Supporting Information

Mito-DCA: A mitochondria targeted molecular scaffold for efficacious delivery of metabolic modulator dichloroacetate

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Materials and instrumentation: All chemicals were received and used without further purification unless otherwise noted. Dichloroacetic (DCA) anhydride, tris(hydroxymethyl)aminomethane (Tris), triphenylphosphine (TPP), 6-bromohexanoic acid, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), 3-bromopropanol, 4-dimethylaminopyridine (DMAP), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and adenosine 5'-triphosphate (ATP) disodium salt hydrate were purchased from Sigma-Aldrich. Tetramethyl rhodamine methyl ester (TMRM) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide or JC-1 were procured from Invitrogen. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was purchased from Sigma-Aldrich. Alexa Fluor® 488 Annexin V/Dead cell apoptosis kit was purchased from Invitrogen. Lactate assay kit was obtained from
BioVision, CA, USA. CellTiter-Glo® luminescent cell viability assay kit from Promega was used to quantify cellular ATP content. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from R&D systems. Interleukin (IL)-6, IL-2, IL-10, interferon-gamma (IFN-γ), IL-12, IL-4, and tumor necrosis factor (TNF)-α cytokines were tested using BD OptEIA mouse enzyme-linked immunosorbent assay (ELISA) kits. IL-6, IL-2, IL-10, IFN-γ, IL-12, IL-4, and TNF-α cytokines on human cancer cell supernatants were tested using R&D systems human ELISA kits. Ultra-pure lipopolysaccharide (LPS) was purchased from Invivogen, CA, USA. CD11c microbeads for dendritic cell (DC) purification was purchased from Miltenyi Biotech, CA USA. Mesenchymal stem cell basal medium recombinant human fibroblast growth factor-basic, recombinant human fibroblast growth factor-acidic, and recombinant human epithelial growth factor were purchased from ATCC.

Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 MΩ) containing a 0.22 μm filter. ¹H, ¹³C spectra were recorded on a 400 MHz and ³¹P NMR spectra recorded on a 500 MHz Varian NMR spectrometer, respectively. High-performance liquid chromatography (HPLC) analyses were made on an Agilent 1200 series instrument equipped with a multi-wavelength UV-visible and a fluorescence detector. Flow cytometry studies were performed on a BD LSRII flow cytometer equipped with digital acquisition using FACSDiva v6. Plate reader analysis was performed on a Bio-Tek Synergy HT microplate reader. Confocal images were recorded in a Nikon A1 confocal microscope. X-ray intensity data were collected at 100 K on a Bruker APEX CCD diffractometer. Electrospray ionization mass spectrometry (ESI-MS) and high-resolution mass spectrometry (HRMS)-ESI were recorded on Perkin
Elmer SCIEX API 1 plus and Thermo scientific ORBITRAP ELITE instruments, respectively. Bioenergetics assays were carried out using a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA, USA).

**Methods**

**Synthesis of TPP-(CH$_2$)$_5$-COOH:** A mixture of 6-bromohexanoic acid (2.0 g, 10.3 mmol) and TPP (2.8 g, 10.8 mmol) was heated to reflux for 24 h in acetonitrile. The solvent was evaporated to dryness. The resulting residue was washed with diethyl ether (3 x 30 mL) followed by vacuum drying to afford a white solid as a pure product. Yield: 91 % (4 g). Melting point: 200-205 °C; $^1$H NMR (CDCl$_3$): $\delta$ 9.3 (s, 1H), 7.6-7.8 (m, 15H), 3.5 (t, 2H), 2.3 (t, 2H), 1.6 (m, 6H) (Figure S1). $^{13}$C NMR (CDCl$_3$): $\delta$ 175, 135, 133.6, 130.6, 118.5, 34.2, 29.37, 23.9, 22.8, 22.29, 21.9 (Figure S1). $^{31}$P NMR (CDCl$_3$) 24.34 ppm (Figure S1). HRMS-ESI (m/z): [M-Br]$^+$ calcd. for C$_{24}$H$_{26}$O$_2$P$^+$, 377.1665; found, 377.1629 (Figure S2).

**Synthesis of TPP-Tris-(OH)$_3$:** TPP-(CH$_2$)$_5$-COOH (0.50 g, 1.1 mmol), Tris (0.15 g, 1.2 mmol), and EEDQ (0.32 g, 1.3 mmol) were dissolved in ethanol. This mixture was stirred at 50 °C for 12 h followed by drying under vacuum. The crude mixture was recrystallized 3-4 times using ethanol/CH$_2$Cl$_2$/diethyl ether (5:5:90) to give a white solid of TPP-Tris-(OH)$_3$ in 86 % yield (0.52 g). Melting point: 115-120 °C; $^1$H NMR (CDCl$_3$): 7.7 (m, 15H), 3.7 (s, 6H), 3.5 (t, 2H), 2.4 (t, 2H), 1.7 (m, 6H) ppm (Figure S3). $^{13}$C NMR (CDCl$_3$): $\delta$ 175.6, 135.2, 133.6, 130.6, 118.4, 113.2, 64.1, 62.3, 36.2, 29.3, 24.9, 22.5, 21.5 ppm (Figure S3). $^{31}$P NMR (CDCl$_3$) 24.28 ppm (Figure S3). HRMS-ESI (m/z): [M-Br]$^+$ calcd. for C$_{28}$H$_{35}$NO$_4$P$^+$, 480.2298; found, 480.2243 (Figure S4).
**Synthesis of TPP-(CH$_2$)$_3$-OH:** A mixture of 3-bromopropanol (1.0 g, 7.2 mmol) and TPP (2.1 g, 7.9 mmol) in toluene (25 mL) was heated to reflux for 24 h. The resulting white precipitate was filtered through fritted glass followed by the washing with diethyl ether (3 x 30 mL). The white solid was dried under vacuum to get the pure product of TPP-(CH$_2$)$_3$-OH. Yield, 61 % (1.7 g). Melting point: 235-240 °C; $^1$H NMR (CDCl$_3$): 7.65-7.80 (m, 15H), 4.94 (t, 1H), 3.70-3.83 (m, 4H), 1.81 (m, 2 H) ppm (Figure S9). $^{13}$C NMR (CDCl$_3$): δ 135.09, 133.47, 130.51, 118.72, 60.40, 25.92, 20.43 ppm (Figure S9). $^{31}$P NMR (CDCl$_3$) 24.77 ppm (Figure S9). HRMS-ESI (m/z): [M-Br]$^+$ calcd. for C$_{21}$H$_{22}$OP$^+$, 321.1403; found, 321.1403 (Figure S10).

**HPLC Studies:** HPLC studies were carried out using an Agilent 1200 series instrument to explore the purity of the samples. A 5 µL of 5 mM solution of Mito-DCA in DMSO was injected using a Zorbax C18 column and a 50:50 acetonitrile:isopropanol-1% trifluoroacetic acid as a mobile phase (Figure S8). The wavelength used for these experiments was 268 nm.

**Solubility and Stability Studies:** In order to confirm the solubility of Mito-DCA in the aqueous medium solubility check experiments were performed by diluting DMSO stock solution (5 mM) of Mito-DCA by phosphate buffered saline (PBS) to get the concentrations similar to that of MTT experiments. For stability study, a 5 mM solution of Mito-DCA or TPP-DCA in 10% DMSO in PBS was incubated for 4-12 h at room temperature. This solution was used in ESI-MS experiments to check the degradation behavior of TPP compounds (Figures S13 and S14).

**X-Ray Crystallographic Study:** Colorless crystals of Mito-DCA suitable for X-ray diffraction were grown by slow diffusion of diethyl ether into a saturated solution of Mito-
DCA in dichloromethane at 25 °C. A single crystal was mounted on the top of a glass fiber. Intensity data were collected at 100 K on a Bruker APEX CCD diffractometer using the SMART software, with Mo-Kα radiation (λ=0.71073 Å) using ω-scan technique. The data were collected in 1464 frames with 10 sec exposure. The SAINT software was used for data integration. The structure was solved by direct methods using the SHELXTL 6.1 software package.\(^1\) Non-hydrogen atomic scattering factors were taken from the literature tabulations.\(^2\) Non-hydrogen atoms were located from successive difference Fourier map calculations. Empirical absorption corrections were applied with SADABS.\(^3\) One O atom and two Cl atoms in the cation of molecule were found disordered in two sets, for the O atom labeled as O(5) (one set) and O(5′) (another set), for the two Cl atoms labeled as Cl(3), Cl(4) (one set) and Cl(3′), Cl(4′) (another set), respectively. Each of these two sets is divided using the PART commands with proper restraints and refined occupancies. The set of O(5) has 73.379% occupancies while the other (O(5′)) has 26.621% occupancies. The set of Cl(3), Cl(4) has 81.997% occupancies while the other (Cl(3′), Cl(4′)) has 18.003% occupancies. In the final cycles of each refinement, all the non-hydrogen atoms were refined in anisotropic displacement parameters. The hydrogen atom positions were calculated and allowed to ride on the carbon to which they are bonded assuming a C–H bond length of m Å (m = 1.00 for CH groups, m = 0.99 for CH\(_2\) groups, m = 0.98 for CH\(_3\) groups, m = 0.95 for Ph-H groups). Hydrogen atom temperature factors were fixed at n (n = 1.2 for CH and CH\(_2\) groups, n = 1.5 for CH\(_3\) groups, n = 1.2 for Ph-H groups) times the isotropic temperature factor of the C-atom to which they are bonded. A perspective view of Mito-DCA was obtained with ORTEP (Figure S7). A summary of crystal data,
intensity collection and refinement parameters, geometry, and important bond angle and bond distances are given in Table S1 and S2. Crystal structure data for Mito-DCA can be accessed from the Cambridge Crystallographic Data Centre (CCDC; www.ccdc.cam.ac.uk) with accession number CCDC 940383.

**JC-1 Assay:** PC3 cells were cultured on a live cell imaging glass bottom dish at a density of 1x10^6 cells/mL and allowed to grow overnight at 37 °C. Cells were treated with 150 µM Na-DCA, 150 µM TPP-DCA, 50 µM TPP-Tris-(OH)_3, and 50 µM Mito-DCA for 6 h at 37 °C. A solution of JC-1 reagent (10 µg/mL in RPMI) was added and incubation was carried out at 37 °C for 20 min. The cells were washed 3 times with PBS, and live cell imaging was performed in phenol red free RPMI media. Quantification of fluorescence intensities and corrected total cell fluorescence (CTCF) values were carried out using ImageJ. Mitochondrial uncoupling by FCCP (50 µM, 1 h) was carried out as a control to cause mitochondrial depolarization and collapse of the mitochondrial membrane potential prior to JC1 assay (Figure 2 and Figure S15). PC3 cells were cultured on a six well plate at a density of 1x10^6 cells/mL and allowed to grow overnight at 37 °C. Cells were treated with 150 µM Na-DCA, 150 µM TPP-DCA, 50 µM TPP-Tris-(OH)_3, and 50 µM Mito-DCA for 6 h at 37 °C. A solution of JC1 reagent (10 µg/mL in RPMI) was added and incubation was carried out at 37 °C for 10 min. The cells were washed 3 times with PBS and trypsinized for 15 min at 37 °C. The cells were isolated and washed 3x by centrifugation (1,800 rpms for 3 min at 4 °C). The resulting cell pellet was resuspended in 100 µL PBS and analyzed by flow cytometry using 488 nm and 633 nm excitations with 530 nm and 660 nm bandpass filters (Figure 2).
**TMRM Assay:** PC3 cells were cultured on a live cell imaging glass bottom dish at a density of $1 \times 10^6$ cells/mL and allowed to grow overnight at 37 °C. Cells were treated with 50 $\mu$M TPP-Tris-(OH)$_3$, 150 $\mu$M Na-DCA, 150 $\mu$M TPP-DCA, and 50 $\mu$M Mito-DCA for 6 h at 37 °C. A solution of TMRM was added and incubation was carried out at 37 °C for 20 min. The cells were washed 3 times with PBS, and live cell imaging was performed in phenol red free RPMI media (Figure S16).

**MTT Assay and Data Analysis:** The cytotoxic behaviors of Mito-DCA, TPP-DCA, Na-DCA, and TPP-Tris-(OH)$_3$ were evaluated using the MTT assay against PC3, DU145, LNCaP, and MSC cells. Cells (2000 cells/well for PC3, DU145, and MSC cells; 5000 cells/well for LNCaP) were seeded on a 96-well plate in 100 $\mu$L of desired medium and incubated for 24 h. The cells were treated with different DCA compounds and TPP-Tris-(OH)$_3$ at varying concentrations and incubated for 72 h at 37 °C. The cells were then treated with 20 $\mu$L of MTT (5 mg/mL in PBS) for 5 h. The medium was removed, the cells were lysed with 100 $\mu$L of DMSO, and the absorbance of the purple formazan was recorded at 550 nm using a Bio-Tek Synergy HT microplate reader. Each well was performed in triplicate. All experiments were repeated three times. Cytotoxicity was expressed as mean percentage increase relative to the unexposed control ± SD. Control values were set at 0% cytotoxicity or 100% cell viability. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a three parameters logistic model used to calculate the IC$_{50}$, which is the concentration of chemotherapeutics causing 50% inhibition in comparison to untreated controls. The mean IC$_{50}$ is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent measurements that were
reproducible and statistically significant. The IC$_{50}$ values were reported at ±99% confidence intervals. This analysis was performed with GraphPad Prism (San Diego, U.S.A).

**Apoptosis Detection by Flow Cytometry:** PC3 and MSC cells were seeded at a density of 1x10$^6$ cells/mL on each well of a six well plate and allowed to grow overnight. Cells were treated with 150 $\mu$M Na-DCA, 150 $\mu$M TPP-DCA, and 50 $\mu$M Mito-DCA for 12 h at 37 °C. As positive controls, etoposide (100 $\mu$M, incubation time: 12 h) for apoptosis and H$_2$O$_2$ (1 mM, incubation time: 45 min) for necrosis were used. The cells were trypsinized, repeatedly washed with cold PBS, and centrifuged at 1800 RPM for 3 min, and the supernatants were discarded. Cell density was determined and cells were resuspended in 1X Annexin-binding buffer to ~1x10$^6$ cells/mL preparing a sufficient volume to have 100 $\mu$L per assay. To 100 $\mu$L of cell suspension, 5 $\mu$L Alexa Fluor® 488 Annexin V and 1 $\mu$L (100 $\mu$g/mL) PI working solution were added, and incubated for 15 min at room temperature. After the incubation period, 400 $\mu$L 1X Annexin-binding buffer was added to each sample, samples were gently mixed keeping the samples on ice and the samples were analyzed on the flow cytometer immediately.

**Intra and Extracellular Lactate Determination:** PC3, LNCaP, and MSC cells were seeded at a density of 1x10$^6$ cells/mL on each well of a 12 well plate and allowed to grow overnight at 37 °C under 5% CO$_2$. Cells were treated with 10 or 50 $\mu$M TPP-Tris-(OH)$_3$, 30 or 150 $\mu$M Na-DCA, 30 or 150 $\mu$M TPP-DCA, and 10 or 50 $\mu$M Mito-DCA for 3 or 6 h at 37 °C. The media was removed and the cells were homogenized. The lysate and the media were separately added to the enzyme and substrate working reagent mixture and incubated for 30 min. Lactate concentrations in the cell lysates and media
were measured using Bio-Tek Synergy HT microplate reader at 450 nm and lactate concentration were calculated using a standard curve.

**CellTiter-Glo® Luminescent ATP Quantification:** PC3, LNCaP, and MSC cells were plated at a density of 10,000 cells per well in 96-well plates compatible with the luminometer used in 100 µL media. TPP-Tris-(OH)$_3$ (20 µM), Mito-DCA (20 µM), TPP-DCA (60 µM), and Na-DCA (60 µM) were added to experimental wells, and incubated for 3 h at 37 °C in 5% CO$_2$ atmosphere. Control wells containing medium without cells were prepared to obtain a value for background luminescence. Plates were then equilibrated at room temperature for ~30 min. A volume of CellTiter-Glo reagent equal to the volume of cell culture medium present in each well was added, this mixtures were well mixed for 2 min using a shaker to induce cell lysis. The plates were allowed to incubate at room temperature for 10 min to stabilize luminescent signal. Luminescence was recorded using a plate reader. ATP quantification was carried out from a standard curve prepared in culture media using ATP disodium salt hydrate.

**Seahorse XF24 Bioenergetics Assays:** The three key parameters of glycolytic function: glycolysis, glycolytic capacity, and glycolytic reserve were assessed using a Seahorse XF glycolysis stress kit. Prior to the assay, XF sensor cartridges were hydrated. To each well of an XF utility plate, 1 mL of Seahorse Bioscience calibrant was added and the XF sensor cartridge was placed on top of the utility plate, and kept at 37 °C without CO$_2$ for 14 h. PC3 cells were cultured in XF24-well cell culture microplates (Seahorse Bioscience) at a density of 5x10$^4$ cells/well (0.32 cm$^2$) in 100 µL growth medium and incubated for 1 h at 37 °C in 5% CO$_2$ atmosphere. After the cells were attached, an additional 100 µL growth medium was added and the cells were incubated
for 24 h at 37 °C in 5% CO₂ atmosphere. The cells were treated with TPP-DCA (30 µM), Na-DCA (30 µM), and Mito-DCA (10 µM) for 4 h at 37 °C in 5% CO₂ atmosphere. After 4 h, all but 50 µL of the culture medium was removed from each well and the cells were rinsed two times with 600 µL of XF stress test glycolysis optimization medium pre-warmed to 37 °C and finally 610 µL of optimization medium was added to each well and the plate was placed at 37 °C without CO₂ for 1 h prior to assay. The OCR, a measure of mitochondrial respiration; and the ECAR, a marker for glycolysis, were measured simultaneously for 16 min to establish a baseline rate. Glycolysis, glycolytic capacity, and glycolytic reserve were calculated by subtracting the average rates before and after the addition of glucose (10 mM), ATP synthase inhibitor oligomycin (1.0 µM), and 2-DG (100 mM). These three compounds were injected consecutively with specific time gap and ECAR values were measured after each injection.

Different parameters of respiration: basal respiration, coupling efficiency, and spare respiratory capacity were investigated by using Seahorse XF-24 cell Mito Stress Test Kit. PC3 cells were plated and treated with different constructs (Mito-DCA 10 µM for 3 h or 4 h or 6 h; TPP-DCA 30 µM for 4 h; Na-DCA 30 µM for 4 h) as mentioned above except the final volume was 500 µL. Different parameters of respiration were calculated by subtracting the average respiration rates before and after the addition of the electron transport inhibitors oligomycin (3.0 µM), FCCP (3.0 µM), an ionophore that is a mobile ion carrier, and a mixture of antimycin-A (3.0 µM) which is a complex III inhibitor, binds to the Qi site of cytochrome c reductase, thereby inhibiting the oxidation of ubiquinol in the electron transport chain of OXPHOS and rotenone (3.0 µM), a mitochondrial inhibitor that prevents the transfer of electrons from the Fe-S
center in Complex I to ubiquinone. The parameters calculated included: basal respiration (baseline respiration minus antimycin-A post injection respiration), ATP turnover (baseline respiration minus oligomycin post injection respiration), maximal respiratory capacity (FCCP stimulated respiration minus antimycin-A post injection respiration) and reserve respiratory capacity (FCCP stimulated respiration minus baseline respiration). All test articles had five replicates.

**Generation of Bone Marrow Derived Dendritic Cells (BMDCs):** BMDCs were isolated from 6-8 weeks old C57BL/6 mice. After euthanization, bone marrows were isolated by flushing mouse femurs in RPMI. The harvested cells were centrifuged at 1250 rpm for 10 min and the pellet was resuspended in ice-cold 2 mL of buffer to lyse erythrocytes. The cells were counted, resuspended, and transferred to petri dishes at the final concentration of $1.5 \times 10^6$ cells/mL. To this culture, granulocyte-macrophage colony-stimulating factor (20 ng/mL) was added to generate BMDCs. Media was changed on days 2 and 4. On day 6 cells were processed further to obtain pure DC population by subjecting cells to MACS bead purification using anti-CD11c antibody as per manufacturer’s instructions. DC purity was tested by incubating BMDCs with lipopolysaccharide (LPS) (100 ng/mL) and measuring the surface expression of CD11c.

**Anti-tumor Immunity Study by ELISA.** PC3 cells were plated at a concentration of $0.5 \times 10^6$ cells/mL in six well plates and allowed to grow for 12 h. The cells were incubated with 150 $\mu$M Na-DCA, 150 $\mu$M TPP-DCA, and 50 $\mu$M Mito-DCA for 24 h at 37 °C. PC3 cell supernatants were added to freshly prepared BMDCs ($0.5 \times 10^6$ cells/mL) and incubated at 37 °C for 24 h. Additionally, lipopolysaccharides (LPS) alone (100 ng/mL) as a control was added to the DC cultures. The DCs were centrifuged at 1,800
RPM for 3 min and ELISA was performed on the supernatants against the cytokines IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ according to the manufacturer’s protocol. Briefly, antibody coated plates were blocked with 10% FBS in PBS for 1 h at room temperature followed by washings. BMDC supernatants were incubated on the plates for 1 h at room temperature. This was immediately followed by washings and sequential incubations with the cytokine-biotin conjugate and streptavidin working solution. Finally, the substrate reagent containing 3,3',5,5' tetramethylbenzidine (100 µL) was added to each well, incubated for 30 min, the reaction was stopped by adding 50 µL stop solution containing 0.1 M H₂SO₄. The absorbance was recorded at 450 nm using a BioTek Synergy HT well plate reader.
Figure S1. $^1$H, $^{13}$C, and $^{31}$P NMR of TPP-(CH$_2$)$_5$-COOH in CDCl$_3$. 
Figure S2. Positive ion ESI-MS spectrum of TPP-(CH$_2$)$_5$-COOH and isotopic pattern analysis.
Figure S3. $^1$H, $^{13}$C, and $^{31}$P NMR of TPP-Tris-(OH)$_3$ in CDCl$_3$.
Figure S4. Positive ion ESI-MS spectrum of TPP-Tris-(OH)₃ and isotopic pattern analysis
Figure S5. $^1$H, $^{13}$C, and $^{31}$P NMR of Mito-DCA in CDCl$_3$. 
Figure S6. Positive ion ESI-MS spectrum of Mito-DCA and isotopic pattern analysis.
Figure S7. ORTEP diagram showing 50% probability thermal ellipsoids with all atom labels for Mito-DCA.
Figure S8. HPLC chromatograms of TPP compounds.
Figure S9. $^1$H, $^{13}$C, and $^{31}$P NMR of TPP-(CH$_2$)$_3$-OH in CDCl$_3$. 
Figure S10. Positive ion ESI-MS spectrum of TPP-(CH$_2$)$_3$-OH and isotopic pattern analysis.
Figure S11. $^1$H, $^{13}$C, and $^{31}$P NMR of TPP-DCA in CDCl$_3$. 
Figure S12. Positive ion ESI-MS spectrum of TPP-DCA and isotopic pattern analysis.
Figure S13. Photographs of solubility test of Mito-DCA in water-DMSO.
Figure S14. Stability of Mito-DCA and TPP-DCA by ESI-MS.
Figure S15. Changes in the $\Delta\psi_m$ by the JC-1 assay. Top: Treatment of PC3 cells with 50 $\mu$M TPP-Tris-(OH)$_3$ for 6 h. Bottom: Treatment of PC3 cells with 50 $\mu$M of FCCP for 1 h. Cells were stained with JC-1 for 15 min. Green fluorescence, depolarized (J-monomer) mitochondria; red fluorescence, hyperpolarized (J-aggregates). DIC: Differential Interference Contrast.
**Figure S16.** Reversal of mitochondrial membrane potential by TMRM live cell imaging assay. Mito-DCA significantly depolarized the mitochondria of PC3 cells at a concentration of 50 µM but Na-DCA (150 µM), TPP-DCA (150 µM), and TPP-Tris-(OH)₃ (50 µM) had no effect on these cells.
**Figure S17.** Cytotoxicity of TPP-Tris-(OH)$_3$ in PC3, LNCaP, DU145, and MSC cells as determined by the MTT assay.
Figure S18. FACS analysis using annexin V-alexa fluor 488/PI staining for apoptosis detection in MSC and PC3 cells. Cells in the lower right quadrant indicate Annexin-positive/PI negative, early apoptotic cells. The cells in the upper right quadrant indicate Annexin-positive/PI positive, late apoptotic or necrotic cells. As positive controls, etoposide (100 \( \mu \text{M} \), incubation time: 12 h) and \( \text{H}_2\text{O}_2 \) (1 mM, incubation time: 45 min) treated cells were used to determine the early apoptotic and late apoptotic population.
Figure S19. (A) Intracellular lactate levels and lactate in the media in PC3 cells after treatment with 10 μM TPP-Tris-(OH)₃, 30 μM Na-DCA, 30 μM TPP-DCA, and 10 μM Mito-DCA for 3 h at 37 °C. (B) Intracellular lactate levels in LNCaP and MSC cells after treatment with 50 μM TPP-Tris-(OH)₃, 150 μM Na-DCA, 150 μM TPP-DCA, and 50 μM Mito-DCA for 6 h at 37 °C. (C) Changes in intracellular ATP content associated with the lactate fluctuation in the LNCaP and MSC cells after treatment with 60 μM Na-DCA, 60 μM TPP-DCA, and 20 μM Mito-DCA for 3 h at 37 °C. Statistical analyses were performed using GraphPad Prism software 5.00. A one-way ANOVA with a post-hoc Tukey test was used to identify significant differences among the groups.
Figure S20. Intracellular ATP levels in PC3 cells after treatment with TPP-Tris-(OH)$_3$ (50 µM), Mito-DCA (50 µM), TPP-DCA (150 µM), and Na-DCA (150 µM) for 6 h. Statistical analyses were performed using GraphPad Prism software 5.00. A one-way ANOVA with a post-hoc Tukey test was used to identify significant differences among the groups.
**Table S1.** Summary of crystal data, intensity collection and refinement parameters, and geometry of Mito-DCA

| Parameter                          | Value                      |
|------------------------------------|----------------------------|
| Formula                            | C_{38}H_{45}BrCl_{6}NO_{8}P |
| Molecular mass, g.mol^{-1}         | 967.33                     |
| Morphology                         | Colorless plate            |
| Dimension, mm                      | 0.24 × 0.20 × 0.03         |
| Crystal system                     | Orthorhombic               |
| Space group                         | Pca2_{1}                   |
| a                                  | 24.304(3) Å               |
| b                                  | 13.1642(19) Å             |
| c                                  | 14.2312(2) Å              |
| V, Å³                              | 4553.3(11) Å              |
| Z                                  | 4                          |
| ρ_{calc}, g.cm^{-3}                | 1.411                      |
| µ, mm^{-1}                         | 1.337                      |
| F(000)                             | 1984                      |
| θ range, deg.                      | 2.28 to 25.68              |
| Total/unique reflection            | 49.524/8,640               |
| Completeness to 2θ, %              | 99.9                      |
| Data/restraints/parameters         | 8,640/25/525               |
| GoOF                               | 1.002                     |
| R₁ *                               | 0.0629                     |
| wR₂ †                              | 0.1556                     |
| Largest diff. peak and hole, e Å³  | 0.523 and -0.495          |

*R₁ = \sum |F_o| - |F_c| / \sum |F_o|

†wR₂ = \sqrt{\sum[w(F_o^2-F_c^2)^2]/\sum[w(F_o^2)^2]}^{1/2}
Table S2. Bond distances [Å] and angles [deg.] for Mito-DCA.

| Bond lengths [Å] and angles [deg] |  |
|-----------------------------------|---|
| P(1)-C(29)                       | 1.780(7) |
| P(1)-C(23)                       | 1.786(8) |
| P(1)-C(1)                        | 1.796(7) |
| P(1)-C(17)                       | 1.795(7) |
| N(1)-C(6)                        | 1.358(9) |
| N(1)-C(7)                        | 1.460(9) |
| O(1)-C(6)                        | 1.222(9) |
| O(2)-C(9)                        | 1.329(9) |
| O(2)-C(8)                        | 1.447(9) |
| O(3)-C(9)                        | 1.198(8) |
| O(4)-C(12)                       | 1.312(10) |
| O(4)-C(11)                       | 1.432(8) |
| O(5)-C(12)                       | 1.251(15) |
| O(5)-C(12)                       | 1.202(19) |
| O(6)-C(15)                       | 1.336(9) |
| O(6)-C(14)                       | 1.461(8) |
| O(7)-C(15)                       | 1.220(8) |
| Cl(1)-C(10)                      | 1.769(7) |
| Cl(2)-C(10)                      | 1.772(8) |
| Cl(3)-C(13)                      | 1.767(9) |
| Cl(4)-C(13)                      | 1.801(10) |
| Cl(5)-C(13)                      | 1.728(15) |
| Cl(4')-C(13)                     | 1.763(14) |
| Cl(5)-C(16)                      | 1.756(8) |
| Cl(6)-C(16)                      | 1.793(7) |
| C(1)-C(2)                        | 1.543(10) |
| C(2)-C(3)                        | 1.524(9) |
| C(3)-C(4)                        | 1.525(10) |
| C(4)-C(5)                        | 1.529(10) |
| C(5)-C(6)                        | 1.519(11) |
| C(7)-C(14)                       | 1.516(10) |
| C(7)-C(11)                       | 1.547(9) |
| C(7)-C(8)                        | 1.539(10) |
| C(9)-C(10)                       | 1.518(11) |
| C(12)-C(13)                      | 1.507(13) |
| C(15)-C(16)                      | 1.489(10) |
| C(17)-C(18)                      | 1.382(9) |
| C(17)-C(22)                      | 1.390(9) |
| C(18)-C(19)                      | 1.416(11) |
| C(19)-C(20)                      | 1.358(11) |
| C(20)-C(21)                      | 1.408(10) |
| O(2)-C(9)-C(10)                  | 108.8(6) |
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