Inhibition of Copper Transport Induces Apoptosis in Triple Negative Breast Cancer Cells and Suppresses Tumor Angiogenesis

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SUPPLEMENTARY INFORMATION

Supplementary Data

Figure S1 – Western blot images showing ATOX1 and CCS levels in TNBC cells, corresponding to data presented in Figure 1.

Figure S2 – Dose-response profiles of TM in TNBC cells and DCAC50 in HMEC; caspase-3/7 activity after DCAC50 treatment of HMEC.

Figure S3 – Copper content, copper distribution and iron and zinc content for DCAC50-treated TNBC cells, supplementing Figure 3.

Figure S4 – Protein levels of copper transporters, GSH levels, GSH depletion experiments and SOD1 activity levels in TNBC cells in response to DCAC50 treatment, supplementing Figure 4.

Figure S5 – Dose-response profile for DCAC50 and caspase-3/7 activity after DCAC50 treatment in HuVEC.

Figure S6 – Evaluation of DCAC50 treatment on tumor volumes and angiogenesis in MDA-MB-468 and MDA-MB-231 xenograft mouse models, supplementing Figure 5.

Supplementary Methods – Information about the antibodies and detailed procedures used for Western blot, immunofluorescent staining and SOD activity assay.
Supplementary Figures

Figure S1. Representative Western Blot Images Used for Analysis of Protein Levels in Cell Lysates. Equal amount of total protein was loaded for each cell lysate and analyzed using SDS-PAGE to perform quantitative analysis. All proteins were analyzed on the same blot.

Figure S2. Response to 72h Treatment with TM (TNBC cells) and with DCAC50 (HMEC). A-B. Representative dose-response profiles of TM (TNBC cell lines, A) and of DCAC50 (HMEC, B) evaluating cell proliferation. C. Caspase-3/7 activity in HMEC after 72 h treatment with DCAC50. P-value = 0.46 for 20 μM DCAC50 versus DMSO control, according to two-tailed Student’s t-test.
Figure S3. Copper Content and Distribution in TNBC Cells. A. Copper content was determined by ICP-MS of bulk cell pellets after treatment with DMSO, 20 µM DCAC50 or 30 µM ATTM for 72 h. Mean ± SD of four to six biological replicates. *P < 0.05, compared to DMSO control, according to two-tailed Student’s t-test. B. The ratio of cytoplasmic to nuclear copper concentration in HCC1395 cells (top). Visible light microscope images and XFM elemental maps (bottom) of P, Fe, Cu and Zn are shown for representative cells within each sample, matched to the corresponding data points in the graphs by a square or star. Mean ± SD for n = 9-10 cells. *P < 0.05, compared to DMSO control, according to two-tailed Student’s t-test; ns, not significant. A 10 µm scale bar is included in each Zn map and the relative elemental concentration within each cell is indicated by the intensity scale. C-D. The ratio of cytoplasmic to nuclear iron (C) and zinc (D) concentrations in cells treated with DMSO or 20 µM DCAC50 for 24 hours.
Figure S4. A. Representative Western Blot images used for analysis of protein levels in cell lysates. Cells were treated with 20 µM DCAC50 or DMSO (control) for indicated times and total or fractionated cell lysates were prepared. Equal amount of total protein was loaded for each cell lysate and SDS-PAGE was performed followed by Western Blot. β-Tubulin or Histone H3 (H3) were used as loading controls and were probed from the same experimental samples on the same gel. B. Relative GSH and GSSG levels determined by a luminescence-based assay after 72 hour treatment with DMSO or 20 µM DCAC50. Data are presented as fold-change relative to control, with mean ± standard deviation of three biological replicates. *P < 0.05, compared to DMSO control, according to two-tailed Student’s t-test. C. GSH level after 24 h treatment with 200 µM BSO presented as relative luminescence (mean ± SD of three biological replicates). D. Proliferation of MDA-MB-436 and HCC1395 cells presented as a percentage of confluence after 72 h treatment with 300 µM BSO, 20 µM DCAC50 or their combination. *P < 0.05, according to two-tailed Student’s t-test. E. A semi-quantitative SOD1 activity assay. The SOD1 band in the native PAGE gel was identified by the inhibition of SOD1 activity after soaking a sample in 8 mM H2O2 for 15 minutes prior to staining and comparing to the same sample assayed in the absence of H2O2. F. Representative full native PAGE gel after SOD1 in-gel activity assay. G. Representative Western Blot, corresponding to the native gel shown in (F).
Figure S5. Effect of DCAC50 on cell proliferation and apoptosis in endothelial cells, HuVEC. A. Representative dose-response profile of DCAC50 after 72 h treatment. B. Caspase-3/7 activity assay. Percent change of relative luminescence signal proportional to caspase-3/7 activity after treatment with 20 μM DCAC50 in comparison to DMSO (control) is reported. Mean ± SD of three technical replicates is reported.
Figure S6. Changes in tumor volumes in MDA-MB-468 (A) and MDA-MB-231 (B, C) xenograft mouse models and effect of DCAC50 on angiogenesis in MDA-MB-231 mouse model (D). A-B. Each line represents tumor volume changes during treatment in each animal. C. Tumor volumes at the end of treatment in MDA-MB-231 model. Data are expressed as Mean ± SD. D. Quantification of CD31+ and CD31+CD105+ angiogenic blood vessels in the 400 µm tumor macrosections for DMSO and DCAC50 treatment groups. Data are expressed as Mean ± SD for three biological replicates. *P < 0.05. Z-stack projections of CD31+ and CD105+ angiogenic blood vessels in a representative macrosection. Scale bars are 500 µm.
Supplementary Methods

Western Blot. The membrane was probed with ATOX1 (Abcam, ab54865), CCS and CTR1 (Santa Cruz Biotechnology, sc-55560 and sc-66847, respectively), SOD1 (Cell Signaling, 2770S), β-Actin or β-Tubulin or Histone H3 (Cell Signaling, 8457S, 2128S and 4499S respectively), primary antibodies, followed by IRDye® 800CW Goat anti-Mouse IgG or IRDye® 680RD Goat anti-Rabbit IgG secondary antibodies (LiCOR, 926-32210 and 926-68071, respectively).

Immunofluorescence Staining and Cell Imaging. ATP7B (Santa Cruz Biotechnology, sc-373964), Goat anti-Mouse IgG, FITC antibodies (ThermoFisher, A16073) and 1 µg/mL DAPI were used for staining.

Immunofluorescence Staining of Tumors. The macrosections were stained with DyLight550-anti-CD105 (30 µg/60 µl) and DyLight633-anti-CD31 (2.5 µg/5 µl) in 0.5 mL PBS (pH 7.4, 10 mg/ml BSA, 0.3% Triton X-100) at 4 °C overnight. 3D scanning was performed in a defined X/Y/Z (1.78/1.78/7.5 µm) volume with 10 frame averaging and bidirectional scanning using 550 nm excitation and 557-580 nm filters for DyLight550 and 633 nm excitation and 637-655 nm filter for DyLight633.

In-Gel SOD Activity Assay. For SOD1 activity, the gel was stained with nitro blue tetrazolium (1.23 mM in 50 mM phosphate buffer) for 15 minutes, then incubated with riboflavin (28 µM) and TEMED (28 mM) in phosphate buffer (15 minutes), all in the dark (1). The gel was washed and exposed to ambient light for superoxide generation. Achromatic bands indicated superoxide dismutase activity; the SOD1 band confirmed by soaking in 8 mM H2O2 before staining. Gel imaging was performed with BioRad Gel Doc EZ imager and analyzed with Image Studio Software.

References

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