Synthesis of ^{64}Cu-ATSM

In vitro binding of ^{64}Cu-ATSM was determined using tracer synthesized in-house according to previously published methods (*1*). Briefly, ATSM stock solution was prepared by dissolving 1 mg of ATSM powder in 1 mL of dimethyl sulfoxide (DMSO). For radiolabeling, 10-20 μL of ATSM stock solution (10-20 μg) to 37-74 MBq of ^{64}Cu with 200 μL of 3M HCl aqueous acid as a buffer. The mixture was stirred (1000 rpm) for 5 min at room temperature. The product was purified using a C18 light Sep-Pack (Waters Corporation), which was pre-conditioned with ethanol and distilled water (Milli-Q water filtration system, 18 MΩ/cm2, Millipore Corp.). Excess water was passed through the column to remove any unreacted ^{64}Cu and DMSO. The purified radiolabeled complex was then eluted with ethanol (100%, Sigma). Purity was determined with thin-layer chromatography (TLC). Silica Gel plates (60 F254, 10 x 5 cm, EM Science) were used as the stationary phase and eluted with ethyl acetate (Fisher Scientific) as the mobile phase. Radiochemical yield was 75-85% and purity above 95%.

^{64}Cu-ATSM Uptake In Vivo

All *in vivo* studies were conducted according to protocols approved by the Washington University Division of Comparative Medicine and the Institutional Animal Care and Use Committee. Animals were housed 3-5 per cage, with free access to food and water and a 12-hour light/dark cycle.

Mice with orthotopic tumors were used for imaging experiments. Orthotopic tumors were implanted in 6-8 week old female nude mice (Charles River Laboratories) as follows. First, mice were anesthetized with a ketamine/xylazine cocktail and 3-4 million luciferase-expressing SiHa (n=4) or ME180 (n=5) cells were slowly injected intravaginally into the cervix in 30 μL of a serum-free media/matrigel mixture. Implantation of tumors was confirmed with bioluminescent imaging on a PerkinElmer IVIS50 imaging system 1-and 2-weeks following implantation.

Mice with xenograft tumors were used for bio-distribution experiments following redox active drug treatment. Xenograft tumors were likewise implanted in 6-8 week old female nude mice. After being anesthetized ketamine/xylazine, 3-4 million tumor cells were injected slowly in 30 μl of a serum-free media/Matrigel mixture subcutaneously into the flank using a 27-guage tuberculin syringe.

Mice with orthotopic tumors underwent a series of imaging experiments. Magnetic resonance imaging (MRI) experiments were performed 3, 4, and 5 weeks post-implantation, and PET/CT data with ^{64}Cu-ATSM were collected 4 weeks post-implantation. MRI was performed on an Agilent/Varian DirectDrive 4.7T small-animal MRI, with a 2.5 cm quadrature birdcage RF coil. Animals were placed supine within the coil and secured in an animal tray with tape. Animals were scanned using with T1-, T2-, and diffusion-weighted sequences. Animals were anesthetized with isoflurane in 100% O_2 (1-3%, v:v) at 1 L/min, and kept warm using heated air. All CT scans were performed on an Inveon microCT scanner (Siemens Healthcare), and PET scanning was performed on either an Inveon microPET (Siemens Healthcare), or a Focus 220 (Siemens Healthcare). PET and CT imaging experiments were performed with a custom-built imaging chamber that holds 4 mice. PET imaging was performed from 0-60 minutes following tail vein injection of ^{64}Cu-ATSM (3.92 ± 0.11 MBq). For the ^{64}Cu-ATSM imaging, isoflurane was mixed with 21% O_2 at 1 L/min . Animals were kept warm during tail vein cannulation with a heating pad, and during PET/CT scanning with a heating lamp. Following the final MRI, animals were sacrificed and tumors were harvested, weighed, measured, and flash frozen.

PET images were reconstructed using the scanners' commercial algorithm, (ordered subset expectation maximization (OSEM)-3D/*Maximum a posteriori Probability* (MAP)) with scatter correction, using the CT for attenuation correction. Images were reconstructed to a 128x128 matrix. The dynamic data acquired form 0-60 minutes were initially reconstructed into the following image time frames: 1x3 seconds, 6x2 seconds, 9x5seconds, 6x10 seconds, 4x30 seconds, 2x60 seconds, 2x120 seconds, 10x300 seconds. After examining the dynamic data, the final 20 minutes of the scans were summed into a single

image for analysis. Image analysis was performed using the Inveon Research Workplace software (Siemens Healthcare). Tumor locations were determined using the combination of the T2W and diffusion-weighted MRI, and the fused PET/CT. Volumes of interest (VOIs) were drawn on the PET images for the cervical tumor, musculature of the right thigh, heart, and liver. Tumor volumes were defined using a 40% of the maximum SUV threshold, using the MRI and fused CT to edit the volume off of surrounding tissues (e.g. bowel and rectum) if needed. Tracer activity was examined from 0-60 minutes, and the ratio of tracer activity between tumor/muscle was measured between 40-60 minutes. The mean tumor standardized uptake value (SUV; tumor activity (Bq/mL) * animal mass (g) / injected activity (Bq)) was also determined.

Mice with xenograft tumors were treated with β-lapachone or vehicle starting 4-5 weeks after tumor implantation. Animals were treated with β-lapachone (30 mg/kg) via intraperitoneal injection on 5 consecutive days prior to and including the day of sacrifice for bio-distribution.

Biodistribution of ^{64}Cu-ATSM was performed on all animals with xenograft tumors. Animals were anesthetized using isoflurane and room air and then injected with ~20 μCi of ^{64}Cu-ATSM via tail vein. After an uptake period of 90 minutes, animals were sacrificed and blood, heart, liver, muscle, and tumor were harvested, weighed, and radioactivity was counted in a Beckman 8000 gamma counter. Tumors were rinsed in 4ºC PBS immediately after harvesting and then flash frozen.

Measurement of Redox Metabolites by LC/MS-based Metabolomics

Intracellular levels of NADH/NAD+ and NADPH/NADP+ were determined using mass spectrometry for both *in vitro* cell samples and animal tumor samples. Cells were grown in 100-mm dishes to 90% confluence. For each condition four dishes were cultured and one reserved for cell counting. Cells were washed twice with cold phosphate-buffered saline (PBS) and extracted as previously described (*2*).

Tumors were frozen in liquid nitrogen and pulverized with a mortar and pestle. Pulverized tumors were weighed for normalization using an analytical balance. Tumors were extracted by the same method used to extract cells (*2*). Briefly, tumors (or cells) were put on dry ice and treated with 460 μL 2:2:1 acetonitrile:methanol:water with 0.1 M formic acid for 5 minutes. The final volume was brought to 500 μL by adding 2 M ammonium bicarbonate and adjusting the pH to 8. Samples were subjected to freeze-thaw cycles, vortexing, and sonication prior to being centrifuged at 15,000g for 30 minutes at 4 ºC. Finally, 50 μL of supernatant was transferred to LC/MS vials for analysis.

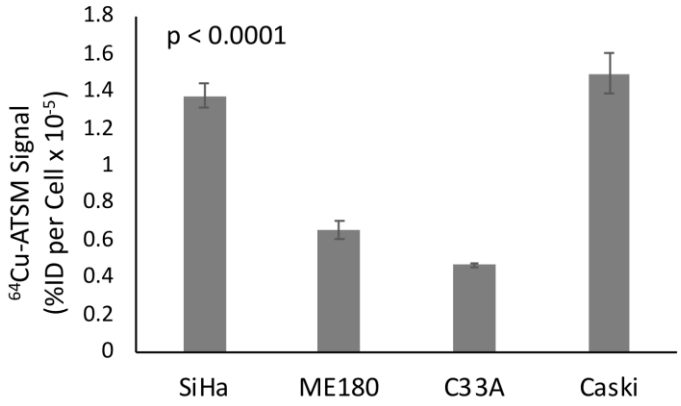
Absolute quantitation of NAD(P)H was performed on an Agilent 6460 triple-quadruple mass spectrometer with an electrospray ionization source coupled to an Agilent 1290 UPLC system. Metabolite separation was achieved by using an aminopropyl column (100 mm x 2 mm, 3 μm, 100 Å, Phemomenex) in hydrophilic interaction liquid chromatography mode. Solvent A was 95:5 water:acetonitrile with 10 mM ammonium hydroxide and 10 mM ammonium acetate. Solvent B was 95:5 acetonitrile:water LC separation was accomplished at a flow rate of 200 μL/min and a total gradient length of 20 min. The linear gradient was as follows: 0 min, 95% B; 1 min, 95% B; 5 min, 30% B; 15 min, 0% B; 20 min, 0% B. Metabolite levels were measured by selective reaction monitoring. For NADPH, the quantifier ion transition m/z 371.5->371.5 was used with a fragmentor voltage of 80 V and a collision energy of 0 V and the qualifier ion transitions m/z 371.5->304.0 and 371.5->134.0 were used with a fragmentor voltage of 80 V and a collision energy of 20 V. For NADH, the quantifier ion transition m/z 664.0->664.0 were used with a fragment voltage of 190 V and a collision energy of 0 V and qualifier ion transitions m/z 664.0->408.0 and m/z 664.0 -> 79.0 were used with a fragmentor voltage of 190 V and a collision energy of 30 V. For NAD+, the quantifier ion transition m/z 662.0->662.0 were used with a fragment voltage of 110 V and a collision energy of 0 V and qualifier ion transitions m/z 662.0->540.0 and m/z 662.0 -> 273.0 were used with a fragmentor voltage of 110 V and a collision energy of 40 V. For NADP+, the quantifier ion transition m/z 742.0->742.0 were used with a fragment voltage of 100 V and a collision energy of 0 V

and qualifier ion transitions m/z 742.0->619.0 and m/z 742.0 -> 408.0 were used with a fragmentor voltage of 100 V and a collision energy of 30 V. Absolute quantitation was accomplished using a standard curve and commercial standards obtained from Sigma Aldrich. Samples were normalized by extraction volume and tumor weight, or cell number as appropriate.

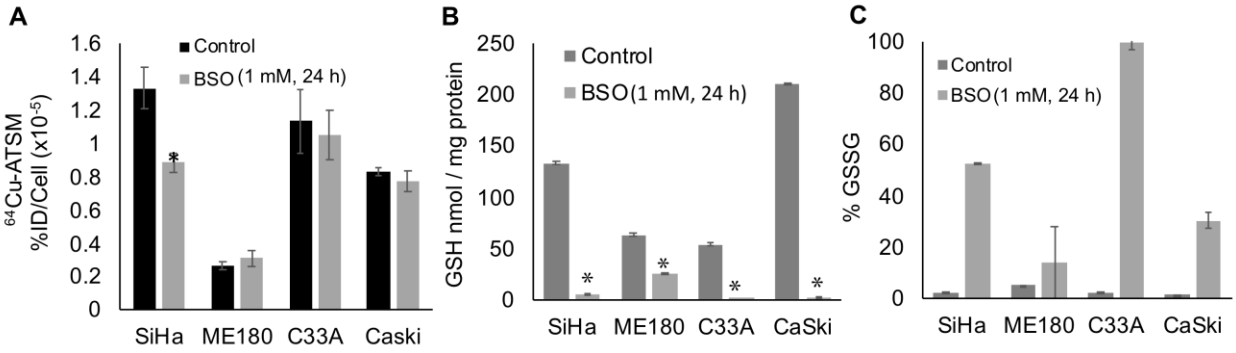
1. Nie X, Randolph GJ, Elvington A, et al. Imaging of hypoxia in mouse atherosclerotic plaques with (64)Cu-ATSM. *Nucl Med Biol.* 2016;43:534-542.

2. Llufrio EM, Wang L, Naser FJ, Patti GJ. Sorting cells alters their redox state and cellular metabolome. *Redox Biol.* 2018;16:381-387.

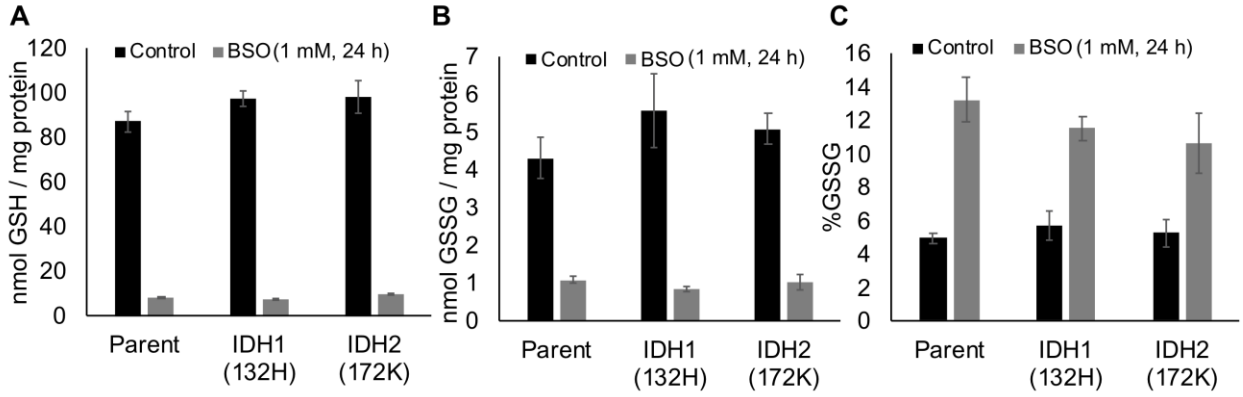
Supplemental Figures



Supplemental Figure 1. Baseline ^{64}Cu-ATSM uptake under normoxic conditions across the four cervical cancer cell lines studied ($p < 0.001$ by ANOVA).



Supplemental Figure 2. Impact of altering glutathione metabolism on ^{64}Cu-ATSM signal. Interfering with glutathione metabolism using BSO (1 mM, 24h) significantly decreased ^{64}Cu-ATSM signal in SiHa cells (A), but changes in reduced glutathione concentration (GSH) (B), and percent oxidized glutathione (C) were seen across all cell lines.



Supplemental Figure 3. Impact of treatment with buthionine sulfoximine (BSO, 1 mM for 24 hours) on levels of glutathione in HCT116 parent and IDH mutant cell lines. BSO significantly decreased levels of reduced glutathione (GSH) (A), and oxidized glutathione (GSSG) (B), and increased the %GSSG (C). (*, $p < 0.05$ relative to controls).