Supplemental Information

Assessing the Mitochondrial Membrane Potential in Cells and In Vivo using Targeted Click Chemistry and Mass Spectrometry

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Figure S1. Synthetic Schemes. Related to Fig 1.

(A) Synthesis of MitoOct (6). Conditions: a) pyridinium chlorochromate, dichloromethane; b) semicarbazide hydrochloride, sodium acetate, ethanol; c) selenium dioxide, dioxane/water (1:1); d) n-butyl lithium, methyl iodide, tetrahydrofuran, −78ºC; e) p-nitrophenylchloroformate, 4-dimethylaminopyridine, triethylamine, dichloromethane; f) 3-(aminopropyl)triphenylphosphonium bromide hydrobromide, triethylamine.

(B) Synthesis of MitoAzido (7). Conditions: a) sodium azide, 1:1 ethanol/water, reflux.

(C) Synthesis of MitoClick (9). Conditions: a) methanol, RT.

Figure S1

A

\[
\begin{align*}
\text{HO} & \rightarrow \text{HO} \\
\text{OH} & \rightarrow \text{OH} \\
\text{OH} & \rightarrow \text{ON} \\
\text{HO} & \rightarrow \text{NH} \\
\text{HO} & \rightarrow \text{NO} \\
\text{H} & \rightarrow \text{Se} \\
\end{align*}
\]

B

\[
\begin{align*}
\text{I} & \rightarrow \text{I} \\
\text{P(Ph)₃} & \rightarrow \text{P(Ph)₃} \\
\end{align*}
\]

C

\[
\begin{align*}
\text{I} & \rightarrow \text{I} \\
\text{P(Ph)₃} & \rightarrow \text{P(Ph)₃} \\
\end{align*}
\]
Figure S2. Analysis of MitoOct, MitoAzido and MitoClick by LC-MS/MS. Related to Fig 2.

(A) Reaction of MitoOct (6) with Tet (8) to form MitoOct<sub>tet</sub> (10). Conditions: a) methanol, RT.

(B) Effective quenching of MitoOct by Tet. Rat liver mitochondria were incubated with 5 µM each of MitoAzido and MitoOct for 45 min and then extracted ± 50 µM Tet. Sample were analyzed by LC-MS/MS and the amount of MitoClick determined. Data are means ± SEM, n = 3.

(C) Typical standard curves for MitoAzido, MitoOct<sub>tet</sub> and MitoClick. For each experiment, standard curves were prepared by spiking 50 mg wet weight mouse liver homogenate with a range of either MitoAzido, MitoOct<sub>tet</sub> or MitoClick concentrations, followed by extraction and generation of standard curves. The equations of the lines are shown and r² is the square of Pearson's product-moment correlation coefficient. The insets to the Figures show the data at lower compound concentrations.

(D) Reaction of MitoAzido and MitoOct analyzed by LC-MS/MS. Equal concentrations of MitoAzido and MitoOct were incubated in 150 µL KCl medium at 37°C. Duplicate incubations were carried out for the indicated times and then 10 µL was taken and added to 0.5 mL 20% ACN/0.1% FA with 50 µM Tet. The concentration of MitoClick formed at each time point was determined by LC-MS/MS and plotted against time.

(E) Rate of click reaction between MitoAzido and MitoOct. From the slopes of the lines in (D), the rates of MitoClick formation at a range of MitoAzido and MitoOct concentrations were plotted against the product of the concentrations. The slope of this plot gave a rate of 162 x 10⁻³ M⁻¹s⁻¹ at 37°C for the reaction of MitoOct and MitoAzido, within the range of 10⁻² to 1 M⁻¹s⁻¹ reported for strain-promoted alkyne-azide cycloaddition reactions (Lang and Chin, 2014).
Figure S3. Application of the MitoClick approach to mice in vivo. Related to Fig 4.

(A, B) MitoAzido, MitoOct$_{tot}$, and MitoClick in mouse kidney (A) and liver (B) in vivo. Mice were administered 50 nmol each of MitoAzido and MitoOct by tail vein injection, and after the indicated times the mice were sacrificed. Kidney and liver were removed, and the contents of MitoAzido, MitoOct$_{tot}$ and MitoClick analysed by LC-MS/MS. Data are mean ± SEM of 3 separate mice per time point.
**SUPPLEMENTAL TABLES**

### Table S1

| Conditions | Reactants (equiv.) | Products (%)<sup>a</sup> |
|------------|-------------------|--------------------------|
| Control    | 6 7 8             | 6 7 8 9 10               |
| + 1xTet    | 1 1 1             | 0 95 5 0 100             |
| + 10xTet   | 1 1 10            | 0 100 25<sup>b</sup> 0 100 |

**Table S1: Inhibition of the click reaction by tetrazine. Related to Fig 2.**

Conditions: A solution of the reactants in ACN containing 0.05% FA was stirred at room temperature for 30 min before solvent removal under vacuum. The residue was re-dissolved in ACN containing 0.05% FA and analyzed by RP-HPLC. 1 equiv. = 82.5 µM, Tet = tetrazine (8).

<sup>a</sup> ± 5% determined by separately measuring standard solutions containing 82.5 µM decyltriphenylphosphonium bromide internal reference.<br>
<sup>b</sup> % of the remaining 9 equivalents of 8 which did not react with 6.

### Table S2

| Compound Name  | Parent (m/z) | Product (m/z) | Cone (V) | Collision (V) |
|----------------|--------------|---------------|----------|---------------|
| MitoAzido      | 360.2        | 263.1         | 38       | 24            |
| d<sub>15</sub>-MitoAzido | 375.3        | 278.2         | 48       | 24            |
| MitoClick      | 415.4        | 262.2         | 62       | 44            |
| d<sub>15</sub>-MitoClick | 430.5        | 277.3         | 70       | 42            |
| MitoOct<sub>tet</sub> | 600.4        | 262.9         | 98       | 74            |
| d<sub>15</sub>-MitoOct<sub>tet</sub> | 615.5        | 278.0         | 88       | 76            |

**Table S2: Transitions and parameters used for LC-MS/MS detection of compounds. Related to Figure 2.**
SUPPLEMENTAL CALCULATIONS

The rate of reaction between MitoAzido and MitoOct to form MitoClick \textit{in vivo} is complicated by a number of factors, in addition to the mitochondrial ($\Delta\psi_m$) and plasma ($\Delta\psi_p$) membrane potentials. These additional factors include the rates of uptake and excretion, and the extents of compound binding within different compartments. Even so, the fact that the azido, cyclooctyne and TPP moieties are not metabolized significantly \textit{in vivo} simplifies interpretation, and enables us to assess the relative changes in the average $\Delta\psi_m$ in cells. To do this, we can consider the rate of reaction of MitoOct with MitoAzido following simultaneous administration of both compounds and then assume that the MitoAzido and MitoOct equilibrate with the $\Delta\psi_m$. The formation of MitoClick within the cell can be simplified as a two compartment model, comprising the cytosol (cyto) and mitochondria (mito), with the distribution between these compartments determined by the $\Delta\psi_m$. The rates of formation of MitoClick in the two compartments are given below:

\begin{align*}
(1) \hspace{1cm} Rate_{\text{mito}} &= k \left[ \text{MitoAzido} \right]_{\text{mito}} \left[ \text{MitoOct} \right]_{\text{mito}} \\
(2) \hspace{1cm} Rate_{\text{cyto}} &= k \left[ \text{MitoAzido} \right]_{\text{cyto}} \left[ \text{MitoOct} \right]_{\text{cyto}}
\end{align*}

To calculate the rates relative to each other in the two compartments, it is necessary to know the relative concentrations, which will be given by the Nernst equation. Relating the mitochondrial concentration to that in the cytosol (where MitoX = MitoOct or MitoAzido) gives:

\begin{equation}
\Delta \psi_m = \frac{2.303RT}{F} \log_{10} \left( \frac{[\text{MitoX}]_{\text{mito}}}{[\text{MitoX}]_{\text{cyto}}} \right)
\end{equation}

These can be rearranged to relate the concentrations in the two compartments

\begin{equation}
[\text{MitoX}]_{\text{mito}} = [\text{MitoX}]_{\text{cyto}} 10^{\varphi \Delta \psi_m / 2.303RT}
\end{equation}

These relationships for MitoOct and MitoAzido, can be substituted into the rate equations 1 and 2 to give the relative rates of formation of MitoClick in the two compartments.
\( \frac{Rate_{\text{cyto}}}{Rate_{\text{mito}}} = 10^{2 \frac{\Delta \psi_m}{2.303RT}} \)

This gives the ratios of these rates between the various compartments.

\( \frac{Rate_{\text{mito}}}{Rate_{\text{cyto}}} = 10^{2 \frac{\Delta \psi_m}{2.303RT}} \)

The actual flux in each compartment will be the rate multiplied by the compartment volume. Therefore the relative fluxes between the compartments are given by:

\( \frac{Flux_{\text{mito}}}{Flux_{\text{cyto}}} = \frac{\text{MitoVol}}{\text{CytoVol}} 10^{2 \frac{\Delta \psi_m}{2.303RT}} \)

If we take HeLa cells the volume of the cell that is mitochondrial is \( \sim 10\% \) (Posakony et al., 1977). If we add in a plausible \( \Delta \psi_m \) of 150 mV (James et al., 1999), then we can calculate the relative flux of MitoClick formation:

\( \frac{Flux_{\text{mito}}}{Flux_{\text{cyto}}} = 7,900 \)

Therefore within a cell the vast majority of MitoClick is formed within the mitochondria.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Overview of chemical syntheses

MitoOct (6) was derived from the known hydroxycyclooctyne (4) as outlined in Figure S1A. The established procedures (Jessen et al., 1986; Meier and Petersen, 1978) for the synthesis of 4-cyclooctyn-1-ol (4) from the diol were slightly modified. Oxidation of cis-cyclooctane-1,5-diol with pyridinium chlorochromate gave the 5-hydroxyketone (1) which was converted to the semicarbazone (2) followed by cyclization with selenium dioxide to form the bicyclic selenadiazole (3). The selenadiazole was treated with n-butyl lithium and iodomethane to form the alcohol (4) (Jessen et al., 1986). The alkyne (4) was unstable under the usual conditions required to produce phosphonium salts directly from triphenylphosphine, therefore the triphenylphosphonium was introduced as an intact function. The alcohol function in 4 was activated by forming the para-nitrophenyl carbonate (5) which was displaced by an amino alkyltriphenylphosphonium salt to form the required cyclooctyne triphenylphosphonium salt MitoOct (6) as the bromide salt. The corresponding iodide salt could be obtained by anion exchange with iodide loaded ion exchange column. MitoAzido (7) (Chen, et al., 2011) was synthesized from the previously reported 4-(iodobutyl)triphenylphosphonium iodide (Lin, 2002) (Figure S1B).

Reaction of MitoOct (6) with MitoAzido (7) proceeded readily (Figure S1C) and the triazole product MitoClick (9) was isolated as a regioisomeric mixture for use as a reference compound. The cycloaddition reaction gave MitoClick (9) as a 1:1 mixture of regioisomers, evident from the $^1$H NMR spectrum, which was satisfactory for the analytical protocol. Deuterated internal standards of MitoOct, MitoAzido and MitoClick were produced from $d_{15}$-triphenylphosphine, as described in Figures S1A-C.

To halt the click reaction between MitoOct and MitoAzido during isolation we used a blocking reagent to rapidly sequester and inactivate MitoOct. Addition of 3-phenyl-1,2,4,5-tetrazine (8) to mixtures of MitoOct (6) and MitoAzido (7) resulted in a rapid reaction with 6 to give the pyridazine product 10 (Figure S2A). The pyridazine (10) was characterized and used as a reference compound during the analysis. As with the formation of the triazole (9), the non-specific cycloaddition reaction of 8 with 6 gave 10 as a regioisomeric mixture as indicated by $^1$H NMR. To confirm that the tetrazine (8) could effectively arrest the click reaction between MitoOct and MitoAzido during extraction, incubations were carried out in the extraction
medium (Figure S2B and Table S1). In the control reaction, 6 and 7 had been entirely consumed to form 9. In contrast, one equivalent of 8 prevented formation of 9 and instead gave quantitative conversion to the pyridazine product 10, while most of 7 was recovered unchanged. Reaction with ten equivalents of 8 showed a similar profile, although only 25% of the expected 9 equivalents of 8 remained suggesting that excess 8 decomposed upon concentration.

**Detailed description of the chemical syntheses**

Unless otherwise stated, all reagents and solvents were purchased from commercial sources and used without further purification. 4-(Iodobutyl)triphenylphosphonium iodide (Lin et al., 2002), 3-(aminopropyl)triphenylphosphonium bromide hydrobromide, (McAllister et al., 1980) and 3-phenyl-1,2,4,5-tetrazine (8) (Birkofer et al., 1982) were prepared according to established procedures. Dry solvents were obtained from a PureSolv solvent purification system and handled using Schlenk techniques. Petroleum ether refers to the fraction boiling in the range 40–60°C. Reactions were followed by TLC on aluminium-backed silica gel 60 F254 sheets from E-Merck, visualized under UV light. Column chromatography was performed using Scharlau silica gel 60, 0.04-0.06 mm, 230-400 mesh. 1H and 13C NMR spectra were recorded on either a 400 MHz Varian 400 or Varian 500 MHz NMR at 298 K. Chemical shifts are reported in parts per million and referenced to residual solvent peaks (CDCl3: 1H δ 7.26 ppm, 13C δ 77.16 ppm; DMSO-d6: 1H δ 2.50 ppm, 13C δ 39.50 ppm, CD3OD: 1H δ 3.31 ppm, 13C δ 49.15 ppm). Coupling constants (J) are reported in Hertz (Hz). Standard abbreviations indicating multiplicity were used as follows: m = multiplet, quint. = quintet, q = quartet, t = triplet, d = doublet, s = singlet, br = broad. IR spectra were recorded on a Bruker Alpha-P diamond anvil system. Microanalyses were performed at the Campbell Microanalytical Laboratory at the University of Otago. All triphenylphosphonium salts were isolated as hydrates and calculation of the molecular structure fits with added water molecules: 1H NMR spectra show a sharp singlet around 1.6 ppm due to associated water molecules. Electrospray mass spectra (ESMS) were acquired on a Bruker microTOF spectrometer. HPLC analysis was carried out using a Shimadzu Prominence system on a C18 column (Phenomenex Prodigy ODS(3) 5 μm 100 Å, 250 x 3 mm) with a 2 x 4 mm C18 guard column, peaks detected at 210 and 254 nm, solvents ACN in aqueous 0.05% TFA: t0=10%, t12.5=100, t15=100, t17=10, t20=10%.

**5-Hydroxycyclooctanone 1.** Pyridinium chlorochromate (5.36 g, 24.96 mmol) was added to a stirred solution of *cis*-cyclooctan-1,5-diol (3.00 g, 20.80 mmol) in dichloromethane
The suspension was stirred at 0°C under nitrogen for 1 h, and then at room temperature for a further 20 h until conversion to the hydroxy-ketone was complete. Diethyl ether (100 mL) was added and the mixture stirred for 30 min. The suspension was filtered through a pad of Celite®, the residue washed with dichloromethane (50 mL) and the combined organic extract was filtered and dried over magnesium sulfate and the solvent removed in vacuo. The crude product was purified by silica flash column chromatography (diethyl ether) to give 1 (Meier and Petersen, 1978) as a white solid (2.33 g, 79%). Rf 0.65 (diethyl ether).

2-(5-Hydroxycyclooctylidene)hydrazinecarboxamide 2. A solution of sodium acetate (3.45 g, 42.10 mmol) and semicarbazide hydrochloride (2.35 g, 21.03 mmol) in ethanol (25 mL) was heated to reflux for 15 mins. After cooling, a solution of 1 (1.30 g, 9.14 mmol) in ethanol (15 mL) was added dropwise and the mixture heated to reflux for 3 h, then cooled and stirred at room temperature for 16 h. The precipitate was filtered off, washed with ethanol (20 mL) and solvent removed from the filtrate in vacuo. The residue was dissolved in dichloromethane (200 mL) and methanol (5 mL) and washed with brine (100 mL). The organic extract was filtered and dried over magnesium sulfate and the solvent removed in vacuo to give 2 (Meier and Petersen, 1978) as a white solid (1.40 g, 77%). Rf 0.32 (1:9 methanol / chloroform).

4,5,6,7,8,9-Hexahydrocycloocta[d][1,2,3]selenadiazol-7-ol 3. A saturated solution of selenium dioxide (227 mg, 2.04 mmol) in water (2.5 mL) was added dropwise to a stirred suspension of 2 (185 mg, 0.93 mmol) in dioxane (2.5 mL). The mixture was stirred in the dark at room temperature for 16 h and the suspension was filtered through a pad of Celite®, the solid residue washed with dichloromethane (100 mL) and the combined organic extracts washed with brine. The organic extracts were filtered and dried over sodium sulfate and the solvent removed in vacuo. The crude product was purified by silica flash column chromatography (1:1 diethyl ether / petroleum ether) to give 3 (Jessen et al., 1986) as a yellow oil (462 mg, 80%). Rf 0.40 (diethyl ether).

4-Cyclooctyn-1-ol 4. n-Butyl lithium (1.35 mL, 2.5 M in hexanes, 3.39 mmol) was added dropwise to a solution of the 3 (270 mg, 1.17 mmol) in dry tetrahydrofuran (5 mL) at -78°C. The solution was stirred for 30 mins before drop wise addition of iodomethane (0.256 mL, 4.09 mmol). The reaction was stirred for 30 mins at -78°C, then at room temperature for 30 mins and then quenched by dropwise addition of ice water (1 mL). The reaction was extracted with dichloromethane (3 x 20 mL). The organic extracts were filtered and dried over sodium sulfate and the solvent removed in vacuo. The crude product was purified by silica
flash column chromatography (0-10% acetone/dichloromethane) to give 4 (Jessen et al., 1986) as a volatile colourless oil (117 mg, 81%). Rf 0.62 (diethyl ether).

\[ \text{Cyclooct-4-yn-1-yl (4-nitrophenyl) carbonate 5.} \]

\[
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{PPh}_3 \cdot \text{Br}
\end{array}
\]

4-Nitrophenyl chloroformate (416 mg, 2.06 mmol) and 4-dimethylaminopyridine (13 mg, 0.10 mmol) in dry dichloromethane (5 mL) were added via a cannula to a solution of 4 (128 mg, 1.03 mmol) in dry dichloromethane (5 mL). Triethylamine (0.72 mL, 5.15 mmol) was slowly added dropwise to the reaction. The solution was stirred under nitrogen at room temperature for 16 h and then the solvent removed in vacuo. The crude product was purified by silica flash column chromatography (5-10% diethyl ether/petroleum ether) to give 5 as a colourless oil (211 mg, 71%). Rf 0.66 (2:3 diethyl ether/petroleum ether); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.28 (d, \(J = 9.2\) Hz, 2H; H3’), 7.38 (d, \(J = 9.2\) Hz, 2H; H2’), 4.87 – 4.80 (m, 1H; H1), 2.59 – 1.95 (m, 10H; H2, H3, H6, H7, H8); \(^1\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 155.76 (C1’), 152.18 (C=O), 145.46 (C4’), 125.42 (C3’), 121.93 (C2’), 94.39 (C4), 93.22 (C5), 85.52 (C1), 40.08 (C2), 38.38 (C8), 30.16 (C7), 20.22 (C6), 17.94 (C3); I.R. \(\nu_{\text{max}}\) (cm\(^{-1}\)) 2938, 2854, 1754, 1522, 1346, 1256, 1196, 1162, 959, 857; Not ionised by MS; Anal. calcd. for C\(_{15}\)H\(_{15}\)NO\(_5\): C 62.28, H 5.23, N 4.84; Found: C 62.48, H 5.33, N 4.77%.

\[ \text{(3-(((Cyclooct-4-ynyloxy)carbonyl)amino)propyl)triphenylphosphonium bromide 6.} \]

\[
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{PPh}_3 \cdot \text{Br}
\end{array}
\]

Triethylamine (0.117 mL, 0.831 mmol) was added to a solution of 3-(aminopropyl)triphenylphosphonium bromide hydrobromide salt (100 mg, 0.208 mmol) in dry acetonitrile (5 mL) and the solution stirred under nitrogen for 10 min. The amine was transferred via a cannula to a separate flask containing 5 (63 mg, 0.218 mmol) in dry
acetonitrile and the solution stirred at room temperature for 16 h followed by removal of solvent in vacuo. The crude product was purified by silica flash column chromatography (0-10% methanol / chloroform). Trituration with diethyl ether (1x 25 mL) followed by removal of the solvent from a dichloromethane solution under high vacuum allowed isolation of 6 as a white powder (92 mg, 81%). Rf 0.64 (1:9 methanol / chloroform); 1H NMR (500 MHz, CDCl3) δ 7.85 – 7.64 (m, 15H; PPh3), 6.46 – 6.38 (m, 1H; NH), 4.59 (dd, J = 9.5, 5.5 Hz, 1H; H1), 3.81 – 3.68 (m, 2H; H3’), 3.55 – 3.45 (m, 2H; H1’), 2.41 – 1.78 (m, 12H; H2, H3, H6, H7, H8, H2’) ppm; 13C NMR (126 MHz, CDCl3) δ 156.91, 135.28 (J = 3.0 Hz; para-PPh), 133.71 (J = 10.0 Hz; ortho-PPh), 118.25 (J = 86.2 Hz; ipso-PPh), 94.21, 93.93 (C4), 80.02 (C5), 40.60 (C2), 40.29 (J = 17.8 Hz; C1’), 38.73 (C8), 30.26 (C7), 23.08 (J = 3.4 Hz; C2’), 21.38 (J = 51.6 Hz; C3’), 20.41 (C6), 18.10 (C3) ppm; 31P NMR (161 MHz, CDCl3) δ 24.76 ppm; I.R. νmax (cm-1) 3256, 3053, 3020, 2933, 2883, 2852, 2792, 1695, 1587, 1485, 1436; HR ESI-MS (Methanol) m/z = 470.2213 [6 - Br]+ (calc. for C33H30NO2P+ 470.2243); Anal. calcd. for C30H33BrNO2P·(0.5 H2O): C, 64.40; H, 6.13; N, 2.50; found C, 64.69; H, 6.28; N, 2.67%; Rf=9.3min (>99% purity). D15 analogue: HR ESI-MS (methanol) m/z = 485.3215 [6 - I]+ (calc. for C30H18D15NO2P+ 485.3185). d14 15%, d15 85%. The iodide salt was obtained by exchange using iodide anion ion exchange resin

4-(Azidobutyl)triphenylphosphonium iodide 7 (Chen et al., 2011).

A mixture of 4-iodobutyltriphenylphosphonium iodide (Lin et al., 2002) (3.00 g, 5.24 mmol) and sodium azide (3.51 g, 54 mmol) in 1:1 ethanol/water (100 mL) was refluxed under nitrogen for 16 h. Water (50 mL) was added to the cooled solution, the organic fraction extracted into dichloromethane (3 x 20 mL). The combined organic extracts were filtered and dried over magnesium sulfate and solvent removed in vacuo. The residue was dissolved in a minimal amount of dichloromethane followed by addition of diethyl ether (25 mL) to precipitate 7 as a white solid (2.44 g, 96%). Rf 0.26 (1:9 methanol / chloroform); 1H NMR (400 MHz, CDCl3) δ 7.91 – 7.65 (m, 15H; PPh3), 3.92 – 3.76 (m, 2H; H1), 3.44 (t, J = 6.1 Hz, 2H; H4), 2.03 (p, J = 6.7 Hz, 2H; H2), 1.75 (dq, J = 15.7, 8.1 Hz, 2H; H3) ppm; 13C NMR (101 MHz, CDCl3) δ 135.27 (d, J = 3.1 Hz; para-PPh), 133.87 (d, J = 10.0 Hz; ortho-PPh), 130.68 (d, J = 12.6 Hz; meta-PPh), 118.17 (d, J = 86.0 Hz; ipso-PPh), 50.75 (d, J = 1.2 Hz; C4), 29.32 (d, J = 16.8 Hz; C3), 22.72 (d, J = 50.8 Hz; C1), 19.98 (d, J = 3.9 Hz; C2) ppm; 31P NMR (CDCl3) δ 25.44 ppm; I.R. νmax (cm⁻¹) 2095, 1435, 1109, 754, 735, 722, 688, 528, 501; HR ESI-MS (Methanol) m/z = 360.1625 [7 - I]+ (calc. for C22H23N3P+ 360.1624); Anal.
calcd. for C_{22}H_{23}IN_3P: C, 54.22; H, 4.76; N, 8.62; found C, 54.54; H, 4.73; N, 8.66%; 
R_t=8.9 min (>99% purity). D_{15} Analogue: HR ESI-MS (methanol) m/z = 375.2535 [7 – I]^{+} 
(calc. for C_{22}H_{8}D_{15}N_3P^{+} 375.2566). d_{14} 8.4%, d_{15} 91.6%.

Triphenyl[4\{6\{3\{triphenylphosphoniy]propylcarbamoyloxy\}\}4,5,6,7,8,9-hexahydro-cyclooctatetraazol-1-yl\}butyl\}phosphonium diiodide 9.

![Chemical Structure](image)

(1:1 mix of 1a-3a isomers)

A mixture of the iodide salts of 6 (25 mg, 0.042 mmol) and 7 (20 mg, 0.042 mmol) in 
methanol (1 mL) were concentrated in vacuo. The process of dissolution then concentration 
was repeated twice more and checked by HPLC to confirm complete conversion. The residue 
was dissolved in minimum ethanol and water, removal of the ethanol in vacuo and freeze-
drying gave 9 as a white powder (45 mg, 100%). ¹H NMR (500 MHz, CDCl₃) δ 7.84 – 7.64 
(m, 30H; PPh₃), 6.80 (t, J = 6.2 Hz, 0.5H; NH), 6.50 (t, J = 6.2 Hz, 0.5H; NH), 4.75 (dd, J = 
10.0, 4.8 Hz, 0.5H; H6), 4.44 – 4.26 (m, 2.5H; H6, H1’), 3.84 – 3.55 (m, 4H; H4’, H3’’), 3.39 
(m, 2H; H1’’), 3.15 – 2.68 (m, 4H; H4, H9), 2.41 – 2.18 (m, 2H; H2’), 2.08 – 1.30 (m, 10H; 
H5, H7, H8, H3’, H2’’) ppm; ¹³C NMR (126 MHz, CDCl₃) δ 156.43 (C=O), 144.59+143.13 
(C1a), 135.43-135.25 (J = 3.0 Hz; para-PPh), 133.87- 133.63 (J = 10.0 Hz; ortho-PPh), 
133.30+132.66 (C3a), 130.82-130.60 (J = 12.5 Hz; meta-PPh), 118.43- 117.53 (J = 86.2 Hz; 
ipsa-PPh), 73.70+73.65 (C6), 47.24+46.76 (C1’), 40.37+40.22 (C1’’), 33.14+33.13 (C5), 
32.09+32.08 (C7), 29.89-29.36 (C2’), 23.84-18.70 (C4, C8, C9, C3’, C4’, C2’, C3’’) ppm; 
³¹P NMR (161 MHz, CDCl₃) δ 24.57, 24.47, 24.39, 24.16 ppm; I.R. ν_{max} (cm⁻¹) 2930, 1699, 
1436, 1245, 1111, 995, 737, 722, 688, 531, 507; HR ESI-MS (Methanol) m/z = 415.1934 [9 - 
2I]^{2+} (calc. for C_{52}H_{56}N_4O_2P_2^{2+} 415.1956); Anal. calc. for C_{52}H_{56}I_2N_4O_2P_2·(2 H₂O): C, 55.72; 
H, 5.40; N, 5.00; found C, 55.79; H, 5.24; N, 4.96%; R_t=8.0 min (>99% purity). D_{30} analogue: 
HR ESI-MS (methanol) m/z = 430.2914 [9 – 2I]^{+} (calc. for C_{52}H_{26}D_{30} N_4O_2P_2^{2+} 430.2875). 
d_{29} 14.9%, d_{30} 85.1%.
Triphenyl[3-(1-phenyl-5,6,7,8,9,10-hexahydro-cyclocta[d]pyridazin-7-yloxycarbonylamino)-propyl]phosphonium iodide 10

A solution of 6 (50 mg, 0.086 mmol) and 3-phenyl-1,2,4,5-tetrazine, 8, (Birkofer et al., 1982) (14 mg, 0.086 mmol) in methanol (1 mL) were concentrated in vacuo. The process of dissolution then concentration was repeated twice more and checked by HPLC to confirm complete conversion. The residue was dissolved in minimum ethanol and water, removal of ethanol in vacuo and freeze-drying gave 10 as a white powder (61 mg, 100%). ¹H NMR (500 MHz, CDCl₃) δ 8.93 (s, 0.5H; H4), 8.89 (s, 0.5H; H4), 7.87 – 7.61 (m, 15H; PPh₃), 7.46 (m, 5H; H2’, H3’, H4’), 6.79 (t, J = 6.3 Hz 0.5H; NH), 6.70 (t, J = 6.3 Hz 0.5H; NH), 4.49 (td, J = 8.5, 4.1 Hz, 0.5H; H7), 4.42 (td, J = 8.5, 4.1 Hz, 0.5H; H7), 3.81 – 3.65 (m, 2H; H1’’), 3.54 – 3.40 (m, 2H; H3’’), 3.13 – 2.63 (m, 4H; H5, H10), 2.13 – 1.49 (m, 8H; H6, H8, H9, H2’) ppm; ¹³C NMR (126 MHz, CDCl₃) δ 162.19+162.12 (C1), 156.58+156.49 (C=O), 151.59+151.39 (C4), 140.70+140.46 (C4a), 138.24+138.22 (C1’), 137.68+137.63 (C1a), 135.31+135.30 (J = 3.0 Hz; para-PPh), 133.66+133.66 (J = 10.0 Hz; ortho-PPh), 130.66+130.66 (J = 12.5 Hz; meta-PPh), 129.10+129.03 (C3’), 128.71+128.64 (C4’), 128.49+128.38 (C2’), 118.16+118.13 (J = 86.2 Hz; ipso-PPh), 73.80+73.60 (C7), 40.27+40.27 (J = 17.5 Hz; C1’’), 36.17+35.46 (C6), 32.84+32.18 (C8), 29.83+29.79 (C9), 27.13+26.76 (C5), 26.06+25.89 (C10), 23.00+23.00 (J = 3.7 Hz; C2’’), 21.50+21.46 (J = 52.5 Hz; C3’’) ppm; ³¹P NMR (161 MHz, CDCl₃) δ 24.70 ppm; I.R. ν max (cm⁻¹) 3275, 2933, 1702, 1518, 1437, 1245, 1111, 996, 738, 723, 689, 533; HR ESI-MS (Methanol) m/z = 600.2771 [10 - I]⁺ (calc. for C₃₈H₃₉N₅O₂P⁺ 600.2774); Anal. calc. for C₃₈H₃₉IN₅O₂P·2H₂O: C, 59.77; H, 5.68; N, 5.50; found C, 59.86; H, 5.43; N, 5.52%; Rf=8.4min (>99% purity).

Characterization of mitochondria-targeted compounds

To analyze the click reaction, in vitro reactions were carried out in 250 μL KCl medium (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2) containing 10 nmol each of MitoAzido and MitoOct. At the end of the incubation 250 μL acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) and 500 μL 0.1% TFA were added, mixed and introduced into a 2 mL sample loop and
assessed by RP-HPLC. RP-HPLC was performed at room temperature using a Jupiter C18 5 µ 300 Å column (250 x 4.6 mm, 5 µM) with a C18 guard column (2 x 4 mm) (both from Phenomenex). The mobile phase consisted of 0.1% (TFA) in water (buffer A) and 100% ACN/0.1% TFA (buffer B) delivered as a gradient as follows: 0-2 min, 5% B; 2-17 min, 5-100% B; 17-19 min, 100% B; 19-22 min, 100-5% B. The flow rate was 1 mL/min. The A220 of the column eluant was detected using a Gilson UV/Vis 151 spectrophotometer.

**Mitochondrial preparation and incubations**

Rat liver mitochondria were prepared by homogenization and differential centrifugation in ice-cold 250 mM sucrose, 5 mM Tris, 1 mM EGTA, pH 7.4. The protein concentration was determined by the biuret assay. Mitochondria were incubated at 1 mg protein/mL in KCl medium (120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2) at 37°C supplemented with 10 mM succinate and 4 µg/mL rotenone. When FCCP was used it was present at 500 nM. An electrode selective for the TPP moiety of MitoAzido, MitoOct and MitoClick was prepared and used as described (Asin-Cayuela et al., 2004) to show uptake of compounds into mitochondria.

Mouse heart mitochondria (MHM) were isolated from wild type and *Ndufs4*-null mice by homogenization and differential centrifugation at 4°C in buffer containing 250 mM sucrose, 5 mM Tris-Cl (pH 7.4), and 1 mM EGTA supplemented with 0.1% (w/v) fatty acid free bovine serum albumin. Protein content of mitochondrial preparations was determined using the biuret assay with bovine serum albumin as a standard. Mitochondrial incubations were performed in isolation buffer and the mitochondrial membrane potential was measured by the uptake of [3H]-TPMP. MHM (2 mg protein/mL) were incubated at 37°C with 10 mM succinate, 500 nM cold TPMP, 50 nCi/mL [3H]-TPMP and test compounds for 2 min. Mitochondria were pelleted by centrifugation at 10,000 x g for 30 s. Pellets were solubilized by the addition of 20% (v/v) Triton X-100, added to scintillant, and incubated for 1 h at room temperature. After 1 h samples were mixed, and the amount of radioactivity quantified using a Packard Tri-Carb 2800-TR liquid scintillation counter (PerkinElmer).

To extract TPP compounds from mitochondria for HPLC analysis, samples were centrifuged (1 min at 16,000 x g) and the pellet was resuspended in 250 µL of 100 % ACN/0.1% TFA, supplemented with 50 µM Tet, vortexed, and centrifuged (10 min at 16,000 x g). The supernatant was transferred to a fresh tube, centrifuged again, 200 µL was passed through a 0.22 µm syringe-driven polyvinylidene difluoride filter (Millex, Millipore) and 800 µL 0.1% TFA added and 1 mL was assessed by RP-HPLC. Extraction of mitochondria for
LC-MS/MS analysis was similar to that for RP-HPLC, with the exception that formic acid (FA) was used in place of TFA, and the addition of IS (100 pmol each of $d_{15}$-MitoOct$_{tet}$, $d_{15}$-MitoAzido and $d_{15}$-MitoClick) to the pellet with the 250 µL 100 % ACN/0.1% FA.

**Cell experiments**

C2C12 cells were cultured at 37°C under humidified 95% air/5% CO$_2$, in medium supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). To assess $\Delta \psi_m$ by fluorescent microscopy, C2C12 cells were plated overnight on glass-bottom cultured dishes (MatTek) and were pre-loaded with 100 nM TMRM (Invitrogen), and 500 ng/mL Hoechst 33342 (Invitrogen) for 15 min in the dark in a 37°C, 5% CO$_2$ humidified incubator. At the end of the incubation cells were washed three times with PBS (pH 7.4) and were maintained in imaging buffer (132 mM NaCl; 10 mM HEPES; 4.2 mM KCl; 1 mM MgCl$_2$; 1 mM CaCl$_2$ adjusted to pH 7.4 with Tris base and supplemented with glucose (1g/L)) for the duration of the experiment. Cells were imaged on a temperature-controlled stage of a Nikon Eclipse Ti microscope using a 63x oil immersion lens. Additions of oligomycin (4 µM) and FCCP (2 µM) were made directly to the cultured cells. Fluorescence intensity was evaluated for 8 cells in three independent experiments using Nikon elements software.

The toxicity of MitoOct, MitoAzido and MitoClick to C2C12 cells was assessed by incubating cells in 96-well plates with the compounds from 0 to 100 µM for 20 h, then the medium was replaced with 100 µL of fresh medium plus 20 µL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) for 2 h at 37°C. The absorbance at 490 nm was measured to assess cell survival. This MTS showed that over 20 h there was negligible toxicity up to 5 µM with mild toxicity apparent at 10 µM and above. For experiments, C2C12 cells were incubated in a 6-well culture dish with 5 µM MitoAzido and MitoOct for up to 4 h. Where required, oligomycin (4 µM) or FCCP (2 µM) was added at the beginning of the incubation. To extract TPP compounds from cells, 1 mL of cell medium was removed to an eppendorf tube containing 1 mL 100% ACN/0.1% FA supplemented with 50 µM Tet and spiked with IS (100 pmol each of $d_{15}$-MitoOct$_{tet}$, $d_{15}$-MitoAzido and $d_{15}$-MitoClick). Samples were vortexed for 1 min, centrifuged (15 min at 16000 x g), then filtered to a fresh tube and dried in a speed vac overnight. The cell layer was scraped into 0.5 mL PBS containing 50 µM Tet and transferred to an eppendorf tube. Cells were pelleted by centrifugation (3 min at 16,000 x g) and supernatant discarded. The pellet was dried and then resuspended in 250 µL of 100% ACN/0.1% FA, spiked with IS as above. Samples were vortexed for 5 min and centrifuged at 16,000 x g for 15 min. 200 µL of each sample was
filtered into a fresh tube and dried in a speed vac for 1-2 h. Once dried, cell pellet and media samples were resuspended in 250 µL 20% ACN/0.1% FA by vortexing for 5 min and then centrifuged for 10 min at 16,000 x g. 200µL was transferred to an autosampler vial for mass spectrometry analysis.

**Mouse breeding and maintenance**

Animal experiments were either carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the University of Cambridge Animal Welfare Policy, and were approved by the UK Home Office under project license 80/2374 and 70/7963, or animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee (IACUC) of the Beth Israel Deaconess Medical Center. For most experiments C57BL/6J male mice (8-10 weeks of age) were used. The heart-specific Ndufs4-null mouse model was a kind gift from the lab of Prof Nils Göran-Larsson, Max Planck Institute for Ageing, Cologne, Germany (Sterky et al., 2012). Previously, these mice were generated by breeding Ndufs4Loxp/+ mice with mice carrying the heart specific cre recombinase, CKM-NLS-cre and established on a C57BL/6 background (Hansson et al., 2004; Kruse et al., 2008). Then double heterozygous offspring were crossed to obtain homozygous Ndufs4Loxp/Loxp mice (Sterky et al., 2012). In order to generate littermate knockout (CKM-NLS-cre Ndufs4Loxp/Loxp) and control mice, the male CKM-NLS-cre knockouts (CKM-NLS-cre; Ndufs4Loxp/Loxp) were crossed to control females (Ndufs4Loxp/Loxp). Mice with genotype Ndufs4Loxp/Loxp were used as controls (Sterky et al., 2012). A mix of CKM-NLS-cre Ndufs4Loxp/Loxp males and females approximately 24 weeks old were used for all experiments. As described previously, at this age there was no discernable difference in health, appearance or behavior between heart-specific Ndufs4-null mice and controls. Tissues, that had been stored frozen until required, were homogenized in 50 mM Tris-HCl, 2 mM sodium citrate, 0.6 mM MnCl2, pH 7 and stored frozen until required. Citrate synthase was assessed as described (Robinson Jr. et al., 1987). Protein concentration was determined by the BCA assay using BSA as a standard.

MitoOct and MitoAzido were administered to mice by intravenous (i.v.) injection via the tail vein in 100 µL 0.9% NaCl. MitoOct and MitoAzido were safe at doses up to at least 100 nmol of each simultaneously. A combined solution of 50 nmol of MitoOct and MitoAzido in 100 µL 0.9% NaCl (~1.6 µmol kg⁻¹ for 25-30 g mice) was administered via the tail vein. Injections took place between 9 am and 12 noon, and the order of administration to control and experimental animals was randomized. Mice were then sacrificed by cervical
dislocation, and tissues including the heart, liver and kidneys were isolated and snap-frozen on liquid nitrogen 10 min, 1 h, 2 h and 3 h post-injection for LC-MS/MS analysis (n=3 per group). For experiments with the heart-specific \textit{Ndufs4}-null strain, mice were injected via the tail vein with 10 nmol of MitoOct and MitoAzido in 100 µL 0.9% NaCl (~ 370 nmol kg$^{-1}$ for 25-30 g mice). Mice were killed after 1 min by cervical dislocation and the heart was rapidly isolated and snap-frozen on liquid nitrogen. For the 2,4-dinitrophenol (DNP) experiments, mice were injected intraperitoneally (i.p.) with DNP (1 mg/kg) as a bolus in 100 µL 0.9% NaCl or administered an oral gavage of DNP (5mg/kg) as a bolus in 200 µL 0.9% NaCl. After 15 min (i.p DNP) or 60 min (oral DNP) mice were injected i.v. via tail vein with 10 nmol MitoAzido and 10 nmol MitoOct in 100 µL 0.9% NaCl. Mice were killed after 1 min by cervical dislocation and the heart was rapidly isolated and snap-frozen on liquid nitrogen.

**Extraction of MitoOct, MitoAzido and MitoClick from mouse tissues**

To extract MitoOct, MitoAzido and MitoClick from mouse tissues 50 mg tissue was homogenized in a 2 mL eppendorf using a bullet blender. Beads were added to the sample with 200 µL 95% ACN/0.1% FA supplemented with 50 µM Tet. The homogenate was spiked with internal standard (IS) (100 pmol each of $d_{15}$-MitoOct, $d_{13}$-MitoAzido and $d_{30}$-MitoClick), homogenized for 3 min at speed 8 and left on ice for 30 min. Samples were then centrifuged (10 min at 16,000 x g). The supernatant was transferred to a filter plate, and the pellet extracted with a further 200 µL 95% ACN/0.1% FA, homogenised for 20 s, centrifuged as before and pooled with the first extraction. Then the supernatants were filtered via a vacuum pump into a 96-well plate, and dried under vacuum using a MiVac quattro SpeedVac (~1-2 h). The dried samples were resuspended in 250 µL 20% ACN/0.1% FA by vortexing for 5 min and transferred to a fresh tube. Finally, the samples were centrifuged (10 min at 16,000 x g) and 200 µL was transferred to an autosampler vial (1.5 mL silanized, from Waters).

**LC-MS/MS analysis of MitoAzido, MitoOct and MitoClick**

The LC-MS/MS system for the analysis consisted of a Waters Xevo TQ-S triple quadrupole spectrometer with an Acquity I-Class LC system (also Waters). Samples and standards in autosampler vials were placed in a refrigerated holder (4°C) while awaiting introduction by the autosampler. Liquid chromatography was performed at 30°C using a Luna 5 µ Phenyl-Hexyl column (1 x 50 mm, 5 µm) with a Phenyl-Hexyl guard column (2 x 4 mm) (both from Phenomenex). The mobile phase consisted of 0.1% FA in water (buffer A) and 95% ACN/0.1% FA (buffer B) delivered as a linear gradient as follows: 0-2 min, 5% B; 2-3 min,
5-25% B; 3-5 min, 25-75% B; 5-7 min, 75-100% B; 7-10 min 100% B; 10-12 min, 100-5% B; 12-20 min, 5% B. The flow rate was 50 µL/min and a 10 µL volume was introduced via a flow-through needle. An in-line divert valve was used to divert eluant away from the mass spectrometer from 0-5 min and 16-20 min of the acquisition time. Electrospray ionisation mass spectrometry in positive ion mode was employed. The instrument parameters were: source spray voltage, 3.1 kV; ion source temperature, 150ºC; cone voltage and collision energy, were optimised for each compound and transition as shown in Table S2. Nitrogen was used as the curtain gas and argon as the collision gas. Multiple reaction monitoring (MRM) in positive ion mode was used to detect the compounds.

The optimum fragment ions to use for quantification were established by direct infusion (10 µL/min) of each compound (50 nM) into the mass spectrometer. Published fragmentation patterns for alkylTPP ions (Claereboudt et al., 1993; Denekamp et al., 1999; Denekamp et al., 2003) and the presence of the deuterium atoms solely on the TPP moiety were used to identify the fragment ions. For LC-MS/MS quantification, the transitions are detailed in Table S2. Data were acquired and analyzed with MassLynx software. Standard curves for the response of MitoOcttet, MitoAzido and MitoClick relative to its deuterated IS against concentration were linear over the range 1–1,000 pmol with R² routinely > 0.99 (Figure 3D).
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