Supplemental Information

Materials and Methods

Reagents

ACS-1 and ACS-2 were synthesized as earlier described (12). D-biotin, DMSO, iodoacetic acid, 2-mercaptoethanol, sodium hydrosulfide and valproic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Okadaic acid was purchased from Millipore (Billerica, MA, USA). Streptavidin-Dynabeads, 4x-LDS buffer and Lipofectamine LTX transfection reagent were purchased from Invitrogen. Recombinant human matrix metalloproteinase-9 (MMP-9) and recombinant human tumor necrosis factor-alpha (TNF-α) were purchased from R&D Systems (Minneapolis, MN, USA) and recombinant human NF-κB p50 was purchased from Active Motif (Carlsbad, CA, USA). Antibodies to actin, HPRT and MMP-9 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to p-IKK-α/β (ser 176/180), IKK-β, IκBα, p-p65 (ser 536), p65 and tata binding protein (TBP) were from Cell Signaling (Danvers, MA, USA). Anti-p50 was purchased from Active Motif and anti-CD14 was from Abcam (Cambridge, MA, USA). The NF-κB-luciferase reporter construct was from Panomics (Santa Clara, CA, USA) and the pGL4.70UC-rluc control plasmid was from Promega (Madison, WI, USA).

Synthesis and characterization of 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl 5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl]pentanoate, ACS-92

Anhydrous dimethylformamide (DMF) and triethylamine were purchased from Sigma-Aldrich (Seelze, Germany); 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride, D-biotin and 1-hydroxybenzotriazole hydrate were purchased from Iris Biotech GmbH (Marktredwitz, Germany). A solution of 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ACS-1) (370 mg; 1.64

mmol) in anhydrous DMF (3 ml) was added dropwise to a solution of D-biotin (400 mg; 1.64 mmol), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (376 mg; 1.968 mmol), 1-hydroxybenzotriazole hydrate (300.7 mg; 1.968 mmol) and triethylamine (1.64 mmol; 0.23 ml) in anhydrous DMF (5 ml). The reaction mixture was stirred for 24 hours at room temperature under a nitrogen atmosphere. The resulting suspension was filtered and the solid residue was washed first with water and finally with diethyl ether. After drying, an orange powder (210 mg) was obtained. Yield: 28%. M.p. (Büchi apparatus): 190-192 °C. Purity was confirmed by HPLC equipped with diode array recorder (Hitachi L-2450); column PUROSPHER STAR RP-18 end capped, 4x250 mm, 5 um. Mobile phase: 25 mM acetate buffer (pH 5.80)/CH3CN (1:1); flow rate: 1.5 ml/min; oven at 40 °C. Analyses were recorded at 325 nm or 430 nm wavelength; ACS 92 was detected at 5.26 min (retention time). Chromatographic purity >97%. ^{1}H NMR (Varian Mercuri 300 VX, DMSO-d_6): = 7.95 (d, J = 8.21 Hz, 2H); 7.81 (s, 1H); 7.29 (d, J= 8.50, 2H); 6.45 (s, 1H); 6.36 (s, 1H); 4.29-4.13 (m, 2H); 3.11-3.02 (m, 1H); 2.83-2.79 (m, 1H); 2.60-2.54 (m, 3H); 1.66-1.39 (m, 6H). Elemental analysis for ACS 92 ($C_{19}H_{20}N_2O_3S_4$ + 0.5 H_2O) calculated %: C 49.43; H 4.58; N 6.07; found: C 49.78; H 4.75; N 6.24.

Genomic Sequence Analyses

The promoter sequence for each gene listed in Table 3 of Glynn et al. (2010) was extracted using ElDorado (version 12-2010) software. NF-κB binding site analysis was performed using the RegionMiner (Release 4.2) software. NF-κB binding site locations are represented as basepairs away from TSS and values indicate the center of the binding region. Both software are part of the commercially available Genomatix Software Suite (V2.1) (Genomatix Software, Inc, Ann Arbor, MI, USA).

Hydrogen sulfide detection

Hydrogen sulfide (H_2S) formation from ACS-1 was measured using a selective chemical potential electrode for H_2S (World Precision Instruments, Sarasota, FL, USA). ACS-1 was added to DMEM containing 10% FBS at a final concentration of 100 μM. The solution was rapidly stirred at 37°C and signal current, which corresponds to H_2S concentration was measured for 30 minutes. Data shown is a representative plot of picoamperes versus time.

Cell Viability and clonogenic survival

Cells were seeded in media containing 10% fetal bovine serum and incubated for 6 h to allow the cells to adhere. The cells are washed (3X) with serum-free media and serum-starved overnight. The cells were then incubated for 24 h in 10% FBS media containing the indicated concentration of ACS-1. Viability was measured as total cellular ATP levels determined by the CellTiter-Glo® luminescent cell viability assay (Promega). The data shown represent the normalized mean (± sem) from six replicate experiments. Clonogenic survival assay was performed as previously described (23).

NF-κB-luciferase assays

NF-κB transcriptional activity was performed by transiently transfecting cells with 750 ng of NF-κB-luciferase reporter plasmid expressing firefly luciferase and 250 ng pGL4.70 plasmid expressing renella luciferase using the Lipofectamine LTX reagent for 6 hours at 37°C. After transfection, cell culture media was replaced with serum-free DMEM containing TNF-α (20 ng/mL), ACS-1, ACS-2, valproic acid, sodium hydrosulfide or DETANO at the concentrations

indicated in the figures. Cells were incubated for 18 hours and luciferase activity was measured using the Dual-luciferase assay kit from Promega. Relative luminescent units (RLU) were measured using a Glomax 96-well plate luminometer (Promega) and data were normalized to fold change from untreated control cells. Data represent mean normalized RLU ± standard deviation.

Western blotting

Western blotting was performed as previously described (18) with minor modification. The lysis buffer used here was Tris-HCl pH 8.0 (50 mM) containing NaCl (150 mM), NP-40 (1%), EDTA (1 mM), NaF (50 mM), Na_3VO_4 (10 mM), PMSF (1 mM) and protease inhibitor cocktail (Calbiochem). Nuclear proteins were isolated using a commercial Nuclear Extract kit (Active Motif) as instructed by manufacturer.

Electrophoresis Mobility Shift Assay

MDA-MB-468 cells grown RPMI + 10% FBS were replaced with serum-free RPMI and TNF-α, ACS-1, ACS-2, ACS-92, biotin and NaSH were added at the indicated concentrations and incubated for 3 hours. Control cells were similarly incubated in serum-free RPMI for 3 hours. Nuclear fractions were harvested and protein content measured using the BCA assay. Nuclear protein (4 µg) was incubated in binding buffer containing Poly d(I-C) (1 µg) for 5 minutes at room temperature. Biotinylated-oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') (10 µg) was added and incubated for 30 minutes at 15°C. Unlabeled probe competition samples were prepared in the same manner including 660 ng of unlabeled oligonucleotide. Loading dye was added after incubation and samples were separated on a non-denaturing gel.

Gels were transferred onto nylon membranes and blocked with BSA in phosphate-buffered saline. Membranes were then incubated with HRP-conjugated streptavidin and oligonucleotide probes were detected by chemiluminescent assay.

Recombinant NF-kB subunit-DNA Binding assay

Recombinant human p50 and p65 were supplied in 20 mM Tris-HCl, 0.2 M NaCl, 2 mM $MgCl_2$, 10% glycerol and 1 M DTT. Prior to reaction with ACS-1, the buffer was exchanged using 10,000 MW cut-off centrifugal filters (Millipore) and recombinant protein was resuspended in tris-buffered saline containing 1 mM EDTA and 2 mM $MgCl_2$. ACS-1 was immediately added to protein aliquots and incubated at room temperature for 60 minutes. Protein binding to NF-κB consensus sequence was measured using an ELISA-based assay (NF-κB TransAm kit, Active Motif) as instructed by manufacturer.

ACS-92 Protein pull-down

MDA-MB-231 whole cell lysate was prepared as above and incubated with either biotin or ACS-92 (100 μM) for 30 minutes at room temperature with gentle agitation. Similarly, human recombinant pro-MMP-9 (R&D Systems) was diluted in TCNB buffer (1 μg/100 μL) and incubated with biotin or ACS-92 (25 μM). Streptavidin-beads were added to samples and incubated for 30 minutes. Beads were washed three times in phosphate buffered saline or TCNB buffer and bound proteins were prepared for western blot analysis by heating beads to 90°C for 10 minutes in 4X LDS buffer (Invitrogen) with or without 5% 2-ME.

MMP-9 activity

The effect of ACS-1 treatment on MMP-9 activity was measured by gel zymography as previously described (24). Conditioned medium from MDA-MB-231 cells were treated with ACS-1 for 24 hours were normalized according to protein content and electrophoresed on 10% gelatin zymogram gels. The gels were washed and incubated in renaturing and developing buffers (Invitrogen) according to the recommendation of the manufacturer and then stained. Gels were imaged and enzyme activity was quantified by densitometry.

IL-6, IL-8, uPA & VEGF measurements

Cells were seeded into 24-well plates (50,000 per well) and grown overnight in 0.5 mL DMEM + 10% FBS. Media was removed and cells were gently washed in PBS before incubating 24 hours in serum-free DMEM containing ACS-1. The conditioned media was removed and cleared of cellular debris by centrifugation. IL-6, IL-8, uPA and VEGF were measured by commercial ELISA kits (R&D Systems) as instructed by the manufacturer.

Cellular Migration and Invasion

Cellular migration and invasion assays were performed as previously described (8). Briefly, MDA-MB-231 cells were seeded into the top chamber of transwell plates with 8 μm pores for migration or transwell plates with a thin film of matrigel for invasion (BD Biosciences, San Jose, CA, USA) in serum-free DMEM containing the indicated concentration of ACS-1 and allowed to migrate or invade towards DMEM containing 5% FBS for 24 hours. Cells were then fixed in cold methanol, stained with crystal violet, rinsed and counted. Data is expressed as the mean percentage of migrating/invading cells relative to untreated controls (± sd).

Matrigel outgrowth assay

Cellular proliferation, migration and invasion were measured using the matrigel outgrowth assay as previously described (25). Briefly, a matrigel matrix was formed in 24-well plates and MDA-MB-231 cells (10,000 cells in 100 μL) were seeded onto the matrix. The cells were then covered with another layer of matrigel. After the top layer of matrigel formed a matrix, DMEM containing 10% FBS and the indicated concentration of ACS-1 were added to the wells. Cells were incubated at 37°C for 14 days and the media was replenished every 3 days. Cellular outgrowth was photographed at 10X magnification.

Animals

Female athymic nude (NCr) nu/nu mice were housed in a sterile environment and given *ad libitum* water and standard chow. All animal procedures were performed in accordance with NIH guidelines and regulation and were approved by NCI Animal Care and Use Committee. Xenografts were implanted by injecting the fourth inguinal mammary fat pads with $2e^6$ MDA-MB-231-GFP cells suspended in 50% matrigel. Tumors were grown for 1 week prior to drug administration. Tumor volumes were measured weekly and final gross tumor volumes are shown as mean volume (± sem). ACS-2 prepared in DMSO (20 mg/kg) or DMSO were administered via interperitoneal injections (20 μL per dose).

Tumor xenograft analyses

Tumor GFP fluorescence in anesthetized mice were measured after 5 weeks of treatment using a Xenogen ISIS imaging system. Tumors were resected from euthanized mice and divided into two portions. One portion was preserved in formalin and 8 μm slices were prepared onto glass slides.

H&E staining was performed by American HistoLabs, Inc. and images were captured using a Nikon inverted microscope. Histopathology examination services were provided by the Histology/Pathology Laboratory (HPL), NCI-Frederick and the Histo-Scientific Research Laboratories, Inc (HSRL). The remaining resected tumors were homogenized for protein analysis. IL-6, IL-8, VEGF and uPA concentrations were measured by ELISA as described above. GFP fluorescence of homogenized tumors was measured (ex 492/em 515 nm) and normalized to micrograms of total protein analyzed. Protein content was measured by BCA assay (Thermo Scientific, Rockford, IL, USA) and protein content determined from BSA standards.