employing the Mascot search engine and MudPIT scoring with a significance threshold of $P<0.05$.

Actin polymerization assay. A fluorescence based assay with rabbit skeletal muscle actin (Cytoskeleton, Inc.) was used to determine the effect of compound of interest on actin polymerization. Fluorescence was measured using a microplate reader (SpectraMAX Gemini XS, Molecular Devices; λ_{ex}=350 nm λ_{ex}=410 nm).

Western blot analysis. Proteins were separated by 10% SDS-PAGE and transferred onto 0.2 μm nitrocellulose (Bio-Rad). Densitometrc analysis of band intensity was carried out using Image J 1.54s software (National Institutes of Health, USA).

siRNA-mediated gene silencing. Cells were seeded in 6-well plates at a cell density of 1.5 x 10^6 cells/well, after sieving through a 40 μm mesh (BD Biosciences). 24 h after seeding, cells were transfected with siRNA, following the manufacturer's protocol (Santa Cruz Biotechnology). Cells were used for experiments 48 h post-transfection. Enolase expression knockdown by siRNA treatment was confirmed by western blotting (supplementary information figure 10).

Reverse transcription polymerase chain reaction analysis of mRNA expression. Cells were treated with drug of interest in a 6-well culture plates. To induce PEPCK expression, cells were treated with 1 μM dexamethasone for 24 h, as previously described.(*8*) Total RNA was isolated with the TRI-Solution™ (Sigma), in accordance with the manufacturer's instructions. RT-PCR was performed with AccuPower® RT PreMix (purchased from Bioneer) in accordance with the manufacturer's instructions. 1 μg of mRNA was used as a template for each RT-PCR. cDNA was amplified using the AccuPower® PCR PreMix (purchased from Bioneer), employing the following reaction conditions for each gene of interest:

1) PEPCK RT-PCR: melting at 94℃ for 1 min, annealing at 60 ℃ for 30 s and extension at 72 ℃ for 10 s (35 cycles).
2) AMPK RT-PCR: melting at 92℃ for 3 min, annealing at 59 ℃ for 30 s and extension at 72 ℃ for 30 s (10 cycles); followed by melting at 92℃ for 3 min, annealing at 60 ℃ for 30 s and extension at 72 ℃ for 30 s (10 cycles); followed by melting at 92℃ for 3 min, annealing at 59 ℃ for 30 s and extension at 61 ℃ for 30 s (20 cycles).
3) G6Pase and cyclophilin RT-PCR: melting at 94℃ for 1 min, annealing 59 ℃ for 30 , and extension at 72 ℃ for 30 s (35cycles).

The primer sequence of each gene was as follows:

1) Human AMPK: forward 5'- AAAGAAAGTCGGCGTCTGTTCC-3'; reverse 5'-CTTCTGGTGCAGCATAGTTGGG-3' (211 bp product).
2) Human PEPCK: forward 5'-CCCAGGGTGCATGAAAGGTC-3'; reverse 5'-CGTACCCACTGCCAAAGGAGA-3' (317 bp product).

3) Human G6Pase: forward 5'-GACGGATGCAGAAGGAGATGG-3'; reverse 5'-CTCACCTTCTCCCACCTTCACC-3' (296 bp product).
4) Human cyclophilin: forward 5'-GGCAAATGCTGGACCCAACACAA-3'; reverse 5'-CTAGGCATGGGAGGGAACAAGGA-3' (355 bp product).
5) Zebrafish PEPCK: forward 5' ACC AAC CTG GCC ATG CTG AAG C 3'; reverse 5' CGA AGA TGA TGG CCT CGA TGG G 3' (387 bp product).

Cancer cell invasion assay and migration assay. To evaluate invasive activity, we used a modified transwell invasion assay.(*9*) Using 24-transwell plates (Corning), the inserts containing 8 μm pore size filters were coated with collagen Type I (45 μg/30 μL/well) for invasion assay. Briefly, 2×10^4 /well CAF were seeded in the bottom wells for inducing cancer cell invasion. 24 h later, 1×10^5/well OSCC cells were placed in the upper transwell chambers with porous filters. The cancer cells that penetrated the filter were fixed, stained with 0.25% crystal violet. Cell invasion was quantified by counting the invaded cells, which were counted in five separate microscopic fields (100X) per filter, and the mean values per filter (±SD) were calculated from three replicate filters.

To evaluate cancer cell migration, transwells were coated with 50 μL of 50 μg/ml fibronectin solution for 12 hr and washed with cell grade PBS. Cells were treated with drug for 24 h before seeding on the transwell. To induce migration, 300μl DMEM containing 10% FBS and 1% P/S was added to the lower chamber. The cancer cells that penetrated the transwell were fixed with 3.7% formaldehyde for 1 h and stained with 0.25% crystal violet for 40 min. After PBS washing, the migration rate was quantified by counting the migrated cells in five separate 100X microscopic fields.

Zebrafish care and maintenance. Zebrafish were maintained in accordance with standard protocols.(*10*) Care and treatment of zebrafish were conducted following the guidelines established by the Animal Care and Ethics Committees of the Gwangju Institute of Science and Technology, Republic of Korea.

RNA isolation from drug-treated zebrafish. Adult fish were transferred to a T25 cm^2 cell culture flask containing 15ml E3 fish water. The water was aerated using a pneumatic pump. Drug of interest was added for 3 h and fish were then killed by transfer into iced water slurry. The liver was dissected under microscopy and chilled in ice-cold PBS. The liver was then transferred transfer into 15 mL PBS, centrifuged at 800 rpm for 5 min. 400 uL Triazole (Bio Science Technology) was added for 15 min and the liver was also manually disrupted using forceps. RNA was then isolated by following the Traizole manufacturer's instructions.

Toxicological assessment of ENOblock. Our protocol for toxicity analysis was based upon a previous report.(*11*) After spawning, 8-cell stage fertilized eggs at were incubated with compounds for 72 h in a 24 well plate containing 8 embryos per well. Embryos were maintained at 31°C. Compounds were added at the 8-cell stage (5/4 hpf) for 72 h. E3 water containing each compound was replenished daily. Larvae were checked at each 24 h time point after compound addition using stereo microscopy (Zeiss Stemi 2000-C) and the following developmental and anatomical parameters were monitored: 1) somite development, 2) tail detachment 3) development of otic vesicles, otoliths and eyes, 4) heartbeat, 5) circulation, 6) delayed hatching, 7) presence of skeletal deformities and 8) ability to swim. 50 larvae were incubated in identical conditions, without compound treatment, to check the initial mortality rate (IMR). After assessment of developmental and anatomical parameters, larvae were anaesthetized in E3 water containing 0.02 % tricaine for 10 min incubation and placed on glass microscope slides in plastic chambers (1.6 cm diameter and 3 mm depth) containing 3% methylcellulose

dissolved in E3 water. Images of treated larvae were taken with differential interference contrast (DIC) microscopy (DM2500, Leica DMRBE upright) equipped with a digital camera (DFC425C, Leica). Images were processed using the Lieca Application Suite software and Photoshop CS4 (Adobe Systems Incorporated).

Measurement of cellular glucose uptake. Cells were seeded in 96-well plates at a cell density of 5 x 10^3 cells/well in low glucose (5 mM) culture media. 24 h later, cells were treated with compound of interest for 24 h. Cells were then washed with PBS and further incubated with a 100 μL solution of test compound and 100 μM fluorescent tagged glucose (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG); Molecular probes) for 30 min at 37 ºC. After washing with PBS, cells were lysed by adding 100 μL potassium phosphate buffer (pH 10) containing 1% Triton X-100, with shaking for 10 min at room temperature, followed by addition of 50μL DMSO. 100 μL of the lysed solution was transferred to a black-walled 96-well microtiter plate, and fluorescence was measured in a fluorescence microplate reader (SpectraMAX Gemini XS, Molecular Devices; λ_{ex}=466 nm λ_{ex}=540 nm).

Induction and quantification of adipocyte formation. To induce adipogenesis, 3T3-L1 pre-adipocytes were seeded in a 6-well cell culture dishes at 70% confluence. 24 h later, culture media was changed to 10% FBS DMEM with an adipogenic cocktail (1 μg/ml insulin, 2 μM dexamethasone, and 0.5 μM IBMX) and incubated at 37ºC in 5% CO_2. 2 days later, the culture media was changed to 10% FBS DMEM with 1 μg/ml insulin for 8 days, plus compound of interest, and media was changed every two days.

After 3T3-L1 adipocyte preparation, cells were washed with PBS two times. Fixation was performed using 500 μL 3.7% paraformaldehyde solution (1 h at room temperature). Cells were

gently rinsed with distilled water one time and wash two times with PBS. After washing, 500 μL 60% isopropanol was added for 5 min and cells were stained with 500 μL Oil-Red-O working solution (0.2% Oil Red O in 60% isopropanol) for 15 min. Stained cells were washed with PBS. To measure lipid accumulation in the cell cytoplasm, stained cells were dissolved with 500 μL DMSO and 80 μL was transferred to a 96-well plate. Dissolved Oil-Red-O stained lipid intensity was measured with a microplate reader (Molecular Devices; λ=490 nm).

Induction of macrophage foam cells. THP-1 human monocytes were induced to form macrophages by 72 h treatment with 200 nM phorbol 12-myristate 13-acetate. Macrophage numbers were determined by counting cells that had attached to the culture dish, which is an indicator of monocyte differentiation into macrophages.(*12*) To induce foam cell formation, macrophages were treated with 5 mg/mL oleic acid complexed to bovine serum albumin (2:1 ratio; Sigma) for 48 h, as previously described.(*13*) For foam cell counting, Oil Red O stained cultures were photographed at X200 magnification and foam cells were defined as attached macrophage cells displaying lipid accumulation.

SUPPLEMENTARY TABLE 1:

Target identification data from the MALDI-TOF for compound AP-III-a4 (ENOblock)

Band number	Identification	Accession number	Matched signals	Sequence coverage	Mascot score
1	a) IGK@ IGK@ protein	IPI00784985	3	28.936%	184
	b) IGKC Anti-RhD monoclonal T125 kappa light chain	IPI00853045	3	21.795%	184
2	No proteins identified				
3	a) Putative uncharacterized protein ENSP00000382160	IPI00022434	5	8.534%	216
	b) Gene Symbol=ALB 23 kDa protein	IPI00878282	3	5.832%	179
4	No proteins identified				
5	No proteins identified				
6	a) Gene Symbol=ENO3 Isoform 1 of Beta-enolase	IPI00218474	2	11.06%	170
	b) Gene Symbol=ENO1 Isoform alpha-enolase of Alpha-enolase	IPI00465248	2	5.53%	160
	c) Gene Symbol=ENO2 Gamma-enolase	IPI00216171	2	5.53%	160
7	Gene Symbol=ENO2 Gamma-enolase	IPI00216171	2	4.148%	155
8	No proteins identified				
9	a) Gene Symbol=ACTA1 Actin, alpha skeletal muscle	IPI00021428	5	25.464%	146
	b) Gene Symbol=ACTG2 Actin, gamma-enteric smooth muscle	IPI00025416	5	23.936%	146
	c) LOC653269;POTEI;POTEM;LOC727848;POTEJ;POTEKP;POTEE;LOC653781 Isoform 1 of POTE ankyrin domain family member E	IPI00479743	6	11.721%	146
	d) POTEF POTE ankyrin domain family member F	IPI00739539	4	7.535%	146
	e) ACTBL2 Beta-actin-like protein 2	IPI00003269	3	14.628%	104
	f) LOC653269;POTEI;POTEM;LOC727848;POTEJ;POTEKP;POTEE;LOC653781 actin, beta-like 3	IPI00887316	6	27.711%	104
10	No proteins identified				

SUPPLEMENTARY SCHEME AND FIGURE LEGENDS

Supplementary scheme 1: Synthesis of the AP linker library. **a)** General scheme for orthogonal synthesis reagents and conditions: (a) R_1NH_2 or R_1NH_2OH (5 eq), 2% acetic acid in THF, rt, 1 hr, followed by $NaB(OAc)_3H$ (7 eq), rt, 12 hr. (b) Building block II (4 eq) in THF 60ºC, 1hr, DIEA. (d) R_2R_3'NH, DIEA, NMP:n-BuOH=1:1, 120ºC, 3 hr. (e) 10% TFA in dichloromethane, 30 min. **b)** Linker **c)** Amines and amino alcohols used for construction of building blocks I and **d)** Amines used for construction of building blocks III.

Supplementary Figure 1a-b: AP-III-A4 (ENOblock) NMR and HRMS data:

^{1}**H NMR** (500 MHz, $CDCl_3$): δ = 8.48 (s, 1H), 7.49 (d, *J* = 5.58 Hz, 2H), 7.27 (t, *J* = 7.98 Hz, 2 H), 7.17 (d, *J* = 7.92 Hz, 2H), 6.99 (t, *J* = 8.55 Hz, 2H), 6.75 (*br.* s, 2H), 5.56 (*br.* s, 3H), 4.52 (s, 2H), 3.61-3.31 (m, 12H), 3.21 (s, 2H), 2.95 (s, 2H), 1.81-1.46 (m, 6H), 1.18 (m, 3H), 0.94 (m, 2 H);

^{13}**C NMR** (125 MHz, $CDCl_3$): δ = 172.1, 164.3, 163.5, 160.3, 134.7, 129.5, 129.1, 129.0, 121.2, 120.5, 115.3, 115.0, 70.0, 69.9, 69.7, 67.1, 47.0, 43.8, 42.6, 39.3, 39.1, 37.9, 30.8, 26.3, 25.8;

HRMS (C31H43FN8O3): Calc. $[M+H]^+$: 595.3442, Found $[M+H]^+$: 595.3538

Supplementary figure 2: Target study for compound AP-III-a4. Proteins with significant Mascot scores above 100 are shown and peptides identified as keratins are not included. Note: IGK protein molecular weight is approximately 26 kD (http://www.uniprot.org/uniprot/Q6P5S8), whereas the molecular weight of Band 1 is greater than 100 kD. Thus, an IGK protein was discounted as a candidate protein for Band 1. Putative uncharacterized protein

ENSP00000382160 is a member of the Ensembl Family (Ensembl protein families), which are keratins (http://www.biomyn.de/?mid=A8MW45&ispc=UniProtKB&o=related). Thus, Ensembl Family was discounted as a candidate protein for Band 2. Band 3 is greater than 60 kD. Thus, ALB (human serum albumin) was discounted from our target study. Band 9 was identified as either an ankyrin domain family members or as actin. The molecular weight of POTE ankyrin domain family member E is121 kD (http://www.uniprot.org/uniprot/Q6S8J3) and the molecular weight of POTE ankyrin domain family member F is 121 kD (http://www.uniprot.org/uniprot/A5A3E0). Thus, ankyrin family members were discounted from our target study. An actin polymerization assay was carried out to determine if actin is a functional target of compound AP-III-a4 (ENOblock). It was found that this compound does not modulate actin ploymerization (supplementary information figure 3). Thus, actin was discounted from our target study.

Supplementary Figure 3: Compound AP-III-a4 (ENOblock) cannot modulate actin polymerization. A) The rates of actin polymerisation was not affected by treatment with 5 μM or 10 μM ENOblock.. B) Actin polymerisation was enhanced by treatment with 4 μM phalloidin, which reacts stoichiometrically with actin to promote polymerisation (Proc Natl Acad Sci USA. 1977 Dec;74(12):5613-7.). Treatment with 10 μM ENOblock did not affect polymerisation. C) Actin polymerisation was enhanced by treatment with 1 μM cytochalasin D, which shortens actin filaments by blocking monomer addition at the fast-growing end of polymers (Biochem Pharmacol. 1994 May 18;47(10):1875-81.). In contrast, treatment with 10 μM ENOblock did not affect actin polymerisation

Supplementary Figure 4: ENOblock inhibited the activity of purified enolase in a concentration-dependent manner. The IC_{50} for enolase inhibition by ENOblock was calculated as 0.576 mM. Error=SD.

Supplementary Figure 5: siRNA-mediated knockdown of enolase expression in HCT116 cancer cells increased susceptibility to hypoxia. Cells were treated with increasing concentrations of enolase (ENO1) siRNA or two types of negative control siRNA: 1) 80 pmols scrambled or 2) 80 pmols p57 (a cyclin dependent kinase inhibitor). 24 h post-transfection with siRNA, cells were transferred to a 96-well culture plate at a density of 10^4 cells/well and, 24 h later, cultured in normoxia or exposed to hypoxia by treatment with 150 mM $CoCl_2$. Cytotoxicity was assessed by MTT assay 24 h later. Increasing concentrations of ENO1 siRNA induced dose-dependent cancer cell death under hypoxia. Error=SD; *=P<0.05 for increased cytotoxicity under hypoxia compared to normoxia.

Supplementary Figure 6: ENOblock treatment of HCT116 cells under hypoxia for 24 h induced cell death, as measured by counting cells that were stained for trypan blue. Staurosporine, a known inducer of apoptosis(J Biomol Screen. 2008 Jan;13(1):1-8.), was used as a positive control. Error=SD; *=*P*<0.05 compared to untreated cells.

Supplementary Figure 7: Micrographs of crystal violet stained transwell inserts show that treatment with 10 μM compound AP-I-f10 does not reduce HCT116 colon cancer cell invasion. Treatment with 5 μM LY294002 was used as a positive control and produced a noticeable reduction in invaded cancer cells (scale bar=100 μm).

Supplementary Figure 8: ENOblock can reduce PEPCK protein expression. A) Treatment of HEK kidney cells with 10 μM ENOblock (abbreviated as ENO) for 48h reduced PEPCK expression. 48 h treatment with 10 μM GAPDS or 10 μM rosiglitazone (abbreviated as ROSI) did not reduce PEPCK expression. B) Treatment of Huh7 hepatocytes with 10 μM ENOblock or 10 μM rosiglitazone for 48 h reduced PEPCK expression. 48 h treatment with 10 μM GAPDS did not reduce PEPCK expression. Fold-change in PEPCK expression was calculated using densitometry from three independent western blot experiments. Error=SD; *=$P<0.05$ compared to the untreated group.

Supplementary figure 9: Treatment of HCT116 cancer cells with 150 μM $CoCl_2$ for 4 h produced a hypoxic environment, as shown by increased expression of the hypoxia-responsive gene, enolase. In contrast, a different protein from the glycolysis pathway, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) did not show increased expression after inducing hypoxia, which is consistent with previous reports (Said, *et al*, BMC Mol Biol. 2007 Jun 27;8:55.). Red numbers are densitometry analysis to calculate the fold-change in expression.

Supplementary figure 10: Immunoblot analysis to confirm enolase expression knockdown via siRNA treatment in HEK kidney cells and Huh7 hepatocytes Cells were transfected with 80 pmols siRNA for 48 h.

REFERENCES FOR SUPPLEMENTARY INFORMATION

1. Khersonsky, S. M., Jung, D. W., Kang, T. W., Walsh, D. P., Moon, H. S., Jo, H., Jacobson, E. M., Shetty, V., Neubert, T. A., and Chang, Y. T. (2003) Facilitated forward chemical genetics using a tagged triazine library and zebrafish embryo screening, *J Am Chem Soc 125*, 11804-11805.
2. Lee, L. L., Ha, H., Chang, Y. T., and DeLisa, M. P. (2009) Discovery of amyloid-beta aggregation inhibitors using an engineered assay for intracellular protein folding and solubility, *Protein Sci 18*, 277-286.
3. Piret, J. P., Mottet, D., Raes, M., and Michiels, C. (2002) CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2, *Ann N Y Acad Sci 973*, 443-447.
4. Maher, J. C., Krishan, A., and Lampidis, T. J. (2004) Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions, *Cancer Chemother Pharmacol 53*, 116-122.
5. Sedoris, K. C., Thomas, S. D., and Miller, D. M. (2010) Hypoxia induces differential translation of enolase/MBP-1, *BMC Cancer 10*, 157.
6. Gorsich, S. W., Barrows, V., Halbert, J., and Farrar, W. W. (1999) Purification and properties of gammagamma-enolase from pig brain, *J Protein Chem 18*, 103-115.
7. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J Immunol Methods 65*, 55-63.
8. Elo, B., Villano, C. M., Govorko, D., and White, L. A. (2007) Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds, *J Mol Endocrinol 38*, 433-440.

9. Youngs, S. J., Ali, S. A., Taub, D. D., and Rees, R. C. (1997) Chemokines induce migrational responses in human breast carcinoma cell lines, *Int J Cancer 71*, 257-266.

10. Nusslein-Volhard, C., and Dahm, R. (2002) *Zebrafish: A Practical Approach (Practical Approach Series)*, OUP, Oxford.

11. Selderslaghs, I. W., Blust, R., and Witters, H. E. (2012) Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds, *Reprod Toxicol 33*, 142-154.

12. Theus, S. A., Cave, M. D., and Eisenach, K. D. (2004) Activated THP-1 cells: an attractive model for the assessment of intracellular growth rates of Mycobacterium tuberculosis isolates, *Infect Immun 72*, 1169-1173.

13. Schnoor, M., Buers, I., Sietmann, A., Brodde, M. F., Hofnagel, O., Robenek, H., and Lorkowski, S. (2009) Efficient non-viral transfection of THP-1 cells, *J Immunol Methods 344*, 109-115.