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SUPPLEMENTARY INFORMATION

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Inhibition of human copper trafficking by a small molecule significantly attenuates cancer cell proliferation

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Contents

Supplementary Methods

Expression and Purification of Atox1 and Full Length CCS

The *E. coli* strain BL21 was transformed with pET28a-Atox1 and full length CCS or mutants of Atox1 and full length CCS, cultured in LB medium containing 50 mg/mL kanamycin at 37 ºC to an absorbance of 0.6 at 600 nm, and induced with 1 mM IPTG for 16 hours at 16 ºC before being harvested by centrifugation. The cell pellets were suspended in lysis buffer (10 mM Tris, pH 7.5, 200 mM NaCl, 1 mM DTT) and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-NTA column and proteins were eluted with elution buffer (10 mM Tris, pH 7.5, 200 mM NaCl, 1 mM DTT, and 400 mM Imidazole). The 6-His-tag was removed by digestion with thrombin. The samples were exchanged and further purified with the buffer using size-exclusion chromatography (S200 Sephacryl column, GE) in 50 mM HEPES, 200 mM NaCl and 1 mM DTT. Fractions containing the protein were

analyzed using SDS-PAGE and fractions showing a single band corresponding to the expected molecular weight were pooled, resulting in >95% pure protein samples.

Expression and Purification of eCALWY3

The protein was expressed in *E. coli* strain BL21 and purified according to the published method. Expression was induced using 0.1 mM IPTG, and bacteria were subsequently grown at 16 ºC for 16 h. Lysis of bacteria was obtained by sonication and the soluble protein fraction was purified using nickel affinity chromatography. The 6-His−tag was subsequently removed by digestion with thrombin and the protein was purified with a second, additional purification step using size-exclusion chromatography (S200 Sephacryl column, GE) in 50 mM Tris, 100 mM NaCl, and 1 mM DTT, pH 7.5. Fractions containing protein were analyzed using SDS-PAGE and fractions showing a single band corresponding to the expected molecular weight were pooled, resulting in >95% pure protein samples.

Virtual Screening for Compounds that Bind the Copper Transfer Interface of Atox1

A hierarchical virtual screening strategy was adopted to identify hit compounds targeting the copper transfer interface of Atox1. DOCK4.0 was used for initial screening on a Specs database that contains more than 200,000 commercial available compounds. The Atox1 crystal structure (PDB entry: 1FEE) was used as the docking receptor and residues in the copper transfer interface was defined as the binding site. Standard DOCK scoring function was used to rank the result list and the CSCORE module of SYBYL rescored the top ranked 10,125 candidates. Autodock 4.0 further evaluated the compounds with the consensus score of 4 or 5. Per estimates of empirical binding free energy generated by Autodock 4.0, 301 compounds were chosen and then structurally clustered using Pipeline Pilot 7.5. Lastly, 127 compounds were selected based on their structure features, physical chemistry properties, drug-like characters, and scaffold diversity and subjected to the FRET assay.

Based on the structures of the initial hits identified by the first round assays, we conducted 2D similarity search and scaffold hopping using FP2 and SHAFTS methods implemented in ChemMapper web server. After water-solubility prediction

and structural cluster of the 520 newly screened hits, 110 candidates were purchased for the second round of FRET assay which identified six hits that inhibit Atox1.

Compounds Stock

All compounds are soluble in DMSO and stock solutions of 50 mM were made before dilution with water in various assays. The concentration of DMSO is less to 5% for all *in vitro* assay buffers.

eCALWY3 FRET Measurement

FRET with eCALWY3 (1 μM) was performed in 150 mM HEPES, 100 mM NaCl, 1 mM DTT and 10% glycerol (pH 7.1). Zn^{2+} titration was performed by mixing 0.9 mM of Zn^{2+} from a slightly acidic stock solution of $ZnCl_2$ with buffering systems consisting of 1 mM DHPTA. Subsequently, we treated the FRET probe with 100 μM of small molecules identified from the computational screen. The volume of DMSO was less than 5 μL in 200 μL HEPES buffer. Fluorescence spectra were recorded on a Varian Cary Eclipse spectrometer. Protein concentration was determined by measuring the citrine absorbance at 515 nm using an extinction coefficient of 77,000 $M^{-1}cm^{-1}$. The eCALWY3 excitation is at 433 nm and the Citrine/Cerulean emission ration was calculated by dividing the emissions at 527 nm and 475 nm, respectively.

Surface Plasmon Resonance (SPR) Assay

SPR technology-based binding assays were performed on Biacore T200 instrument (GE Healthcare) at 25 ºC with running buffer HBS-EP (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20) at 25 ºC. A portion of Atox1 (5 μM) or full length CCS protein (1 μM) was covalently immobilized on a CM5 chip using a standard amine-coupling procedure in 10 mM sodium acetate (pH 5.0). The chip was first equilibrated with HBS-EP buffer overnight. DC_CA50 was serially diluted and injected at a flow rate of 30 μL/min for 120 s (contact phase) followed by 120 s (dissociation phase). The concentration of DMSO is less than 5%. The KD value of DC_CA50 was determined using Biacore T200 evaluation software (GE Healthcare).

Fluorescence Anisotropy (FA) Measurement of Kd

DC_AC50 is a self-fluorescence compound with excitation at 294 nm and 355 nm and emission at 494 nm. We measured the Kd of DC_AC50 (1 μM) to Atox and CCS in 50 mM HEPES, 200 mM NaCl, 1 mM DTT (pH 7.1). We applied excitation at 355 nm with titrations of varied concentrations of Atox1 and full length CCS (1-100 μM). Fluorescence emission at 494 nm was recorded. Results are represented as mean and s.e.m. of at least three independent experiments.

A FRET_Based Kd Measurement

DC_AC50 is a fluorescent compound with an excitation peak partially overlapping with emission peaks of Atox1/full length CCS and DC_AC50. The FERT assay was measured in 50 mM HEPES, 200 mM NaCl, 1 mM DTT (pH 7.1). Atox1 or full length CCS (1 μM) displayed the maximum fluorescent signal at 335 or 350 nm in the absence of DC_AC50. With the addition of DC_AC50, the peak at 335 or 350 nm, corresponding to the emission of Tyr or Trp, was reduced, whereas the emission of DC_AC50 at 494 nm was elevated (maximum 2.5% DMSO). From these fluorescence data, we were able to calculate the Kd values of the small molecule with these proteins. Results are represented as mean and s.e.m. of at least three independent experiments.

Cancer Cell Culture

H1299, MDA-MB231 and K562 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). 212LN cell lines were cultured in DMEM/Ham's F-12 50/50 mix medium in the presence of 10% FBS. 293T cells were cultured in Dulbecco Modified Eagle Medium (DMEM) with 10% FBS.

Generate Stable Cell Lines

Stable knockdown of endogenous Atox1 and CCS was achieved using lentiviral vector harboring shRNA construct that were purchased from Open Biosystems, Huntsville, AL. We used five lentiviral shRNAs for H1299 cells to make stable knock down cell lines and selected by antibiotic puromycin and confirmed by western blot. After that, we chose the best one and performed relevant knockdown assays. PLKO.1 is the name of the lentiviral vector as a control of stable knockdown assay. Stable knockdown of overexpressed Atox1 and CCS was achieved using transfection by lipofectamine 2000 under selection G418.

Cell Proliferation and Viability Assays

Cell proliferation assays were performed by seeding 5 x 10^4 cells in a 6-well plate and culturing the cells at 37 ºC in normoxia (5% CO2 and 95% air). Cell proliferation was determined by cell numbers recorded at 72 h after being seeded, and normalized to that of each of the cell lines at the starting time (t = 0 h). For cell viability assays, 5 x 10^4 cells were seeded in 6-well plates and incubated with increasing concentrations of Physcion or S3 at 37 ºC for indicated times. Relative cell viability at each experimental time point up to 72 h was determined using either CellTiter 96® Aqueous. One solution proliferation kit (Promega) or by trypan blue exclusion using a TC10 Automated Cell Counter (BioRad). The rescue assay with NAC (1 mM) and compound C (200 nM) were performed by cell counting in H1299 cells, which were normalized to the control vector cells without small molecule treatment. Results are represented as mean and s.e.m. of at least three independent experiments.

Xenograft Studies

Nude mice (nu/nu, male 6-8 week old, Charles River Laboratories) were subcutaneously injected with 20 x 10^6 H1299 cells or 10 x 10^6 K562 cell on their right flanks. For evaluation of DC_AC50 using xenograft mice, the molecule was administered daily by i.p. injection with a dose of 100 mg/kg from 6 days after subcutaneous injection of H1299 cells on the right flank of each mouse. Tumor growth was recorded by measurement of two perpendicular diameters of the tumors over a 3-week period using the formula $4\pi/3$ x $(\text{width}/2)^2$ x (length/2). The tumors were harvested and weighed at the experimental endpoint, and the masses of tumors (g) treated with vehicle control (DMSO) and DC_AC50 was compared by a two-tailed unpaired Student's test.

Briefly Description of Put-back (rescue) Assay

H1299-Atox1/CCS stable knockdown cells were seeded in the 6-well plates, then transfected with pCDNA3.0-Atox1/CCS after 24 h, respectively. 4 x 10^4 cells were reseeded in a 6-well plate after 24 hour transfection. For cell proliferation assays, cell numbers were recorded at 24, 48 and 72 h after reseeding. Relative cell numbers were

determined and normalized after treatment compared to PLKO.1 lentiviral vector control cells.

Cellular Thermal Shift Assay

In brief, H1299 cells were seeded into 10 cm plates. After 24 h, cells were treated with DMSO (2 µL) in cell media or 10 µM DC_AC50 (2 µL DMSO) for 12 h. Cells were harvested and spun down. For cellular thermal shift assay in H1299 tumor issue, the nude mice were subcutaneously injected with 10 x 10^6 H1299 cells. After the development of tumor, DMSO and DC_AC50 with two concentrations (10 mg/kg/day and 50 mg/kg/day) were injected for 7 day, and then collected tumor tissue. These cells and tumor tissue were suspended in lysis buffer and incubated on ice for 20 min. Cells were then centrifuged at 14,000 rpm for 15 min at 4 ºC. The cell suspension was placed into PCR tubes and heated for 3 min to 42-58 ºC of H1299 cells and 42-76 ºC of tumor tissue followed by cooling for 3 min at room temperature. The heated lysates were centrifuged at 14,000 rpm for 20 min at 4 ºC in order to separate soluble fractions from precipitates. The supernatants were transferred to new microtubes and analyzed by SDS-PAGE followed by western blot analysis. The results of H1299 cells were fitted using a sigmoidal (variables slope) curve fit in Graphpad Prism software and represented as mean and s.e.m. of at least three independent experiments.

Total Copper Content in H1299 Cells by ICP-MS detection

The total copper content of cells incubated for 12 h with media having 10 µM DC_AC50 or Atox1 and CCS siRNA transfection were determined using ICP-mass spectroscopy (Agilent, 7500). We collected 5 x 10^6 cells in 150µL PBS buffer for total Cu uptake experiments and acidified with 200 µL 70% nitric acid incubated 12 h at 80 ºC, diluted to 5 mL by H_2O, and used for analysis.

Measurement of ROS Production

Cells were treated with DMSO and DC_AC50 for 12 h and ROS generation was detected with DCFH-DA. Cells were incubated with 10 µM of DCFH-DA for 30 min at 37 ºC, washed twice with PBS, and immediately analyzed by a FACScan flow cytometer. The antioxidant NAC was treated with 3 mM concentration. Results are represented as mean and s.e.m. of at least three independent experiments.

Measurement of Intracellular ATP Production

An ATP bioluminescent somatic cell assay kit (Sigma) was used to measure intracellular ATP concentration. Cells were treated with DMSO and 10 μM DC_AC50 for 12 h and 1 x 10^6 cells were trypsinized and resuspended in ultrapure water. Luminescence was measured with a spectrofluometer (SPECTRA Max Gemini; Molecular Probe) immediately after the addition of ATP enzyme mix to cell suspension. Results are represented as mean and s.e.m. of at least three independent experiments.

^{14}C-lipid Synthesis Assays

^{14}C-lipids synthesized from ^{14}C-glucose were measured. Subconfluent cells were seeded on a 6-well plate and collect for 12 h. Cells were then incubated in complete medium spiked with 4 μCi/mL of D-[6-^{14}C]-glucose for 2 h, washed twice with PBS, and lipids were extracted by the addition of 500 μL hexane:isopropanol (3:2 v/v). Wells were washed with an additional 500 μL of hexane:isopropanol solution, and extracts were combined and air dried with heat. Extracted lipids were resuspended in 50 μL of chloroform, and subjected to scintillation counting. Scintillation counts were normalized with cell numbers counted by a microscope (x40). Results are represented as mean and s.e.m. of at least three independent experiments.

^{14}C-RNA Synthesis Assays

^{14}C-RNA synthesized from ^{14}C-glucose was measured. Subconfluent cells were seeded on a 6-well plate and collect for 12 h. Cells were then incubated in complete medium spiked with 4 μCi/mL of D-[U-^{14}C]-glucose for 2 h. RNA was then extracted using RNeasy columns (Qiagen) and ^{14}C-RNA was assayed by scintillation counter. ^{14}C counts for each sample were normalized by the amount of RNA. Results are represented as mean and s.e.m. of at least three independent experiments.

Glucose Utilization Assays

1 x 10^6 cells were plated onto a 6 cm dish one day prior to the assay. Media were replaced with phenol-red free RPMI with 1% FBS prior to continuous culture for 3 days. Medium samples were collected each day. Glucose concentrations in media were measured using a colormetric glucose assay kit (Biovision) and normalized with

cell numbers. Results are represented as mean and s.e.m. of at least three independent experiments.

Lactate Production Assay

Cellular lactate production was measured under normoxia with a fluorescence-based lactate assay kit (MBL). Phenol red-free RPMI medium without FBS was added to a 6-well plate of sub-confluent cells, and was incubated for 1 hour at 37 ºC. After incubation, 1 mL of media from each well was assessed using the lactate assay kit. A microscope (x40) was used to count cell numbers. Results are represented as mean and s.e.m. of at least three independent experiments.

Oxygen Consumption Assay

Oxygen consumption rates were measured with a Clark-type electrode equipped with a 782 oxygen meter (Strathkelvin Instruments). 1 x 10^7 cells were resuspended in RPMI 1640 medium with 10% FBS and placed inside a water-jacked chamber RC300 (Strathkelvin Instruments). Recording commenced immediately. Results are represented as mean and s.e.m. at least three independent experiments.

Relative Apoptosis Assay

H1299 cells were seeded in a 24-well plate (5 x 10^4 cells/well). After 24 h, cells were treated with 10 μM DC_AC50 or 100 nM Doxorubicin (DMSO 0.05%). After 12 h, cells were washed twice with PBS to and collected from 24-well plate into FACS tubes. The tubes were centrifuged at 1,500 rpm for 5 min and washed with 1 mL PBS. Cells were centrifuged again at 1,500 rpm for 5 min and prepared in mastermix (1x binding buffer 100 μL, Annexin V/FITC 5 μL and PI 3 μL/each sample). The resuspended cells in 100 μL mastermix were incubated for 15 min at room temperature in the dark, and tested by FACS to evaluate apoptotic cells. Results are represented as mean and s.e.m. of at least three independent experiments.

Caspase 3 Apoptosis Assay

H1299 cells were seeded in a 96-well plate (5 x 10^3 cells/well), treated with 10 μM DC_AC50 or 100 nM Doxorubicin (DMSO 0.05%). After 12 h, cells were equilibrated to room temperature. 100 μl of Caspase-Glo® 3 Reagent was added to each well of a white-walled 96-well plate containing 100 μl of blank, negative control

cells or treated cells in culture medium. The plate was covered with a plate sealer or lid, gently mixed using a plate shaker at 300-500rpm for 30 seconds, incubated at room temperature for 30 min to 3 hours. The optimal luminescence of each sample was measured in a plate-reading luminometer. Results are represented as mean and s.e.m. of at least three independent experiments.

8-oxo-dG Assay by Triple Quad (QQQ) MS-MS with 1290 UHPLC

H1299 cells were seeded into 10 cm plates. After 24h, the cells were treated with DMSO (2 µL) in cell media or various concentrations of DC_AC50 (maximum 2 µL DMSO) for 24 h and 48 h. Cells were harvested and spun down. The genomic DNA samples were extracted by Wizard® Genomic DNA Purification Kit (Promega). The genomic DNA (3 µg) was digested by two unit Nuclease P1 (Sigma) in 0.01 M NH_4Ac (pH 5.3) at 42 ºC overnight and then two units of Alkaline Phosphatase (Sigma) in 0.1 M fresh NH_4HCO_3 at 37 °C for 3 h. The digested DNA was analyzed by QQQ-LCMS with a C18 reverse-phase column equilibrated with buffer A (50 mM ammonium acetate) and buffer B (50 mM ammonium acetate, 0.1% TFA, 60% CH3CN). The 8-oxo-dG standard was purchased from sigma (Cat. H5653). The 8-oxo-dG data were normalized by whole dG in genomic DNA. Results are represented as mean and s.e.m. of at least three independent experiments.

GSH/GSSG Ratio Assay

H1299 cells (5 x 10^3 cells/well) were grown in 96-well luminometer-compatible tissue culture plates. The GSH/GSSG ratio was determined using the GSH/GSSG-Glo assay (Promega) according to the manufacturer's protocol. Results are represented as mean and s.e. m of at least three independent experiments.

NADPH/$NADP^+$ Ratio Assay

NADPH/$NADP^+$ kit (BioAssay Systems) was used to measure the cellular NADPH/$NADP^+$ ratio. Sub-confluent cells seeded on a 10 cm dish. After 24 h, the cells were treated with DMSO (2 µL) in cell media or 10 µM DC_AC50 (2 µL DMSO) for 12 h. Cells were collected with a scraper, washed with PBS, and lysed with 200 µL of $NADP^+$ (or NADPH) extraction buffer for 12 h. Heat extract was allowed to proceed for 5 minutes at 60 ºC before adding 20 µL of assay buffer and 200 µL of the counter NADPH (or $NADP^+$) extraction buffer in order to neutralize the

extracts. The extracts were spun down and the supernatants were reacted with the working buffer according to the manufacturer's protocol. The absorbance at 565 nm from the reaction mixture was measured with a plate reader. Results are represented as mean and s.e.m. of at least three independent experiments.

Glycolytic rate assay by using ^{3}H-glucose

Sub-confluent cells were seeded on a 6-well plate and collect for 12 h. Wash and Krebs buffer incubation without glucose (1mL) for 1h. ^{3}H-glucose (20 μCi = 20 μL) and 10 mM cold glucose incubation in 1mL Krebs buffer for 1 h. Take 50 μL of medium and mix with 50 μL of 2N HCl to stop the reaction. Transfer the 100 μL of samples to uncapped PCR tube overnight and diffusion at 34 ºC and count diffused $^{3}H_2O$ and undiffused solution, then take ratio. Results are represented as mean and s.e.m. of at least three independent experiments.

NADH and NAD^+/NADH Ratio Assay

NAD^+/NADH kit (BioAssay Systems) was used to measure the cellular NADPH/$NADP^+$ ratio. Sub-confluent cells were seeded on a 6-well dish. After 24 h, the cells were treated with DMSO (2 μL) in cell media or 10 μM DC_AC50 (2 μL DMSO) for 12 h. Cells were collected with a scraper, washed with PBS, and lysed with 100 μL of NAD^+ (or NADH) extraction buffer. Heat extract was allowed to proceed for 5 min at 60 ºC before adding 20 μL of assay buffer and 40 μL of the counter NADH (or NAD^+) extraction buffer in order to neutralize the extracts. The extracts were spun down and the supernatants were reacted with the working buffer according to the manufacturer's protocol. The absorbance at 565 nm from the reaction mixture was measured with a plate reader. The data normalized by total isolated proteins. Results are represented as mean and s.e.m. of at least three independent experiments.

SOD Activity Assay

H1299 cells were grown in 10 cm dish. After 24 h, the cells were treated with DMSO (1 μL) in cell media or variety concentration ofDC_AC50 (maximum DMSO 2 μL) for 12 h, 24 h and 48 h. The cells were then washed three times with PBS, scraped from the dish, and pellet. The cell lysates were prepared in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail (Sigma-

Aldrich). The SOD activity was determined using the SOD determination kit (Sigma) according to the manufacturer's protocol. Results are represented as mean and s.e.m. of at least three independent experiments.

In-gel SOD1 Activity Assay

For the in-gel SOD1 activity assay, H1299 cells with and without various concentrations of DC_AC50 treatment were washed once in PBS prior to lyse in lysis buffer supplemented with phosphatase and protease inhibitors cocktail. 50 μg of protein lysates were analyzed by PAGE under native conditions, without boiling of the samples. Gels were incubated for 1 h in the dark on a shaking platform in 50mM potassium phosphate buffer, pH 7.8, supplemented with 0.34 mM nitro blue tetrazolium chloride (Sigma), 14 mM riboflavin (Sigma), and TEMED (Bio-Rad). Subsequently, yellow stainedgels were transferred into deionized H_2O and exposed to bright light to induce a color change by the generation of free radicals, which is inversely correlated to the SOD1 activity.

Cytochrome c Oxidase Activity Assay

Mitochondria were prepared with the Mitochondria Isolation Kit (Pierce). The Cytochrome c Oxidase activity was determined using the cytochrome c oxidase Assay kit (Sigma) according to the manufacturer's protocol. This kit is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. The data normalized by total isolated mitochondria proteins. Results are represented as mean and s.e.m. of at least three independent experiments.

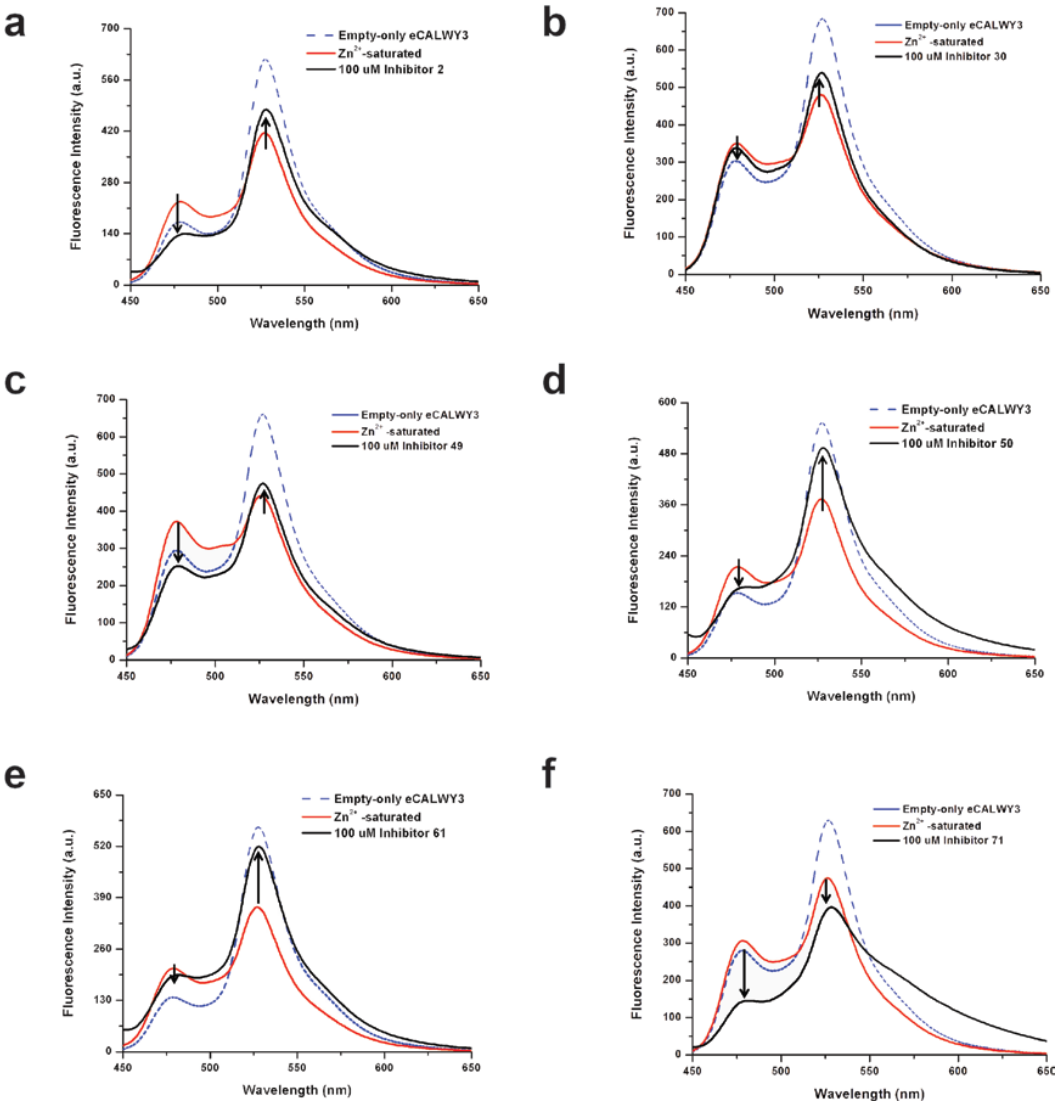
Statistical Analysis

Statistical analysis and graphical presentation were done using GraphPad Prism 4.0. Data shown are from one representative experiment of multiple independent experiments and are given as mean ± s.e.m.. A paired Student's t test determined the p value of the xenograft experiment.

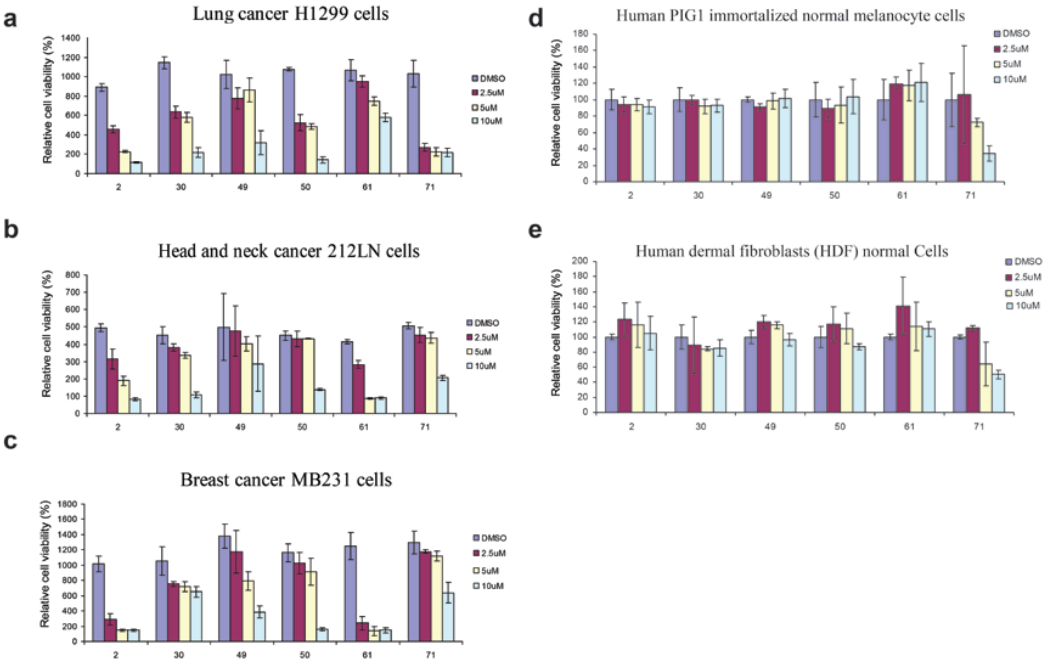
Antibodies

Antibodies used for immune blotting were GAPDH (A00192; GenScript), Atox1 (sc-100557; santa cruz), CCS (sc-20141; santa cruz), SOD1 ((8B10); MA1-105; Thermo

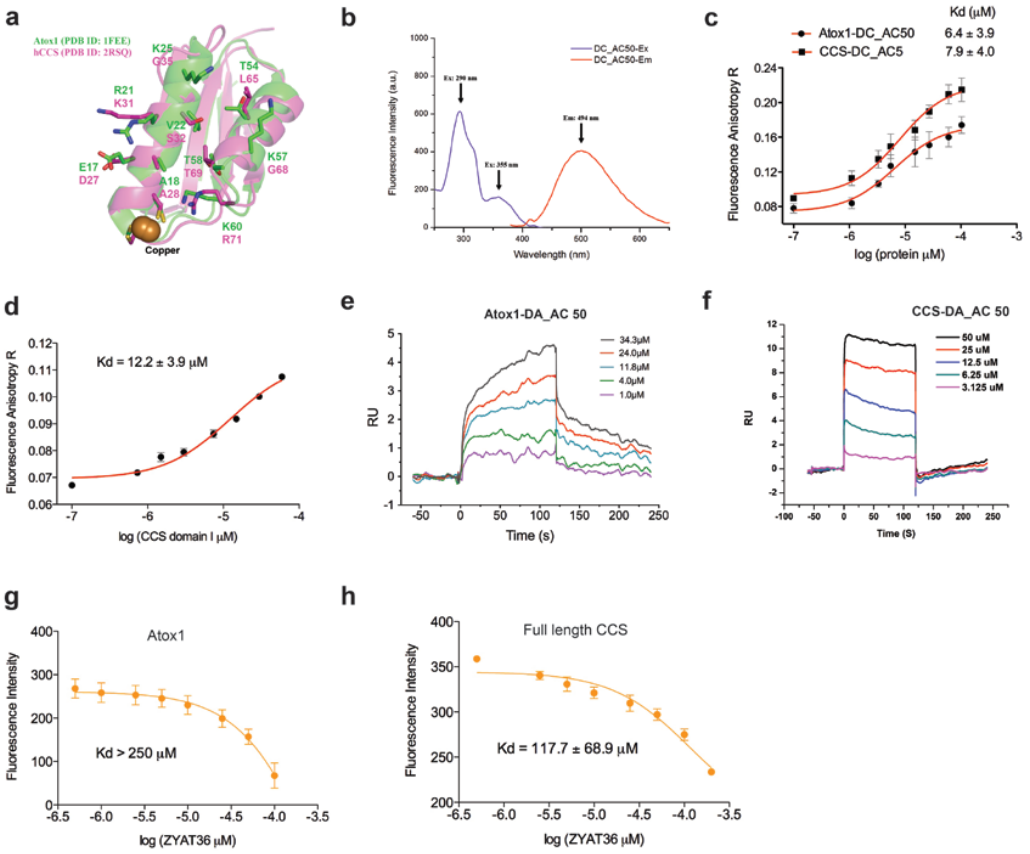
Scientific), SOD2 (PA5-30604; Thermo Scientific), Phospho-AMPKα ((40H9); 2535; Cell signaling), AMPKα (2532; Cell signaling), Phospho-ACC ((Ser79); 3661; Cell signaling), ACC ((C83B10); 3676; Cell signaling), COX I (ab14705; Abcam), COX II (ab79393; Abcam), PARP (9542; Cell signaling), Metallothionein (ab12228; Abcam).



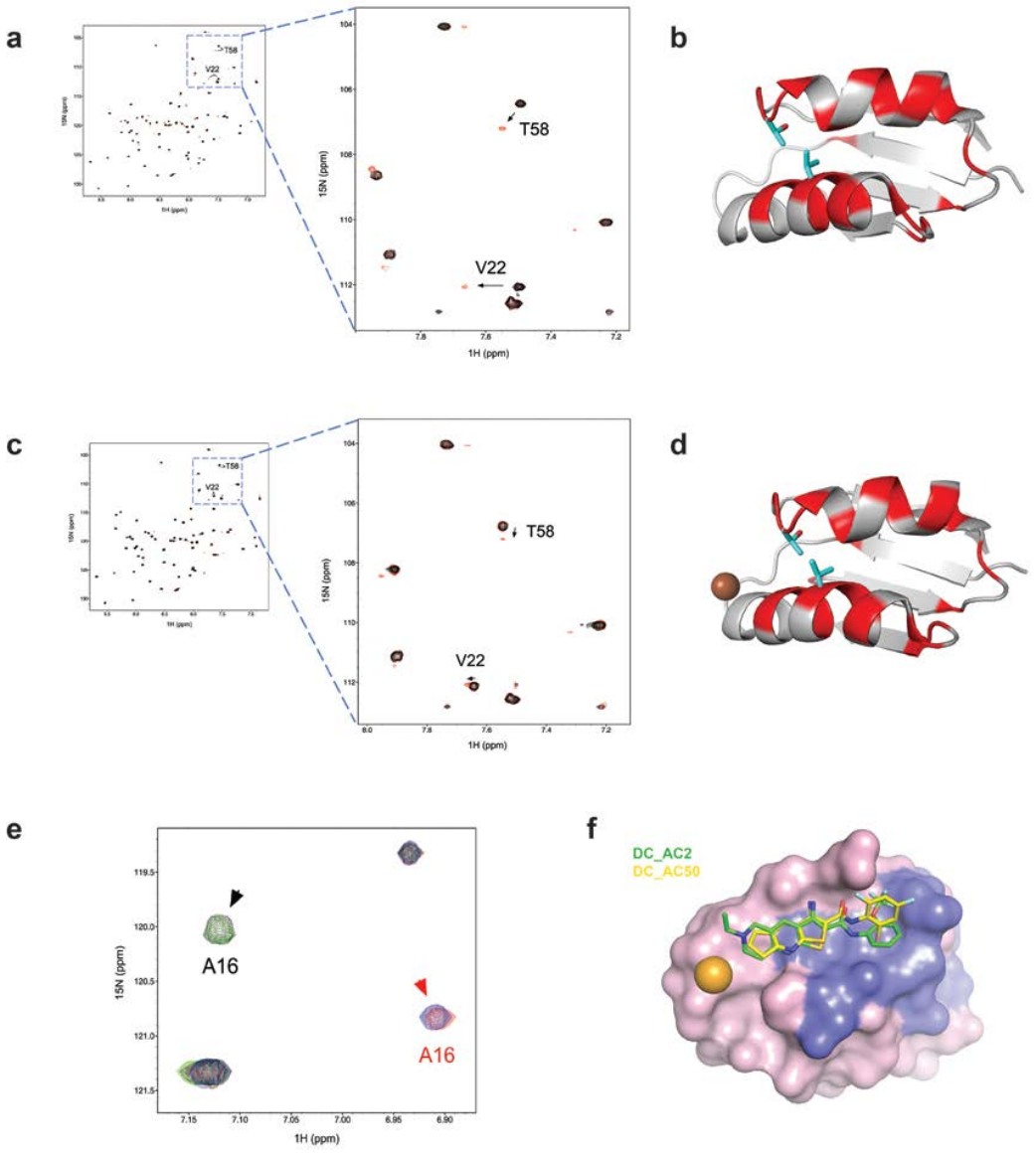
Supplementary Figure 1. FRET response of the 6 representative hits using the eCALWY3 probe. (**a-f**) Binding of zinc(II) induces a 2-fold decrease of the FRET ratio of eCALWY3. Fluorescence responses of the probe before (empty only eCALWY3, dotted line, blue curve) and after (Zn^{2+}-saturated, solid line, red curve) addition of 0.9 mM Zn^{2+} in 1 mM DHPTA are shown, as well as after treatment with 100 µM inhibitors (0.72 µL DMSO in 180 µL buffer) (solid line, black curve). The FRET ratio increased to almost the same level of the apo-form eCALWY3 with the treatment of the six hit compounds (2, 30, 49, 50, 61, 71), indicating inhibition of the Atox1-WD4 interaction in the presence of metal. Zinc was used in the assay as explained previously[1]. Note that DC_AC71 is not very soluble and shows precipitation problems.



Supplementary Figure 2. Initial cell proliferation inhibition screening of several cancer cell lines and normal cell lines with the six selected compounds. (**a-c**) A variety of human cancer cell lines (lung cancer H1299 cells, head and neck cancer 212LN cells and breast cancer MB231 cells) were treated with various concentrations of compounds (2.5-10 μM) or DMSO (control) for 72 h. (**d,e**) Normal human cells, including PIG1 cells and HDF cells, were grown and treated with these compounds at 2.5-10 μM for 72 h. Only DC_AC71 had toxicity to normal cells. Error bars, mean ±s.e.m., $n = 3$, biological replicates.

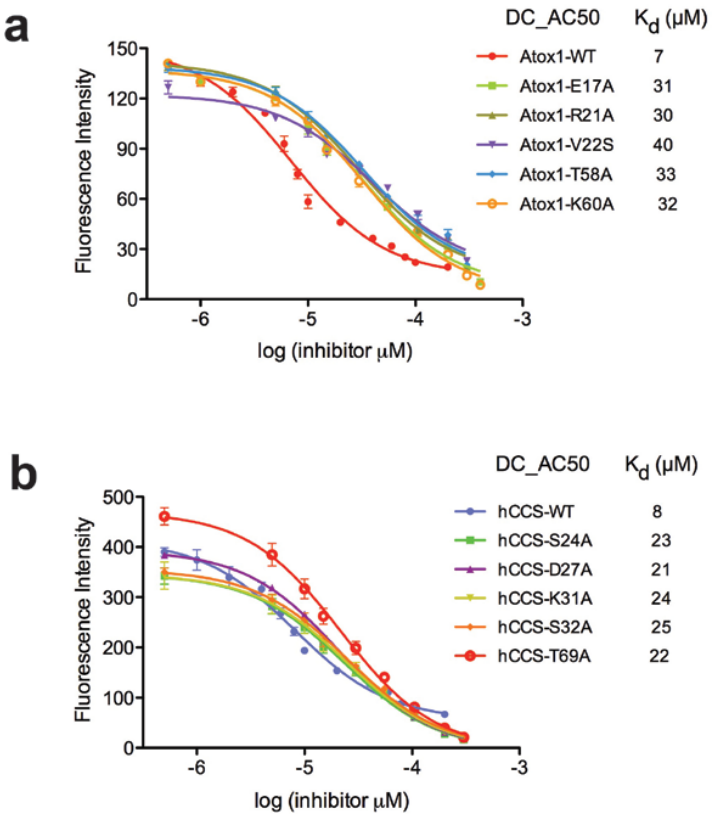


Supplementary Figure 3. Binding of DC_AC50 to Atox1, full length CCS and CCS domain I by FA measurement and surface plasmon resonance (SPR). (**a**) The structure alignment illustrates similar amino acid residues between human Atox1 (green) and Domain 1 of CCS (purple). (**b**) Excitation (purple) and emission (red) spectra for DC_AC50. (**c,d**) Binding curves of DC_AC50 to human Atox1, full length CCS and CCS domain I obtained by using fluorescence anisotropy (FA). Experiments were performed in 50 mM HEPES, 200 mM NaCl, 1 mM DTT (pH 7.1). Excitation at 355 nm with the maximum fluorescence emission recorded at 494 nm for DC_AC50. The concentration of the protein varied from 1 to 100 μM with 1 μM DC_AC50 used. The Kd values obtained are almost identical to those obtained with FRET. (**e,f**) The binding assay was performed in a SPR assay. Purified Atox1 (5 μM) or full length CCS (1 μM) protein was immobilized on CM5P chip. DC_AC50 was added at varied concentrations at a DMSO concentration of 5% in a running buffer composed of HBS-EP 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20. (**g,h**) Binding curves of inactive compound ZYAT36 (1 to 100 μM) to Atox1 (1 μM) and full length CCS (1 μM) obtained by using FRET measurement (from Tyr/Trp to ZYAT36). The results showed that there are no binding between ZYAT36 and Atox1 or full length CCS. Error bars, mean ±s.e.m., n = 3, biological replicates.

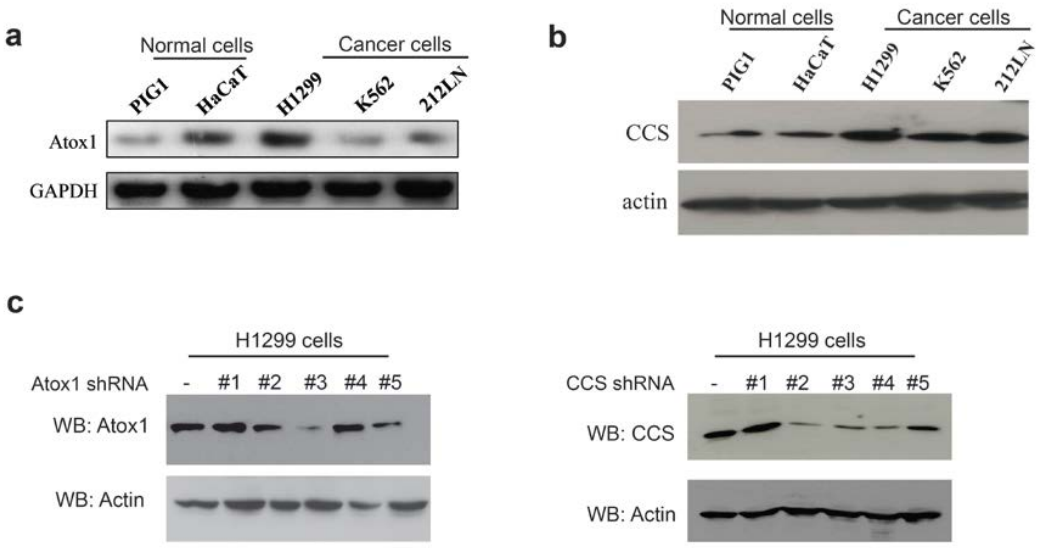


Supplementary Figure 4. Binding of Atox1 to DC_AC2 characterized by NMR spectroscopy. (**a**) Superposition of [^{1}H, ^{15}N] HSQC spectra of Cu(I)-free Atox1 in the absence (black) and presence of DC_AC2 (red, molar ratio of 1:3 Atox1 to DC_AC2) reveals spectral changes upon ligand binding. (**b**) Residues with chemical shift perturbed and attenuated upon DC_AC2 binding as shown in (**a**) are mapped (colored in red) onto 3D model structure of Atox1. Side-chains of selected residues (Val22, and Thr58) for mutation experiments are indicated in green sticks. (**c**) Superposition of [^{1}H, ^{15}N] HSQC spectra of Cu(I)-loaded Atox1 in the absence (black) and presence of DC_AC2 (red, molar ratio of 1:3 Atox1 to DC_AC2) reveals spectral changes upon ligand binding. (**d**) Residues with chemical shift perturbed and attenuated upon DC_AC2 binding as shown in (**c**) are mapped (colored in red) onto 3D model structure of Cu(I)-loaded Atox1. Cu(I) ion is shown as a brown sphere. Side-chains of selected residues (Val22 and Thr58) for mutation experiments are indicated in green sticks. (**e**) Comparison of chemical shifts for Ala16 from the copper-binding motif ^{11}TCGGCA^{16} of Atox1 in different states: Cu(I)-free (black), Cu(I)-loaded (red), Cu(I)-free form in the presence of DC_AC2 (green) and Cu(I)-added form in the

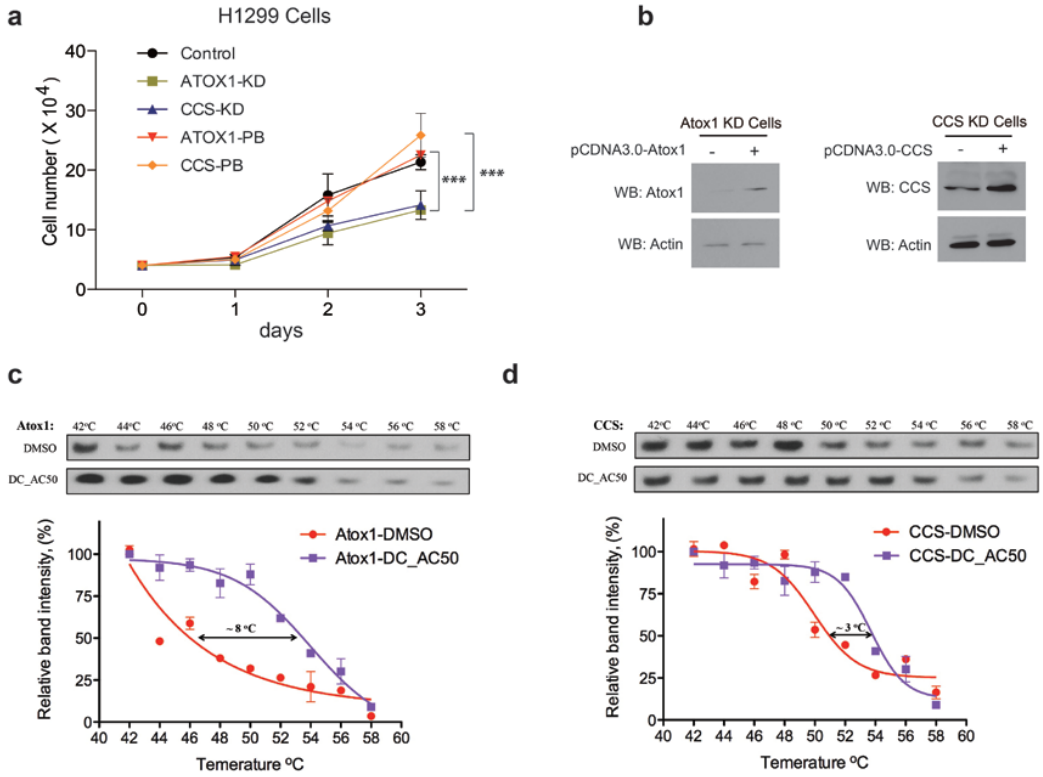
presence of DC_AC2 (blue). NMR resonances of Ala16 in corresponding to four different states of Atox1 clearly group to two positions, which are either from Cu(I)-free forms (indicated by black arrow) or from Cu(I)-loaded forms (indicated by red arrow). (**f**) The modeled binding site of DC_AC2 and DC_AC50 on Atox1. The regions with chemical shift perturbed and attenuated upon DC_AC2 binding are colored by deep blue. DC_AC2 is shown as green sticks and DC_AC50 is shown as yellow sticks. Protein samples were freshly made at a concentration of 20 μM in NMR buffer (50 mM HEPES, 200 mM NaCl, 1 mM DTT, pH 7.0). All NMR Experiments were carried out at 25 ^{o}C on a Bruker Avance III 600 MHz NMR spectrometer equipped with a TCI cryoprobe.



Supplementary Figure 5. Binding curves of DC_AC50 to the wild-type and mutant Atox1 and full length CCS. (**a,b**) Binding curves of DC_AC50 (1 to 100 μM) to Atox1 (1 μM) and full length CCS (1 μM) with mutations. The mutation of these amino acids weakened the binding affinity (3-6 folds) of DC_AC50 to the mutant proteins as compared to the wild-type Atox1 and full length CCS. Error bars, mean ±s.e.m., $n = 3$, biological replicates.



Supplementary Figure 6. Cellular levels of Atox1/CCS in cancer and normal cells and the selection of Atox1/CCS stable knockdown cell lines. (**a,b**) The expression levels of Atox1 and CCS are higher in lung H1299 cancer cells than those in normal cells (human PIG1 cell and human immortal keratinocyte HaCaT cell) by western blot after 12 h. GAPDH and β-actin expression was used as a loading control. (**c**) Stable knockdown cells for Atox1 and CCS using five different lentiviral shRNAs in H1299 cells, selected by antibiotic puromycin, and confirmed by western blot. The best one was chosen for subsequent assays. Error bars, mean ±s.e.m., n = 3, biological replicates.

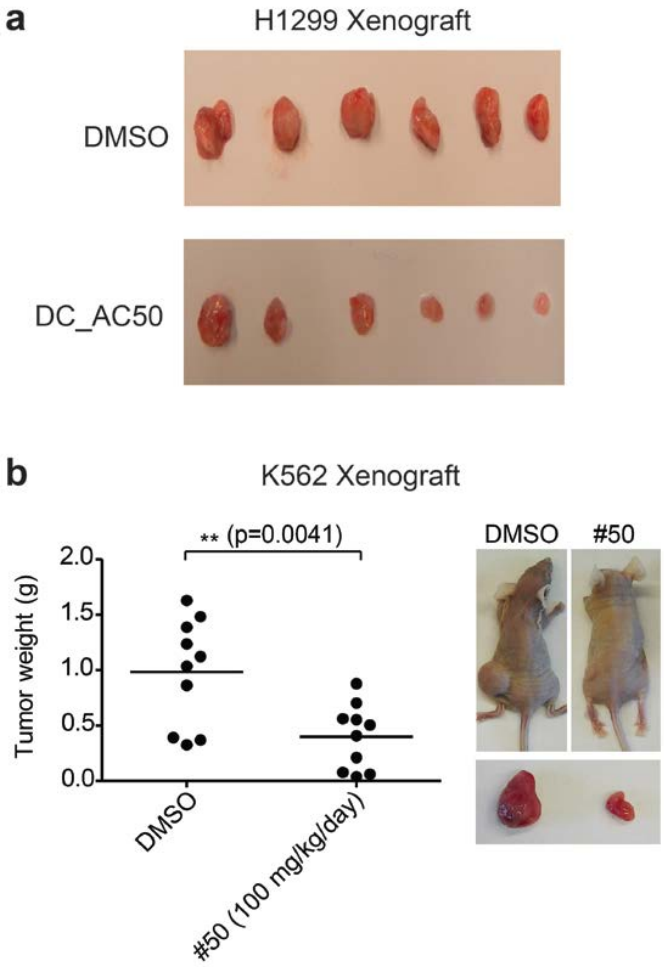


Supplementary Figure 7. R escue assays in Atox1/CCS knockdown cells and cellular thermal shift assays showing binding of DC_AC50 with Atox1 and CCS. (**a**) The cell proliferation in Atox1 and CCS knockdown cells can be rescued by re-expressing Atox1 and CCS in H1299 cells. (**b**) The re-expression efficiency of Atox1 and CCS to their knockdown cells were confirmed via western blotting. (**c,d**) Target engagements of DC_AC50 to Atox1 and CCS inside H1299 cells using cellular thermal shift after 12 h. Atox1 or CCS alone (DMSO as a control) denatures around 46~50 ºC inside H1299 cells. The additional of DC_AC50 (10 μM) stabilizes Atox1 or CCS by 3-8 ºC after 12 h, confirming binding inside H1299 cells. Error bars, mean ±s.e.m., $n = 3$, biological replicates.

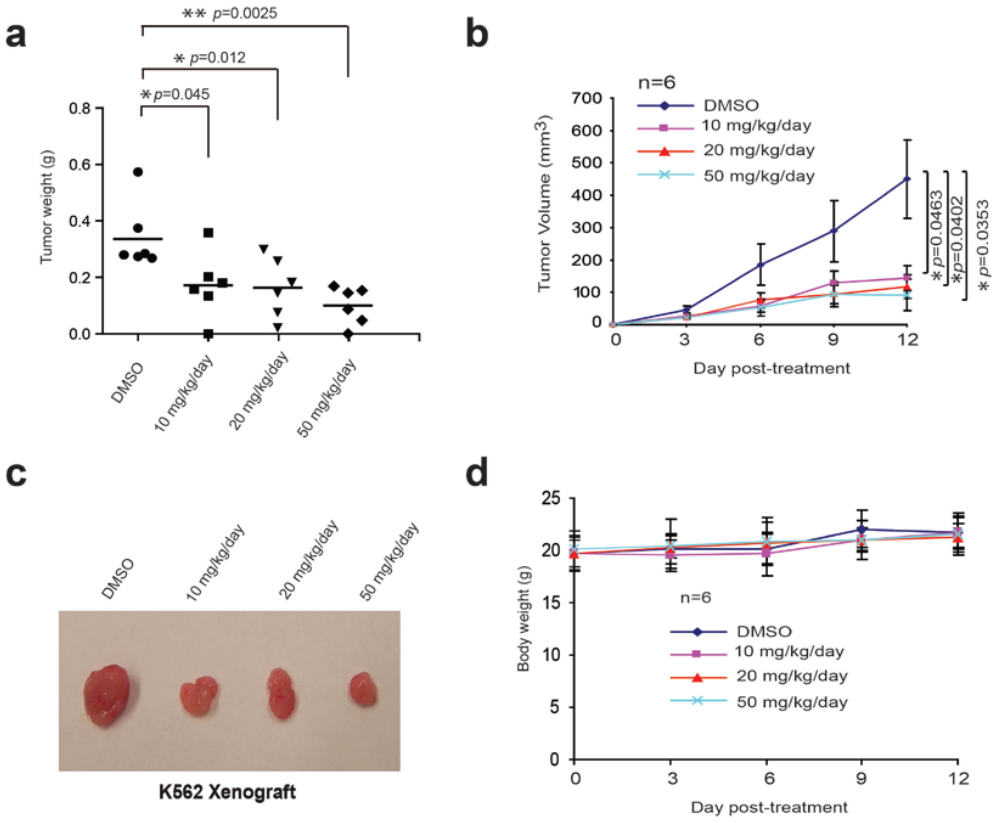
Balb/C Mouse Hematology

Test name (unites)	Reference range (low-high)	Control day 7 (mean)	100 mg/kg day 7 (mean)	p-value
WBC (× 10^3/μL)	2.6-10.1	7.66 ± 1.64	6.89 ± 3.125	0.786868716
LYM (× 10^3/μL)	1.3-8.4	4.685 ± 0.88	4.74 ± 1.556	0.969274582
MONO (× 10^3/μL)	0-0.3	0.475 ± 0.35	0.44 ± 0.17	0.909652973
GRAN (× 10^3/μL)	0.4-2	2.42 ± 0.38	1.58 ± 1.4	0.499040613
HCT (%)	32.8-48	49.6 ± 3.82	30.85 ± 4.879	0.050492932
MCV (fl)	42.3-55.9	58.05 ± 1.06	56.75 ±0.919	0.320479114
RDWa (%)	0-99.9	16.3 ± 0.57	21.5 ± 4.667	0.258201813
HGB (g/dl)	10-16.1	12.85 ± 0.78	7.45 ± 1.768	0.058410031
MCHC (g/dl)	29.5-35.1	25.9 ± 0.42	24 ± 1.98	0.315731714
RBC (× 10^6/μL)	6.5-10.1	8.545 ± 0.81	5.43 ± 0.778	0.059486292
MCH (pg)	13.7-18.1	15.05 ± 0.49	13.6 ± 1.273	0.272036617
PLT (× 10^3/μ)	250-1540	1189.5 ± 38.9	742 ± 684.5	0.453406244
MPV (fl)	0-99.9	6.35 ± 0.49	6.35 ± 0.495	1

Supplementary Figure 8. The hematological data of athymic nude mouse. Nude mice (n=3) were treated with either vehicle control or DC_AC50 for 7 days. CBC analysis showed no significant difference in the hematopoietic properties between the two groups of mice.



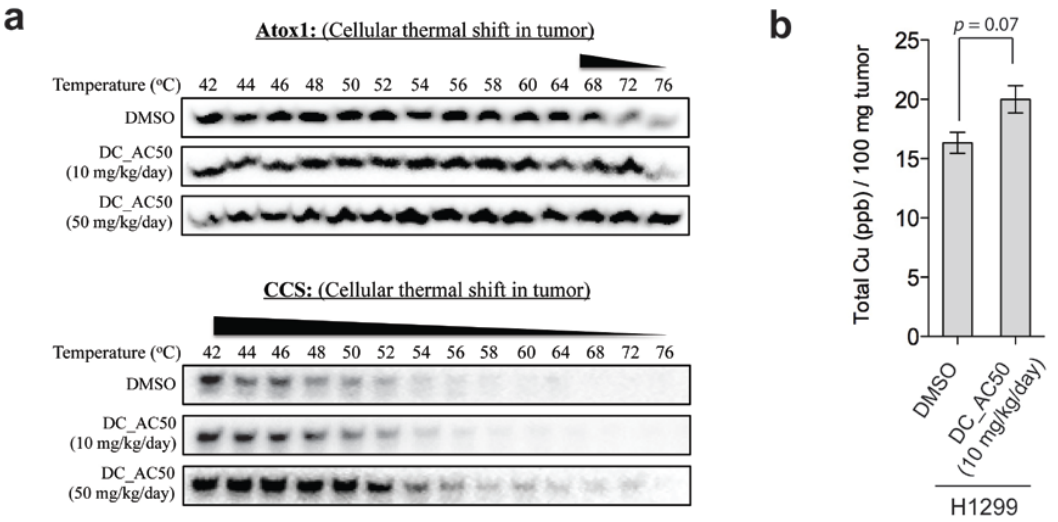
Supplementary Figure 9. Anti-tumor effects of DC_AC50 in lung cancer (H1299) and leukemia cancer (K562) xenograft mouse models. (**a**) Therapeutic activity of DC_AC50 in xenograft tumor mice injected with lung cancer cells. 12 nude mice were subcutaneously injected with 20 x 10^6 H1299 cells. 6 mice were used in each group (DMSO as control and DC_AC50). Mice were analyzed 3 weeks later for tumor formation. Representative images of the mice tumors are shown. (**b**) Tumor growth and tumor size in xenografts nude mice injected with leukemia K562 cells were compared between the group of mice treated with DC_AC50 and the control group treated with vehicle control. 20 nude mice were subcutaneously injected with 10 x 10^6 K562 cells. 10 mice were used in each group (DMSO as control and DC_AC50). Mice were analyzed 3 weeks later for tumor formation. Representative images of the mice are shown. P values were determined by a two-tailed Student's t test. **P < 0.005.



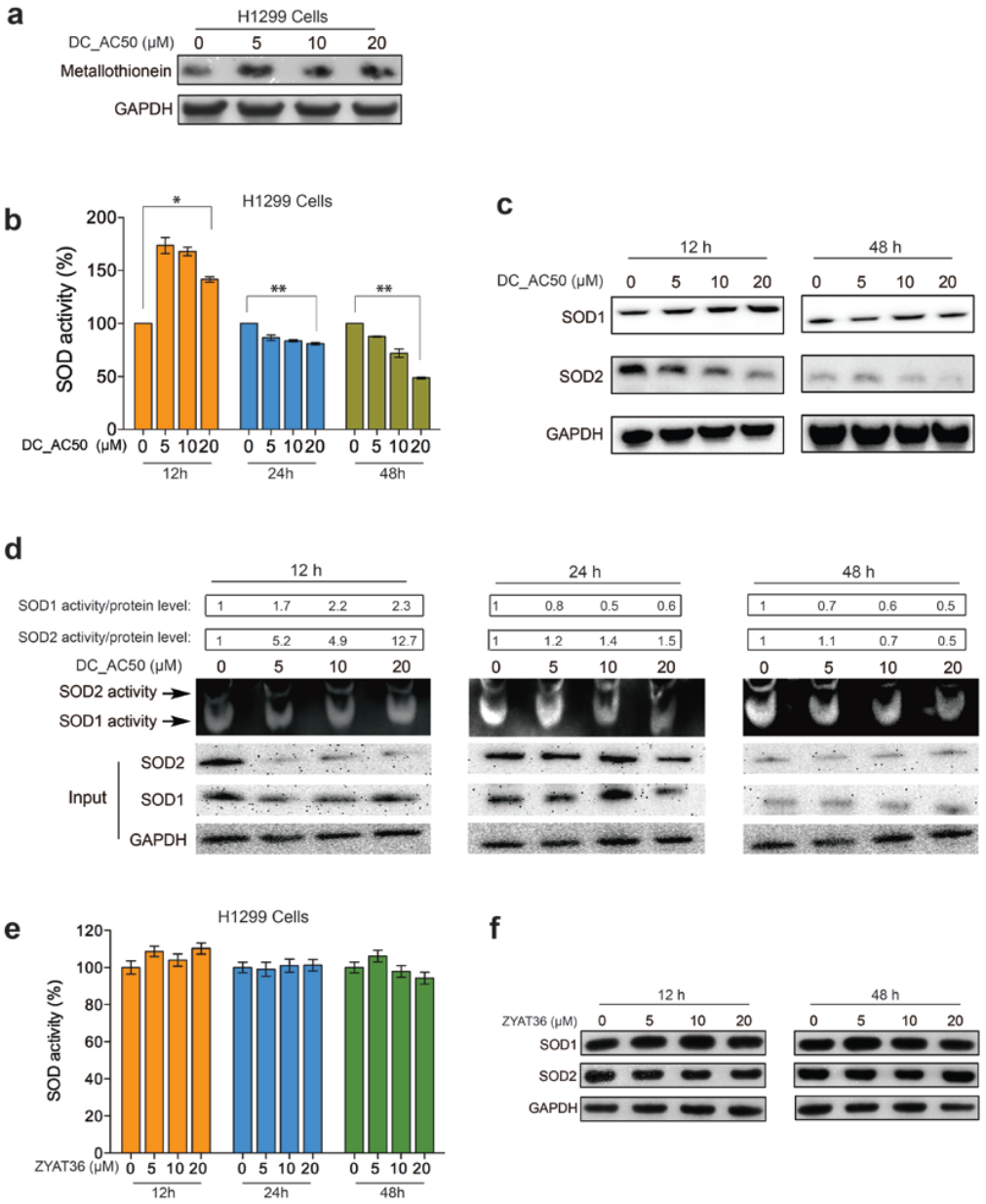
e

Test name	Normal range	DMSO (mean)	10mg/kg/day (mean)	20mg/kg/day (mean)	50mg/kg/day (mean)
RBC(10^6/μl)	6.5 - 10.1	7.9 ± 0.2	7.1 ± 3.5	7.0 ± 1.3	8.5 ± 0.3
MCV(fl)	42.3 - 55.9	53.6 ± 0.4	52.4 ± 0.5	53.6 ± 1.1	48.8 ± 4.5
HCT(%)	32.8 - 48	42.7 ± 0.9	36.8 ± 18.7	38.0 ± 8.1	41.6 ± 5.4
MCH(pg)	13.7 - 18.1	17.1 ± 0.3	17.3 ± 0.4	17.2 ± 0.2	17.1 ± 0.3
MCHC (g/dl)	29.5 - 35.1	31.9 ± 0.3	33.2 ± 1.2	32.1 ± 0.8	35.2 ± 3.7
RDW(%)	0 - 99.9	15.2 ± 0.4	14.8 ± 0.4	15.1 ± 0.8	17.0 ± 1.1
RDWa(fl)	0 - 99.9	30.8 ± 1.0	28.8 ± 1.5	30.3 ± 2.3	29.7 ± 2.1
PLT (10^3/μl)	250-1540	278.3 ± 57.5	279 ± 21.2	280.3 ± 139.1	265 ± 22.5
MPV(fl)	0 - 99.9	5.5 ± 0.1	6.4 ± 0.7	5.9 ± 0.8	5.4 ± 0.1
HGB(g/dl)	10 - 16.1	13.6 ± 0.1	10.7 ± 5.8	12.1 ± 2.4	14.5 ± 0.2
WBC (10^3/μl)	2.6 - 10.1	3 ± 0.6	3.0 ± 1.7	2.7 ± 0.5	3.2 ± 0.3
LYM(10^3/μl)	1.3 - 8.4	1.3 ± 0.5	1.7 ± 1.3	2 ± 0.3	1.9 ± 0.3
MONO(10^3/μl)	0 - 0.3	0.2 ± 0.05	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
GRAN(10^3/μl)	0.4 - 2	0.7 ± 0.05	0.9 ± 0.1	0.5 ± 0.2	0.9 ± 0.1
LYM(%)	0 - 99.9	54.4 ± 7.6	55.2 ± 13.1	60.3 ± 2.1	43.5 ± 8.5
MONO(%)	0 - 99.9	11.4 ± 0.9	9.3 ± 3.5	10.3 ± 2.9	13.5 ± 2.4
GRAN(%)	0 - 99.9	34.1 ± 7.0	35.5 ± 16.6	29.3 ± 2.4	42.9 ± 10.1

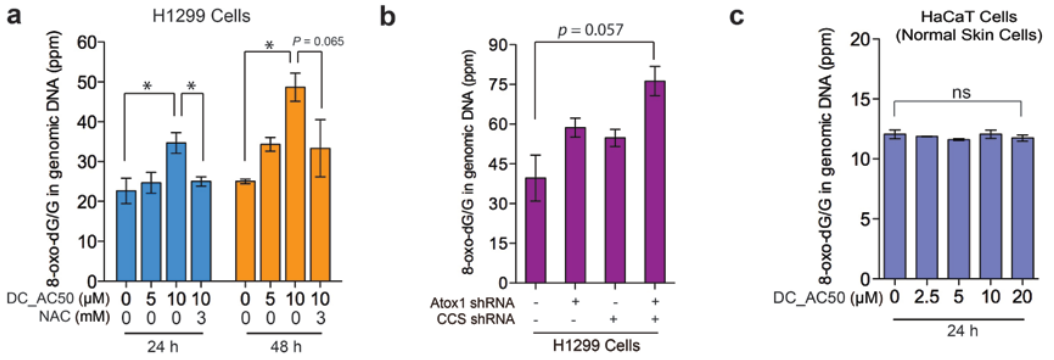
Supplementary Figure 10. Anti-tumor effects of DC_AC50 treatment at different dose in leukemia cancer (K562) xenograft mouse models. (**a-c**) Treatment of DC_AC50 at different doses (10, 20 and 50 mg/kg/day) were injected to mice after inoculation with leukemia K562 cancer cell line, it inhibited growth tumor xenograft. (**d**) There are also no effects of chronic treatment with DC_AC50 or DMSO on body weights of nude mice. (**e**) DC_AC50 didn't cause obvious toxicity in mice at different doses (10, 20 and 50 mg/kg/day). Peripheral blood samples from DC_AC50 or DMSO-treated nude mice (3 representative mice) were examined for hematological properties by using CBC analysis with no obvious effects observed. P values were determined by a two-tailed Student's t test. **P < 0.005, *P < 0.05.



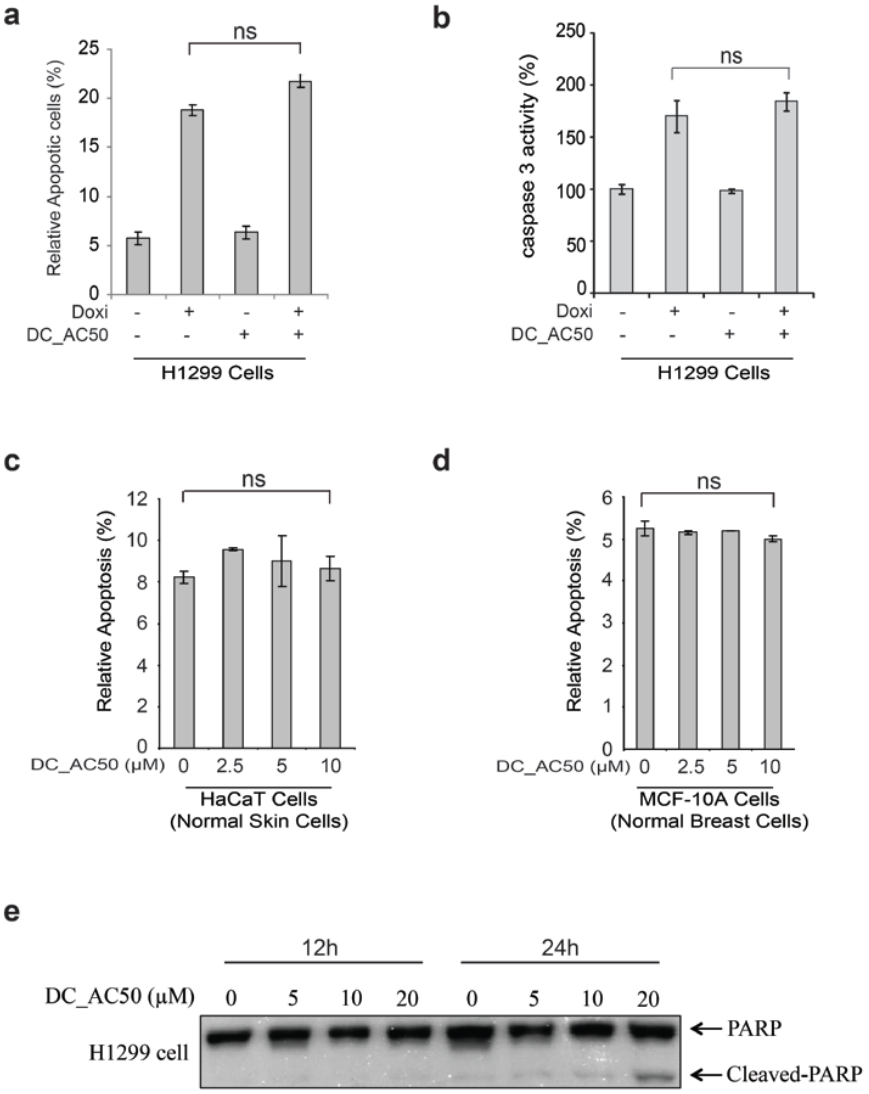
Supplementary Figure 11. Total copper concentration and cellular thermal shift assay to demonstrate target-engagement in mice tumor tissue. (**a**) Target engagements of DC_AC50 to Atox1 and CCS inside mice tumor tissue using cellular thermal shift. The results of Atox1 and CCS cellular thermal shift in mice tumor tissue are from the H1299 nude xenograft mice with DMSO and 10 mg/kg/day and 50 mg/kg/day DC_AC50 treatment for 7 days. Atox1 or CCS alone (DMSO as a control) denatures around 68 ºC and 44 ºC in mice tumor tissue. The additional of DC_AC50 stabilizes Atox1 or CCS at 10 mg/kg/day and 50 mg/kg/day, confirming binding of DC_AC50 to our proposal targets. (**b**) Total copper concentration in tumor tissue isolated from the H1299 nude xenograft mice with DMSO and 10 mg/kg/day DC_AC50 treatment for 7 days. The treatment with DC_AC50 induced ~20% copper accumulation in tumor tissue at 10 mg/kg/day. Error bars, mean ±s.e.m., n = 3, biological replicates.



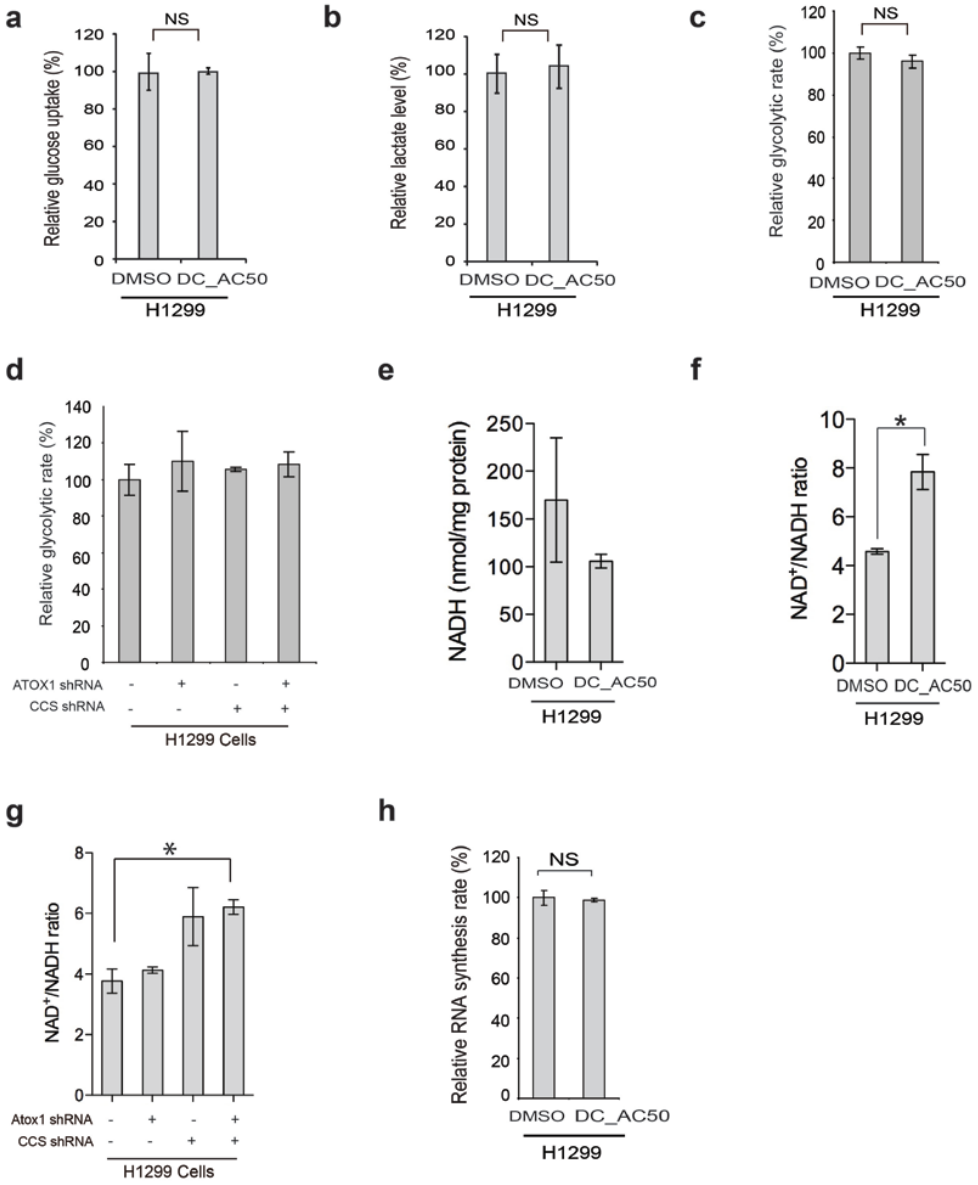
Supplementary Figure 12. DC_AC50 decreased SOD activity and changed protein expression level of metallothionein, SOD1 and SOD2. (**a**) The protein expression level of metallothionein increased in 12 h after DC_AC50 treatment. (**b**) DC_AC50 increased total SOD activity after 12 h in H1299 cells perhaps due to adaptive response to oxidative stress. After 48 h, DC_AC50 (10~20 μM) decreased total SOD activity in H1299 cells. (**c**) The protein expression level of SOD1 increased with DC_AC50 treatment after 12 h. However, the protein expression level of SOD2 decreased with DC_AC50 treatment after 12 h. (**d**) H1299 Cells with and without various concentration of DC_AC50 were lysed, and SOD1 and SOD2 activity was determined in 50 μg of cell lysates by the in-gel activity assay. After 48 h, DC_AC50 (10~20 μM) decreased SOD1 and SOD2 activity significantly in H1299 cells. (**e,f**) Treatment of inactive compound ZYAT36 (0~20 μM) didn't affect total SOD activity and the expression level of SOD1 and SOD2 in H1299 cells. Error bars, mean ±s.e.m., $n = 3$, biological replicates. *P < 0.05, **P < 0.005.



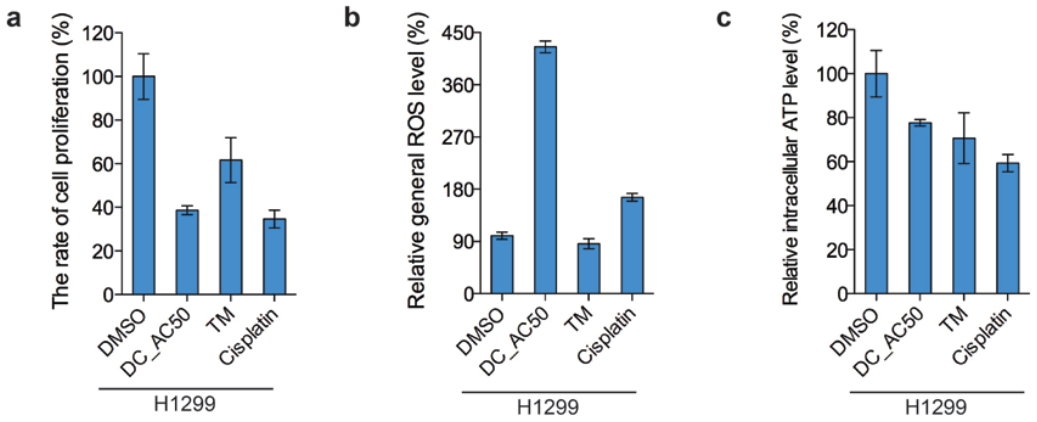
Supplementary Figure 13. DC_AC50 induced DNA damage in H1299 cells. (**a**) The 8-oxo-dG level determined by triple quadrupole mass spectrometry (QQQ) analysis. The cellular levels of 8-oxo-dG increased with DC_AC50 treatment (10 μM) for 24 h and 48 h, which could be rescued by NAC (3 mM) in H1299 cells. (**b**) Knockdown of Atox1/CCS also led to induce DNA damage in H1299 cells. (**c**) The cellular levels of 8-oxo-dG weren't affect by DC_AC 50 treatment in normal HaCaT cells for 24 h. Error bars, mean ±s.e.m., n = 3, biological replicates. *P < 0.05, **P < 0.005.



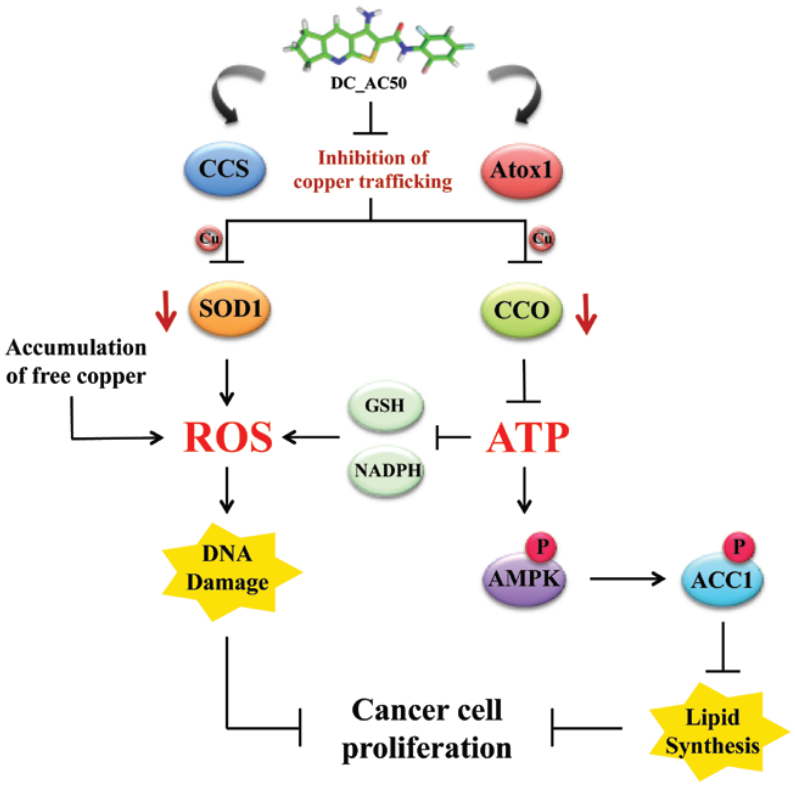
Supplementary Figure14. DC_AC50 didn't induce apoptosis in H1299 cells. (**a,b**) DC_AC50 did not noticeably induce apoptosis or affect caspase-3 activity in H1299 cells after DC_AC50 (10 μM) and Doxorubicin (100 nM) treatment for 12 h. (**c,d**) DC_AC50 also didn't induce apoptosis in normal HaCaT and MCF-10A cells for 12 h. (**e**) DC_AC50 didn't lead to PARP cleavage after 12 h; a weak PARP cleavage was observed after 24 h at higher concentrations of DC_AC50. Error bars, mean ±s.e.m., *n* = 3, biological replicates.



Supplementary Figure 15. DC_AC50 didn't affect glycolysis pathway but decreased cellular levels of NADH. (**a,b**) DC_AC50 (10 μM) did not noticeably affect glucose uptake nor lactate level for 12 h. (**c,d**) Knockdown of Atox1/CCS or treatment of DC_AC50 didn't affect glycolytic rate in H1299 cells for 12 h. (**e-g**) H1299 cells were treated with DC_AC50 (10 μM) or Atox1/CCS KD for 12 h. The NADH level and the ratio of NAD^+/NADH were examined. (**h**) DC_AC50 (10 μM) didn't affect RNA synthesis in H1299 cells for 12 h. Error bars, mean ±s.e.m., $n = 3$, biological replicates. *P < 0.05, **P < 0.005.



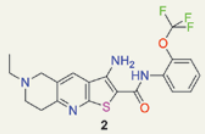
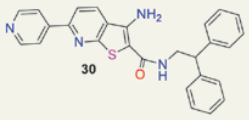
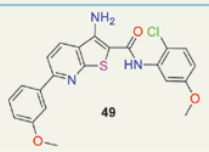
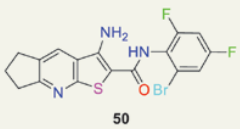
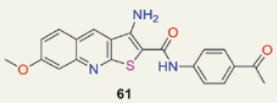
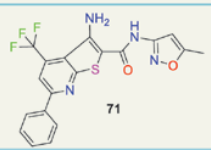
Supplementary Figure 16. Comparison of DC_AC50 with tetrathiomolybdate (TM) and Cisplatin in inhibiting cell proliferation, reducing ATP level and increasing ROS level of H1299 cells. (**a**) The rate of cell proliferation in H1299 cells when treated with 10 μM of DC_AC50, tetrathiomolybdate (TM) and Cisplatin for 72 h, respectively. DC_AC50 is slightly more effective than TM. (**b**) The relative ROS levels in H1299 cells with the treatment of 10 μM DC_AC50, TM and Cisplatin for 12 h, respectively. DC_AC50 caused a significant increase in ROS levels. (**c**) The relative intracellular ATP levels in H1299 cells with the treatment of DC_AC50, TM and Cisplatin for 12 h, respectively. Error bars, mean ±s.e.m., n = 3, biological replicates.



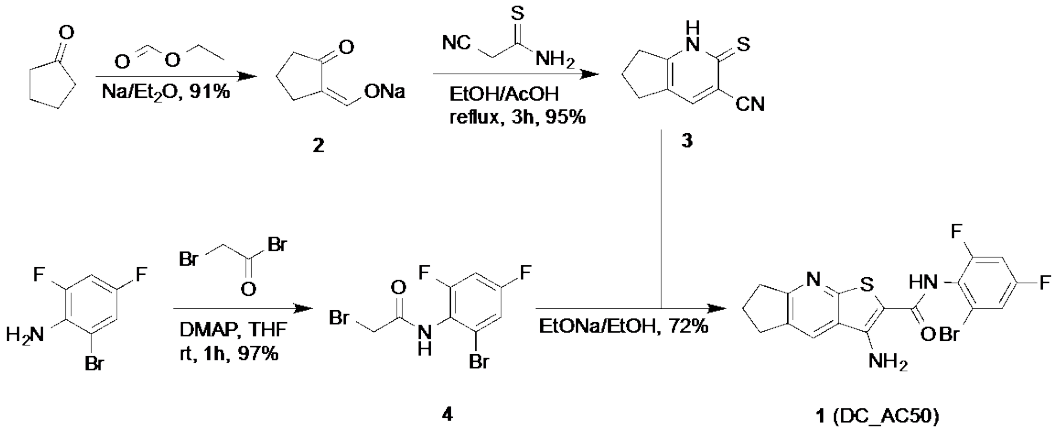
Supplementary Figure 17. A mechanistic model of cancer cell proliferation inhibition through targeting copper trafficking proteins Atox1 and CCS. Selective inhibition of copper trafficking proteins Atox1 and CCS by DC_AC50 elevates cellular ROS level and reduces lipogenesis.

Supplementary Table 1. Representative small molecules that inhibit Atox1-WD4 interaction based on the FRET assay.

Table 1. Representative of small molecular inhibit Atox-WD4 interaction by FRET Assay!

Small Molecular Structures	Fluorescence Intensity Ratio (Citrine/Cerulean)	Recover %
 2	eCALWY3 only: **3.54** Zn(II) saturated: **1.71** 100 μM compound added: **3.05**	**85%**
 30	eCALWY3 only: **2.27** Zn(II) saturated: **1.36** 100 μM compound added: **1.53**	**66%**
 49	eCALWY3 only: **2.24** Zn(II) saturated: **1.13** 100 μM compound added: **1.89**	**84%**
 50	eCALWY3 only: **3.58** Zn(II) saturated: **1.57** 100 μM compound added: 3.**43**	**96%**
 61	eCALWY3 only: **3.68** Zn(II) saturated: **1.76** 100 μM compound added: **2.72**	**74%**
 71	eCALWY3 only: **2.25** Zn(II) saturated: **1.52** 100 μM compound added: **1.86**	**83%**

Supplementary Note. The synthetic route of DC_AC2 and DC_AC50:



Sodium (1.91 g, 83 mmol) was added into 200 mL of dry ether in a 1-L round bottom flask, and the suspension was cooled to 0 ^{o}C in an ice bath. To this cooled suspension was added a mixture of ethyl formate (6.15 g, 0.083 mol) and cyclopentanone (6.89 g, 0.083 mol) in dry ether (100 mL). The temperature of the reaction mixture was allowed to reach room temperature, and stirring was continued overnight. The crude suspension was filtered, and the precipitate was dried under vacuum for 2 h to obtain crude sodium salt **2** (10.2 g, yield 91.4%).

Glacial AcOH (0.57 mL, 10 mmol) was added to a solution of sodium salt **2** (2.68g, 20 mmol) in 10 mL of ethanol, and cyanothioacetamide (2.00 g, 20 mmol) was added at 50 ^{o}C. After additional AcOH (0.57 mL, 10 mmol) was added, the mixture was heated to reflux for 3 h. The precipitate was filtered off, washed with ethanol and hexane, and recrystallized from AcOH to give compound **3** (3.36 g, yield 95.3%).

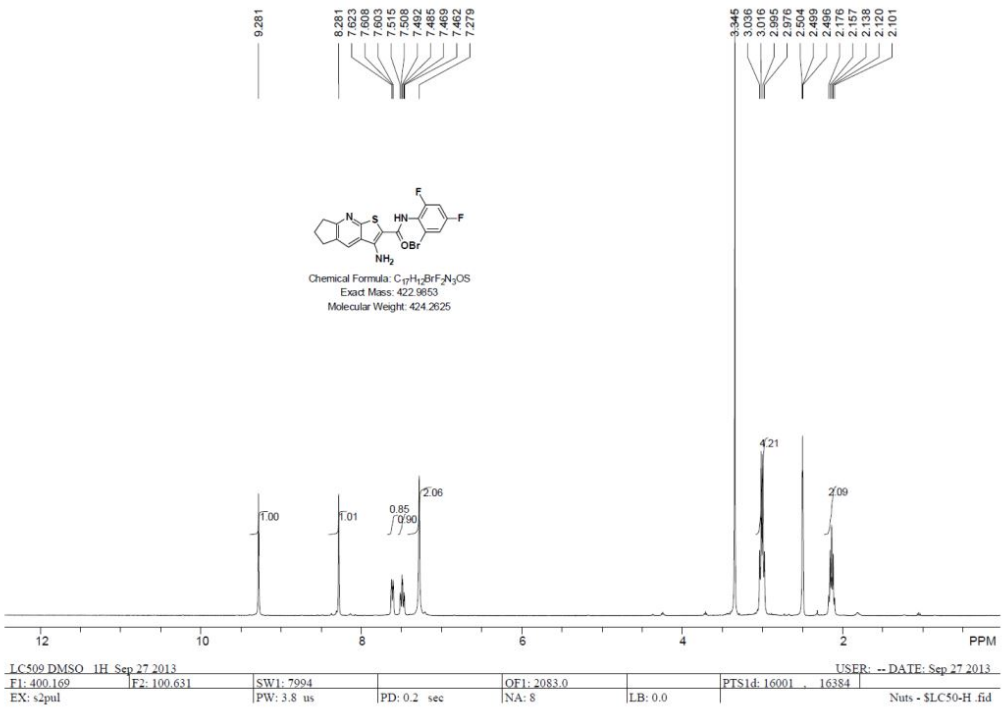
To a solution of aromatic amine (1.54 g, 7.4 mmol) and DMAP (0.45 g, 3.7 mmol) in THF was added dropwise to a solution of bromoacetyl bromide (1.49 g, 7.4 mmol) in THF at 0 ^{o}C. After stirring at 0 ^{o}C for 0.5 h, the mixture was stirred for another 1 h at rt. The reaction was quenched with water and extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated by rotary evaporator. Recrystallized from EtOH to afford compound **4** (2.35g, yield 96.8%).

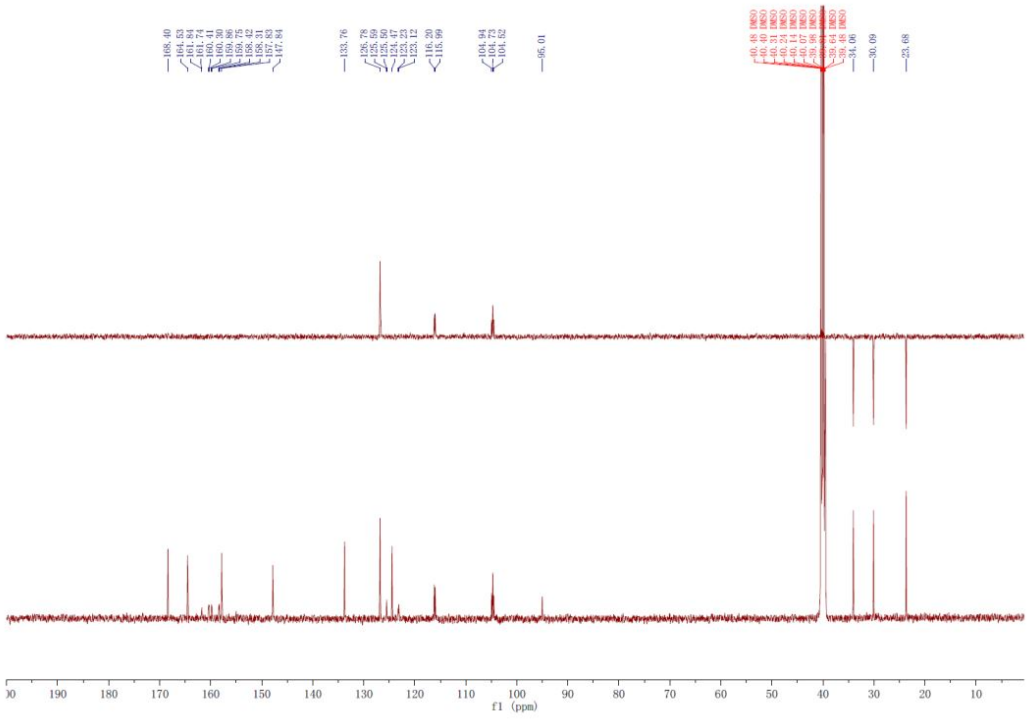
To a solution of compound **3** (441 mg, 2.5 mmol) and compound **4** (822 mg, 2.5 mmol) in EtOH was added EtONa (510 mg, 7.5 mmol), the resulting mixture was refluxed for 1.5 h, the solid was collected and recrystallized from ethanol to give the

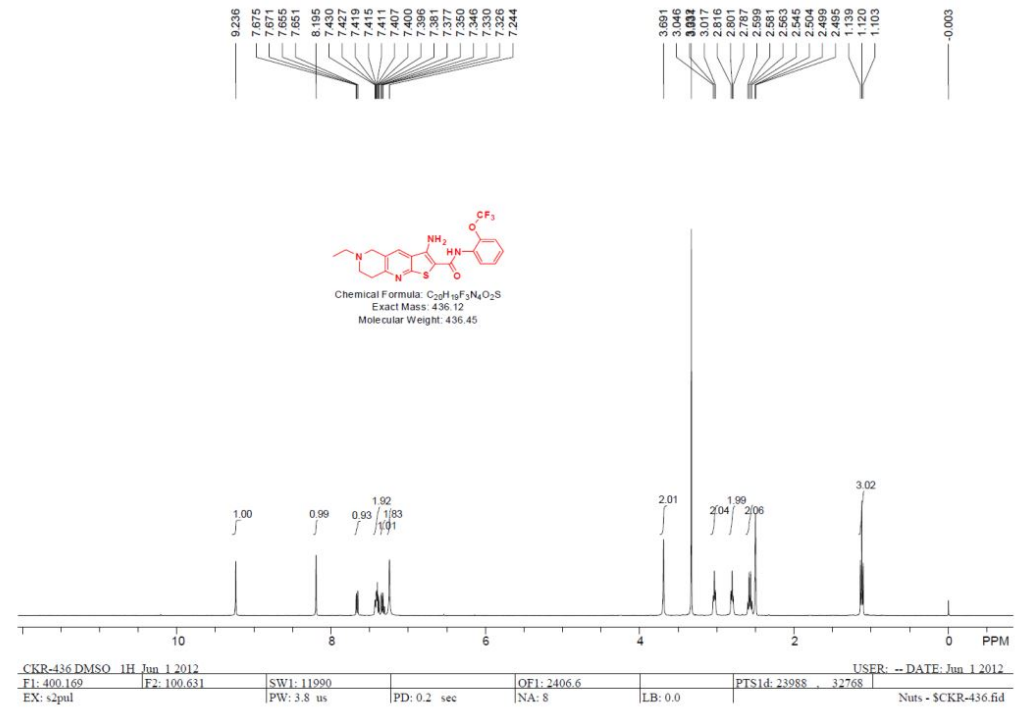
refluxed for 1.5 h, the solid was collected and recrystallized from ethanol to give the target product **DC_AC50** (764 mg, yield 72.0%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.28 (s, 1H), 8.28 (s, 1H), 7.61 (d, J = 7.8 Hz, 1H), 7.49 (td, J = 9.3, 2.8 Hz, 1H), 7.28 (s, 2H), 3.08 – 2.90 (m, 4H), 2.28 – 2.06 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_4) δ 167.82, 164.12, 160.45 (dd, J= 239, 14 Hz), 158.99 (dd, J = 257, 16 Hz), 157.29, 147.38, 133.29, 126.22, 125.10 (d, J = 12 Hz), 123.94, 122.69 (d, J = 15 Hz), 115.67 (d, J = 26 Hz), 104.23 (t), 94.44, 33.61, 29.63, 23.23. LRMS (EI) *m/z*423 $[M]^+$; HRMS (EI) *m/z* calcd $C_{17}H_{12}BrF_2N_3OS$ $[M]^+$ 422.9853, found 422.9851.

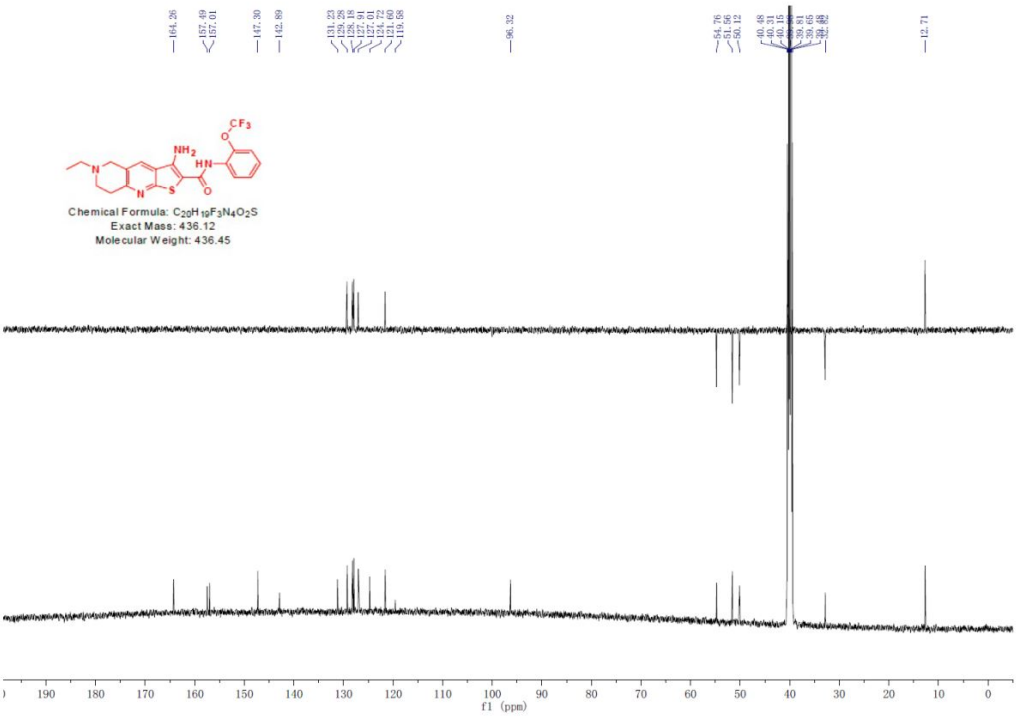
Compound **DC_AC2** was synthesized according to the similar synthetic procedures of **DC_AC50**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (s, 1H), 8.20 (s, 1H), 7.66 (dd, J = 7.9, 1.7 Hz, 1H), 7.45 – 7.37 (m, 2H), 7.35 – 7.30 (m, 1H), 7.24 (s, 2H), 3.69 (s, 2H), 3.03 (t, J = 6.0 Hz, 2H), 2.80 (t, J = 5.9 Hz, 2H), 2.57 (q, J = 7.1 Hz, 2H), 1.12 (t, J = 7.1 Hz, 3H). ^{13}H NMR (125 MHz, DMSO-d_6) δ164.25, 157.48, 156.99, 147.29, 142.88, 131.22, 129.26, 128.17, 127.89, 127.00, 124.71, 121.59, 119.57, 96.31, 54.75, 51.56, 50.12, 32.82, 12.71. LRMS (ESI) *m/z*437 $[M+H]^+$; HRMS (ESI) *m/z* calcd $C_{20}H_{20}F_3N_4O_2S$ $[M+H]^+$ 437.1259, found 437.1260.

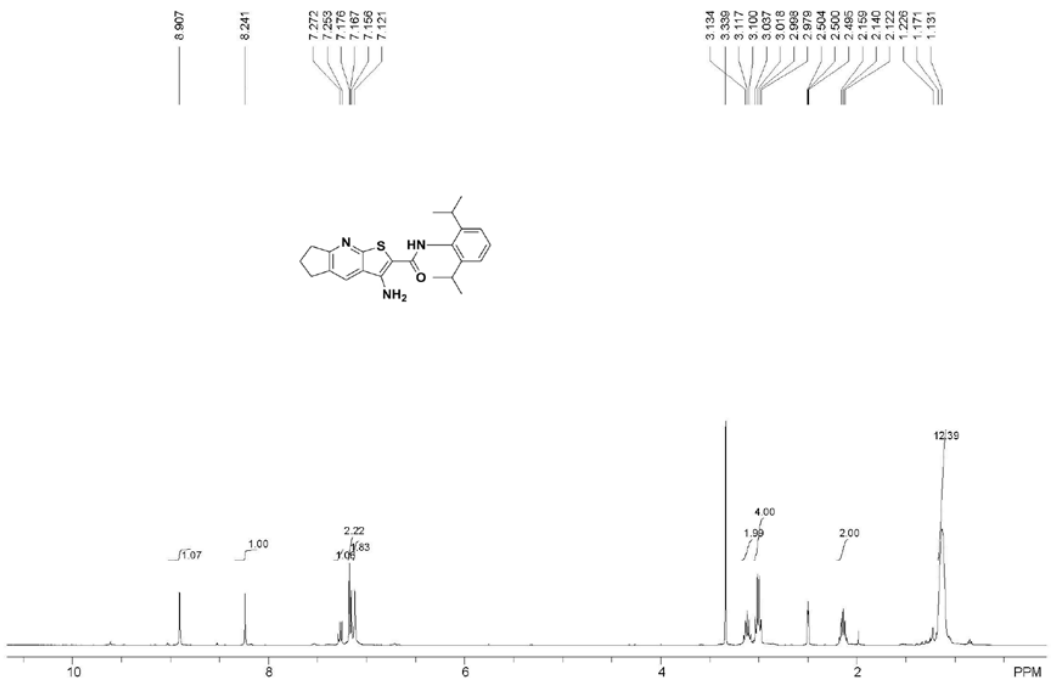
Compound **ZYAT36** was synthesized according to the similar synthetic procedures of **LC-50**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (s, 1H), 8.24 (s, 1H), 7.27 (m, 1H), 7.17 (m, 2H), 7.12 (s, 2H), 3.12 (m, 2H), 3.01 (m, 4H), 2.14 (m, 2H), 1.17 (s, 12H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 168.25, 165.07, 157.25, 146.62, 145.41, 133.98, 130.97, 128.48, 124.79, 123.51, 34.28, 30.33, 28.91, 23.71, 23.68. LRMS (ESI) *m/z*394 $[M+H]^+$; HRMS (ESI) *m/z* calcd for $C_{23}H_{28}N_3OS$ $[M+H]^+$ 394.1948, found: 394.1938.

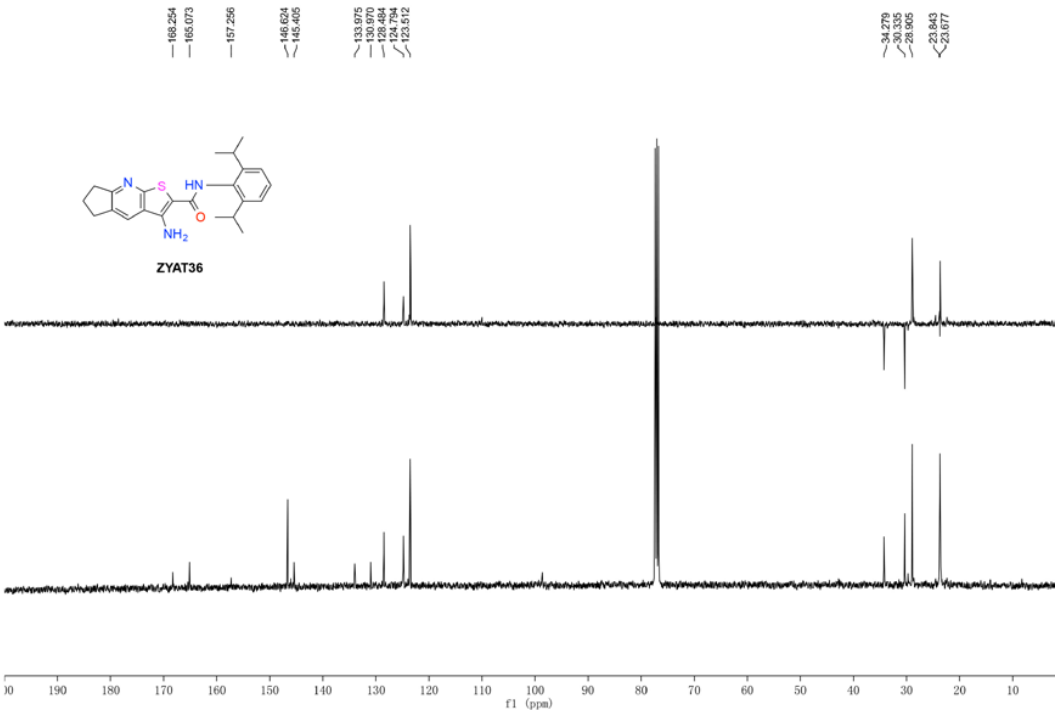












Reference

1. van Dongen, E. M., et al. Variation of linker length in ratiometric fluorescent sensor proteins allows rational tuning of Zn(II) affinity in the picomolar to femtomolar range. *J. Am. Chem. Soc.***129**, 3494-3495 (2007).