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Mitoapocynin, a mitochondria targeted derivative of apocynin induces mitochondrial ROS generation and apoptosis in multiple cell types including cardiac myoblasts: a potential constraint to its therapeutic use

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Abstract

Mitoapocynin is a triphenylphosphonium conjugated derivative of apocynin that specifically locates to the mitochondria. It has been developed as a mitochondrially targeted therapeutic antioxidant. We attempted to attenuate the mitochondrial ROS induced in H9c2 cardiac myoblast cells treated with norepinephrine. Mitoapocynin was a poor quencher of total ROS as detected by the fluoroprobe DCFH-DA. Using mitochondrial superoxide specific probe MitoSoxRed, we found that 5–10 μM mitoapocynin itself induces superoxide over and above that is generated by the norepinephrine treatment. A supposedly control molecule to mitoapocynin, the synthetic compound PhC11TPP, having the triphenylphosphonium group and a benzene moiety with C11 aliphatic chain spacer was also found to be a robust inducer of mitochondrial ROS. Subsequent assays with several cell lines viz., NIH3T3, HEK293, Neuro2A, MCF-7 and H9c2, showed that prolonged exposure to mitoapocynin induces cell death by apoptosis that can be partially prevented by the general antioxidant N-acetyl cysteine. Analyses of mitochondrial electron transport complexes by Blue Native Polyacrylamide gel electrophoresis showed that both mitoapocynin and PhC11TPP disrupt the mitochondrial Complex I and V, and in addition, PhC11TPP also damages the Complex IV. Our data thus highlights the limitations of the therapeutic use of mitoapocynin as an antioxidant.

Keywords Oxidative stress · Mitochondria · Mitoapocynin · NOX

Abbreviations

ROS	Reactive oxygen species
RET	Reverse electron transport
SIRT1	Sirtuin 1
DCFH-DA	Dichloro-dihydro-fluoresceindiacetate
NAC	N-acetyl cysteine
Nox	NADPH oxidase
YFP	Yellow fluorescent protein
PI	Propidium Iodide
Nrf2	The nuclear factor erythroid 2-related factor 2
TPP	Triphenylphosphonium

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Introduction

Mitochondria metabolize carbon sources generating NADH, and then transfer its electrons to molecular oxygen, producing ATP. During this process, it ensure that there is no unwarranted release of electrons or other secondary free radicals that are toxic to the surrounding biomolecules. However, certain electron transporting complexes selectively release electrons at a lower intensity that interact with the

free oxygen, producing superoxide. Superoxide dismutase almost instantaneously converts it to hydrogen peroxide. These reactive oxygen species (ROS) are the mediators of the retrograde and anterograde signalling under physiological conditions [1]. Until date, eleven enzymatic and non-enzymatic sources of ROS have been identified in the mitochondria. These are located in the Complex I, II and III of the electron transport chain [2]. Under physiological conditions, superoxide and hydrogen peroxide generated from these sites play a role in cell growth, differentiation, and metabolic regulations [3]. Certain pathological conditions induce metabolic reprogramming and overproduction of ROS from these sites [4]. Such dysregulated generation of ROS plays a major role in the diseases like cancer, neurodegeneration, diabetes, atherosclerosis, ischemia–reperfusion Injury and other cardiovascular disorders [5–9]. Mitochondria also produce ROS by another mechanism called reverse electron transport (RET) that occurs when electrons from reduced ubiquinone are transferred to NAD+ through the complex I, and reducing it to NADH. RET is involved in physiological processes like the activation of macrophages against bacterial infection, re-organization of the electron transport chain, oxygen sensing etc. [10]. Dysfunctional RET has also been associated with several diseases [11]. Due to the critical role of ROS in health and diseases, the generation and attenuation of mitochondrial ROS remains under tight control. There are several antioxidant enzymes viz., catalase, superoxide dismutase, thioredoxin peroxidase and redox proteins like thioredoxin and glutaredoxin ensuring the homeostatic control of the mitochondrial ROS generation [2].

Since excessive generation of ROS in the mitochondria has been associated with many diseases, their diminution by therapeutic antioxidants have also been explored. Dietary constituents such as vitamin E & C, carotenoids, and polyphenols have long been shown to boost the antioxidant defence [12]. However, these polyphenols from various plant sources are mostly pleiotropic in nature and often exert their beneficial effects through multiple mechanisms. As an example, dietary polyphenols resveratrol, quercetin, catechins etc., exert anti-inflammatory effects via the modulation of signalling pathways and activating SIRT1, a histone deacetylase [13]. Green tea polyphenol epigallocatechin 3-gallate is a widely studied antioxidant that prevents inflammation, weight loss, several heart and brain diseases. It interacts with proteins and phospholipids in the plasma membrane and regulates signalling pathways, mitochondrial function, DNA methylation etc. [14]. Interestingly, in presence of Fe^{3+} it produces hydrogen peroxide, thus acting as a pro-oxidant as well [15]. Therefore, despite the evidences that therapeutic antioxidants are effective for the management of certain degenerative diseases; their utility, especially against complex diseases remains limited. Other issues that also restrict their therapeutic uses are questionable bioavailability, unknown metabolic intermediates, poorly defined target(s) of action and toxicity, if any [16]. Certain non-polyphenolic dietary constituents from fruits and vegetables also boost the cellular antioxidant status by activating various signalling kinases, epigenetic regulators, and microRNAs, which converge through the antioxidant transcription factor Nrf2 [17]. Paradoxically, depending upon their doses and the tissue contexts; several of these dietary phytochemicals exhibit both anti-oxidant and pro-oxidant activities. Therefore, upon indiscriminate consumption, these phytochemicals might scavenge the ROS under certain conditions while facilitating its generation under certain other, severely limiting their therapeutic potential [18].

Due to the immense importance of mitochondrial ROS in the pathobiology of numerous diseases, vis-à-vis the beneficial effects of many phytochemicals; efforts have also been made to deliver of some of those molecules directly to the mitochondria [19]. A common approach to such delivery is to covalently tag the lipophilic cations like triphenylphosphonium [20].

Apocynin is a plant derived nontoxic antioxidant that inhibits nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase or Nox), an enzyme that produces superoxide from oxygen. Human and mice have multiple isoforms of Nox(s) that are widely expressed in various tissues. ROS generated from these Nox(s) regulate the downstream redox signalling associated with neuronal dysfunction, inflammation and other pathological conditions [21]. Apocynin has been extensively studied for its pharmacological properties [22]. In certain cell types, apocynin is enzymatically converted into its dimer Diapocynin, which is more effective than apocynin on the Nox complex. It also inhibits the expression of the mRNA for gp91 (phox) subunit of Nox and suppresses the production of TNF-alpha and IL-10 [23]. When injected intraperitoneally in rats, apocynin is converted to its glycosyl derivative in plasma, liver and brain [24]. Taken together, despite the consensus that apocynin and its derivatives exert their antioxidant effects by the inhibition of Nox, complete mechanism of its action is poorly understood as yet.

Due to the efficacy of apocynin against various neurodegenerative and inflammatory disorders, efforts have also been made for directly delivering it into the mitochondria for quenching the ROS. In several experimental models, mitoapocynin, a triphenylphosphonium derivative of apocynin that easily permeate into the mitochondria, improves the neuronal functions impaired due to oxidative stress [25–28].

We have earlier demonstrated that when H9c2 cardiac myoblasts are treated with norepinephrine (NE), it induces ROS from Nox2 [29]. We further observed that the ROS generated by NE is compartmentalized; while H_2O_2 is generated in the cytosol, superoxide is generated in the mitochondria

[29]. To assess the role of these two ROS in the downstream signalling, we wanted to selectively quench the mitochondrial superoxide load by mitoapocynin. To that purpose, we synthesized mitoapocynin-C11 (apocynin conjugated to a mitochondria-targeting triphenylphosphonium cation moiety [TPP+]) via an alkyl chain consisting of eleven carbon atoms (Fig. 1). The presence of a highly lipophilic and delocalized cationic moiety in mitoapocynin-C11makes it more cell-permeable and selectively target mitochondria [25]. Specifically, its sequestration into mitochondria is facilitated by TPP+ conjugation via long carbon–carbon side chains. We unexpectedly observed that rather than reducing the ROS load, it triggers substantial ROS generation in the mitochondria, followed by apoptosis. Our study thus highlights the serious limitations of the therapeutic use of these mitochondria targeted antioxidant.

Materials and methods

Cell culture and other reagents

H9c2 cardiac myoblasts were procured from Sigma-Aldrich, USA (originally from ECACC, UK). NIH3T3 (murine fibroblast), HEK293 (human embryonic kidney), Neuro 2A (Neuroblastoma) and MCF-7 (human breast cancer) cells were procured from the national cell repository (NCCS), Pune, India (originally from the ATCC, USA).

All reagents used in this study were purchased from Sigma Aldrich, USA unless mentioned otherwise. Foetal bovine serum was obtained from Gibco (USA origin), Hydrogen peroxide sensitive YFP plasmid pHyPer-Cyto was from Evrogen (Moscow, Russia), and MitoSoxRed dye was obtained from Invitrogen, USA. Chemicals used for the synthesis of mitoapocynin were purchased from Sigma-Aldrich, USA; Avra chemicals, India and Spectrochem, India.

General analytical procedures

Silica gel (100–200 mesh) was used for column chromatography. Thin-layer chromatography was performed on Merck-precoated silica gel 60-F254 plates. Chemicals and solvents obtained from commercial sources were purified using standard methods. The 1H and 13C NMR spectra were recorded on a Bruker-Avance 300 MHz/400 MHz/500 MHz (see the Supporting Information). Chemical shifts (δ) are reported in parts per million, using TMS (δ = 0) as an internal standard in CDCl3. Data for1H NMR is reported as follows: chemical shift (δ in ppm), multiplicity (s-singlet, br. s-broad singlet, d-doublet, t-triplet, q-quadruplet, m-multiplet), coupling constant (Hz), integration. ESI mass spectra were recorded on a Finnigan LCQ Advantage max spectrometer. All products reported showed 1H NMR and 13C NMR spectra in agreement with the assigned structures. All tested compounds yielded data consistent with a purity of at least 95% compared with the theoretical values.

Synthesis of (11-Hydroxyundecyl) triphenylphosphonium bromide (2)

A solution of 11-bromoundecane-1-ol (1.0 g, 3.98 mmol) in dry acetonitrile (200 mL) was treated with triphenylphosphine (1.05 g, 4.01 mmol) in round bottom flask. The solution was refluxed with stirring for 48 h. The solvent was removed in a vacuum, and the crude product was subjected to flash column chromatography on silica gel (mesh size

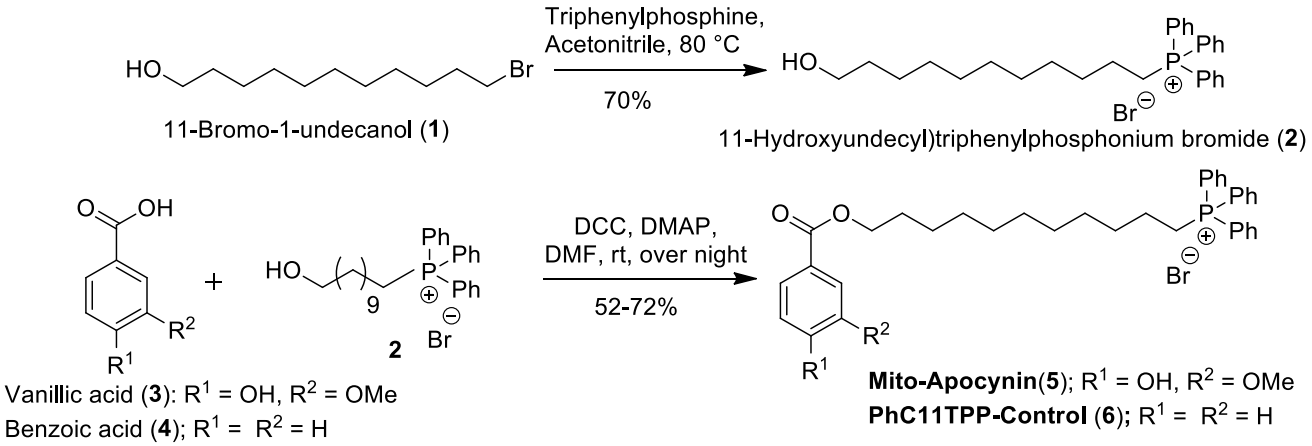


Fig. 1 Synthetic Route to Mitoapocynin and PhC11TPP -Control molecule. In mitoapocynin-C11, apocynin is conjugated to mitochondria-targeting triphenylphosphonium cationic moiety [TPP+]) via eleven carbon aliphatic chain. PhC11TPP, the control molecule is a synthetic compound having a benzene ring conjugated to the triphenylphosphonium cationic moiety via the same eleven carbon aliphatic chain

100–200) with methanol-DCM (5–7%) to yield the compound **2** as a colourless oil (1.43 g, 70%).

1H NMR (300 MHz, MeOD) δ (ppm) 8.14 – 7.66 (m, 15H), 3.60 – 3.43 (m, 4H), 1.83 – 1.45 (m, 6H), 1.30 (s, 12H) 0.13C NMR (75 MHz, MeOD) δ (ppm) 136.34, 136.31, 134.99, 134.85, 131.72, 131.56, 120.65, 119.51, 114.21, 63.06, 55.07, 50.01, 49.72, 49.44, 49.15, 48.87, 48.59, 48.30, 33.74, 31.75, 31.54, 30.74, 30.62, 30.56, 30.43, 30.00, 27.01, 23.68, 23.63, 23.17, 22.50.

Synthesis of mitoapocynin (5)

To a stirred solution of vanillic acid *(3)* (250 mg, 1.48 mmol) in dry dimethylformamide (DMF) (20 mL) was treated with a solution of N, N-dicyclohexylcarbodiimide (DCC; 307 mg, 1.48 mmol) in dry DMF (5 mL) in an argon atmosphere. The mixture was cooled to 0 °C, and a solution of compound **2** (840 mg, 1.63 mmol) in DMF (5 mL) was added slowly. To the resulting mixture, catalytic amounts of N, N-dimethylpyridin-4-amine (DMAP) was added. The reaction was stirred for overnight at room temperature, and any resulting precipitate was removed by filtering. The solvent was then removed, producing a brown residue that was subjected to flash column chromatography on silica gel (mesh size 100–200) with, MeOH-DCM (5–10%) to yield desired compound **5** as a light-yellow gummy oil (511 mg, 52%).

1H NMR (400 MHz, CDCl3) δ (ppm) 7.81 – 7.65 (m, 15H), 7.56 (d, $J = 8.3$ Hz, 1H), 7.51 (s, 1H), 7.02 (d, $J = 8.3$ Hz, 1H), 4.26 (t, $J = 6.4$ Hz, 2H), 3.90 (s, 3H), 3.46 (d, $J = 2.2$ Hz, 2H), 2.04 (s, 1H), 1.78 – 1.67 (m, 2H), 1.62 – 1.45 (m, 4H), 1.45 – 1.35 (m, 2H), 1.33 – 1.26 (m, 2H), 1.28 – 1.14 (m, 8H). 13C NMR (126 MHz, CDCl3) δ (ppm) 166.67, 150.91, 146.79, 135.12, 133.51, 133.43, 130.59, 130.49, 128.85, 123.97, 121.96, 118.63, 117.94, 114.73, 112.22, 77.35, 77.10, 76.85, 71.81, 64.91, 56.11, 30.52, 30.39, 29.70, 29.26, 29.14, 29.01, 28.55, 26.07, 22.63, 22.31, 21.91, 19.18. ESI-MS: m/z [M] + calculated for C37H44O4P+ : 583.29, found: 583.55.

Synthesis of PhC11TPP-control (6)

To a stirred solution of benzoic acid *(4)* (100 mg, 0.818 mmol) in dry dimethylformamide (DMF) (10 mL) was treated with a solution of N, N-dicyclohexylcarbodiimide (DCC; 169 mg, 0.818 mmol) in dry DMF (2.5 mL) in an argon atmosphere. The mixture was cooled to 0 °C, and a solution of compound **2** (462 mg, 0.900 mmol) in DMF (5 mL) was added slowly. To the resulting mixture, catalytic amounts of N, N-dimethylpyridin-4-amine (DMAP) was added. The reaction was stirred for overnight at room temperature, and any resulting precipitate was removed by filtering. The solvent was then removed, producing a colourless residue that was subjected to flash column chromatography on silica gel (mesh size 100–200) with, MeOH-DCM (2–5%) to yield desired compound 6 as a colourless gummy oil (364 mg, 72%).

1H NMR (400 MHz, CDCl3) δ (ppm) 8.03 (d, $J = 6.8$ Hz, 2H), 7.83 – 7.74 (m, 8H), 7.71 (d, $J = 6.9$ Hz, 7H), 7.55 (t, $J = 7.4$ Hz, 1H), 7.43 (t, $J = 7.6$ Hz, 2H), 4.33 – 4.26 (m, 2H), 3.61 (s, 2H), 1.82 – 1.69 (m, 2H), 1.59 (s, 4H), 1.40 (s, 2H), 1.23 (d, $J = 15.6$ Hz, 10H). 13C NMR (126 MHz, CDCl3) δ (ppm) 166.71, 135.07, 133.58, 133.50, 132.83, 130.57, 130.48, 129.82, 129.50, 128.33, 118.69, 118.01, 77.39, 77.13, 76.88, 65.11, 30.51, 30.38, 29.69, 29.39, 29.19, 29.10, 28.69, 25.99, 22.64, 22.42, 22.02. ESI-MS: m/z $[M]^+$ calculated for $C36H42O2P^+$: 537.2917, found: 537.35.

Cell culture

H9c2, MCF-7, NIH3T3, Neuro 2A and HEK 293 cells were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM; 500 mg/l glucose, 2 mmol/l glutamine) supplemented with 10% foetal bovine serum (FBS), 90 units/ml Penicillin, 90 µg/ml Streptomycin and 5 µg/ml amphotericin B at 37 °C in a humidified incubator with 5% CO_2.

Cell viability assay

Cell viability were assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent [30]. Cells were seeded in a 96 well plate at a density of 3×10^3 cells/well and kept overnight, followed by a 12–14 h of serum starvation. The cells were then treated with various concentrations of mitoapocynin or PhC11TPP and incubated for 12 h at 37 °C. In case of checking cell viability in presence of N-Acetyl Cysteine (NAC), selected groups were pretreated with 500 µM or 1 mM of NAC for 1hour, followed by exposure with mitoapocynin or PhC11TPP at 37 °C for 4 h. MTT reagent (20 µL) at concentration of 5 µg/ml was then added to each well and the plates were incubated for 3 h at 37 °C followed by solubilisation of the purple formazan crystals in DMSO. The absorbance was measured at 570 nm in a microplate reader (Thermo Scientific Varioskan Flash).

Measuring apoptosis

DNA content was measured by Propidium Iodide (PI, Sigma-Aldrich P4170) staining. Briefly, cells were seeded in 35 mm culture dishes. Post a 12–14 h of serum starvation, the cells were treated with 5–50 µM M-Apo/PhC11TPP for 8 h. Cells were then harvested and suspended in 1 mL ice cold 1X PBS. After a centrifugation at 1500 rpm for 5 min, PBS was removed and cells were further suspended in 75% ethanol and stored at 4 °C for one overnight. The ethanol was removed post centrifugation at 3000 rpm for 5 min,

followed by 3 washes with 1X PBS. Cells were incubated in dark for 30 min with 500 µL ice-cold FACS buffer (5 mg/mL PI, 10 µg/mL RNase, 0.1% Triton X 100). Cells were analysed by flow cytometer using FACS Aria, Fusion (BD Biosciences, MountainView, CA, USA) at 535 nm excitation maxima.

Monitoring of ROS generation

H9c2 cardiac myoblasts were seeded in 35 mm cell culture dishes at a density of 3×10^5 cells per dish. After a 12–14 h of serum starvation, the cells were labelled with 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 10 µM) along with apocynin or mitoapocynin. After 15 min, the unbound DCFH-DA was washed off with 1X PBS, followed by addition of apocynin or mitoapocynin, together with the adrenergic agonist norepinephrine (2.5 µM) for 10 min. The live cells were then visualized and captured at an excitation maxima of 488 nm under an A1R HD confocal microscope (Nikon). The mean florescence intensity was measured by NIS-Element software (Nikon).

Monitoring superoxide generation in the mitochondria

Cells were seeded at a density of 3×10^5 in 35 mm glass bottom dishes. After an overnight growth, the cells were serum starved for 12–14 h, and then labelled for fifteen minutes with MitoSoxRed (2.5 µM) along with apocynin/mitoapocynin/PhC11TPP/vehicle. After 15 min, the unbound MitoSoxRed was washed off thrice with 1X PBS [31]. Cells were further incubated for an additional 10 min with apocynin/mitoapocynin/PhC11TPP with or without the adrenergic agonist norepinephrine (2.5 µM) for 10 min. Live cell images were captured in a live cell chamber with 5% CO2 at 37 °C with excitation maxima of 510 nm laser line in an A1R HD confocal microscope (Nikon). For the time lapse imaging of superoxide generation, cells were treated with mitoapocynin or PhC11TPP along with MitoSoxRed for 15 min at 37 °C [31]. The excess dye was then removed by washing thrice with 1X PBS. Cells were then replenished with mitoapocynin or PhC11TPP in phenol free media in 5% CO_2 at 37 °C. Imaging was performed at various time points i.e., 0 min, 5 min, 10 min, 30 min, 60 min and 120 min in an A1R HD confocal microscope (Nikon).

Measurement of H_2O_2 generated in the cytosol

Generation of cytosolic H_2O_2 was monitored by the recombinant fluorescent proteins HyPer-Cyto, specifically sensitive to H_2O_2. MCF7 cells grown in 35 mm glass bottom culture dishes to a density of 3×10^5 cells were transfected with 2 µg pHyPer-Cyto plasmid using Lipofectamine 3000 transfection reagent. Twenty-four hours after transfection, cells were kept in serum free media for 12–14 h. Cells were then labelled with MitoSoxRed (2.5 µM) and mitoapocynin or PhC11TPP for 15 min at 37 °C followed by washing thrice with 1X PBS to remove excess MitoSoxRed. Cells were incubated for five more minutes with mitoapocynin or PhC11TPP and live cell images were captured in a chamber with 5% CO_2 at 37 °C in an A1R HD confocal microscope (Nikon) with excitation maxima of 510 nm and 488 nm laser line. The fluorescence intensity was quantified by NIS-element AR-ver 4.000 software.

Estimation of mitochondrial ROS

MCF7 cells were seeded in 96 well black wall plates at a density of 3×10^3 cells per well. Cells were grown overnight and then kept in serum free medium for 12–14 h followed by treatment with mitoapocynin or PhC11TPP at desired doses for various durations at 37 °C. Prior to estimation, cells were treated with MitoSoxRed for 15 min and then washed thrice with 1X PBS and suspended in phenol free media. Absorbance was measured using microplate reader (Thermo Scientific Varioskan Flash) at excitation maxima of 510 nm.

Analysis of mitochondrial respiratory chain complexes by blue native PAGE

The mitochondrial electron transport complexes were analysed by the Blue Native PAGE as described by Konovalova S [32]. Briefly, MCF cells were treated with mitoapocynin or PhC11TPP for 2 and 4 h, cells were harvested and permeabilized by digitonin (1.65 mM). The mitochondrial complexes were released from mitochondrial membrane with the help of 10% lauryl maltoside. The complexes (10 µg of mitochondrial preparation) were then separated on the basis of their mass using a blue native gradient gel and transferred onto a PVDF membrane. To visualize the complexes, the PVDF membranes were incubated with a cocktail of antibodies corresponding to the selected subunits of the five electron transport complexes.

Statistical analysis

Each experiment was performed in triplicate and the results are expressed as mean ± SEM. The experimental groups were compared using one-way ANOVA followed by Tukey's multiple comparison tests using the statistics module of graph pad prism version 7. A value of $P < 0.05$ was considered significant.

Results

Synthesis of mitoapocynin

The published method for the synthesis of mitoapocynin *(5)* involved a large number of steps [25]. We found it difficult and not adaptable to scale-up. We thus optimized and adopted a simple two-step protocol that is economic and gives better yield. The details are given in the Methods and the outline is given in Fig. 1. Briefly, (11-Hydroxyundecyl) triphenylphosphonium bromide *(2)* were synthesized by mixing triphenylphosphine and 11-bromo-undecane-1-ol *(1)* in acetonitrile solvent under refluxing condition. Finally, this salt was used with vanillic acid under standard ester coupling condition to generate target compound mitoapocynin *(5)* with moderate to good yield. We also synthesized a control molecule, PhC11TPP *(6)* where a phenyl ring was inserted instead of vanillin ring. This TPP product was synthesized to compare its bio-activity directly with mitoapocynin. The mitoapocynin and control molecules were characterized by 1H and 13C NMR spectroscopy and ESI-MS mass spectrometry.

Mitoapocynin induces superoxide generation in the mitochondria

In H9c2 cardiac myoblasts, activation of the adrenergic receptors by NE co-stimulates Nox2 that generates ROS in the cytosol and the mitochondria [29, 33]. Considering the importance of mitochondrial ROS in cardiovascular diseases, we wanted to selectively quench it and study the downstream effects [34]. Cells were treated with NE (2.5 µM) along with mitoapocynin (5 and 10 µM) to selectively quench the mitochondrial ROS [22, 25, 35]. Apocynin (5 and 10 µM) was also included as a general antioxidant and an inhibitor of Nox2 for a comparison. Generation of ROS was monitored by the probe DCFH-DA under a fluorescent microscope. As shown in Fig. 2a, apocynin itself slightly increased the ROS levels by 1.5–2.0-fold above baseline but moderately quenched the ROS generated by NE. We consistently observed that the lower dose of apocynin (5 µM)

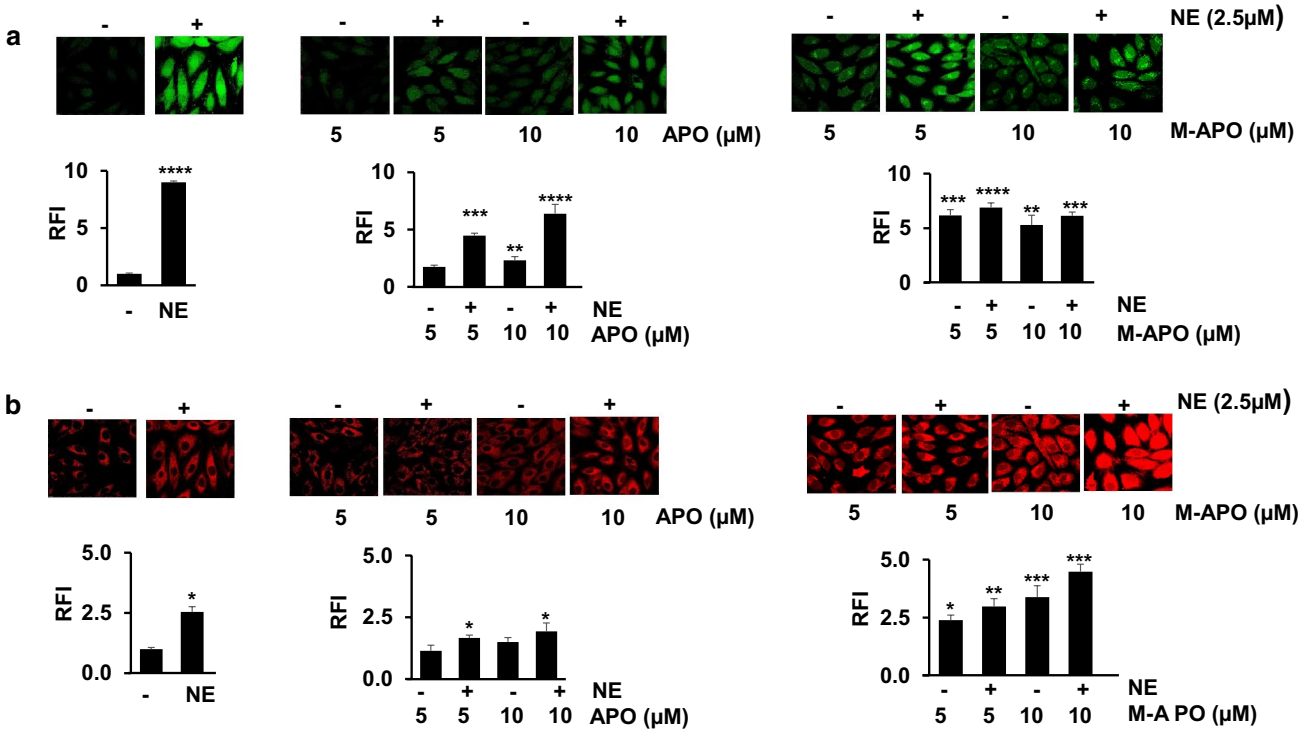


Fig. 2 Mitoapocynin induces superoxide in the mitochondria in H9c2 cells: **a** H9c2 cells were labelled with redox sensitive fluoroprobe DCFH-DA (10 µM) along with apocynin (APO) or mitoapocynin (M-APO) at for 15 min, washed and then treated with the adrenergic agonist NE (2.5 µM) for 10 min. ROS generation were monitored by imaging the live cells under a fluorescence microscope at excitation maxima of 488 nm. **b** Superoxide generation in the mitochondria were monitored by the mitochondria specific fluoroprobe MitoSoxRed (2.5 µM) for 15 min together with APO or M-APO for 15 min. After washing off the florescent probe, cells were treated with NE (2.5 µM) together with APO or M-APO for 10 min. Images of live cells were captured with the excitation maxima of 510 nm laser line at 60X magnification with A1R confocal microscope (Nikon). Relative florescence intensity (fold change) expressed as the Mean ± SEM of three independent experiments performed in duplicate. $^{***}P \leq 0.001$ versus control, $^{**}P \leq 0.01$ versus control, $^{*}P \leq 0.05$ versus control, *RFI* Relative florescence intensity (fold change)

was slightly more effective than the higher dose i.e., 10 µM in quenching ROS (~50% and ~35% respectively). Noticeably, mitoapocynin itself substantially increased the ROS generation with ~6 and 4.5-fold increase above the background, and the lower dose (5 µM) was a stronger inducer. When added together with NE, it was moderately effective in quenching the ROS as ~30–35% reduction was seen for the two concentrations used. The fluoroprobe DCFH-DA detects superoxide, hydrogen peroxide and a several other secondary ROS in the cytosol and other cellular compartments [36]. We thus thought that mitoapocynin might be quenching only a subset of ROS and thus showing lesser efficacy with this probe [25, 26]. We thus further examined the ability of mitoapocynin in selectively quenching the mitochondrial superoxide. Cell treated with NE and apocynin or mitoapocynin were probed with MitoSoxRed, a mitochondria specific probe for superoxide [31]. As shown in Fig. 2b, apocynin itself slightly increased (~1.5-fold) the superoxide level at 10 µM dose and quenched the ROS induced by NE by ~20–30%. Also, in conformity with the data shown in Fig. 2a, the lower dose was slightly more effective quencher than the higher dose. On the contrary, both doses of mitoapocynin alone induced superoxide generation by ~2.5 and 3.5-fold respectively. Quite unexpectedly, when mitoapocynin was added together with NE, the ROS level was even higher than that seen with NE alone. Particularly, 10 µM mitoapocynin together with NE generated twice the amount of superoxide generated by NE alone. This suggest that mitoapocynin and NE generated superoxide in an additive manner. This result was surprising and we further probed whether the generation of superoxide by mitoapocynin in the mitochondria is an intrinsic function of the whole molecule, or it is due to the triphenylphosphonium moiety that is attached to apocynin for targeting it to the mitochondria. As shown in Fig. 3a, superoxide generation by both mitoapocynin and the triphenylphosphonium moiety PhC11TPP was quite robust, but mitoapocynin was slightly stronger than PhC11TPP. Although MitoSoxRed is a specific probe for mitochondrial superoxide, we further confirmed it by treating cells with the general antioxidant N-acetyl cysteine (NAC) and the SOD mimetic MnTMPyP. As shown in Fig. 3b, while 10 µM NAC quenched the ROS generation by mitoapocynin up to ~75%, MnTMPyP completely quenched it even at 25 µM dose. Such result is expected because, while NAC indirectly acts as an antioxidant by increasing the level of glutathione, MnTMPyP mimics SOD that directly destroys the superoxide pool [37, 38].

Mitoapocynin induces apoptosis in multiple cell lines

While experimenting on the generation of superoxide by mitoapocynin, we noticed that after several hours of treatment, cells start rounding up and detaching from the dish, typical signs of apoptosis. We therefore tested whether mitoapocynin induces cell death due to oxidative stress. Number of commonly used cell lines of multiple origins viz., MCF-7 (Human breast cancer), NIH3T3 (mouse embryonic fibroblast), HEK293 (Human embryonic kidney), N2A (Mouse neural crest), and H9c2 (Rat heart) were treated with several concentrations of mitoapocynin till 12 h. Parallel treatments were also done with equivalent doses of PhC11TPP, the triphenylphosphonium tag for mitoapocynin. As shown in Fig. 4a, at the lower doses (5 and 10 µM), both mitoapocynin and PhC11TPP killed the cells in a progressive manner with increasing time. Also, in MCF-7 and NIH3T3 cells, mitoapocynin was more toxic as compared to PhC11TPP. With the higher doses i.e., 20 and 50 µM, the death rate was faster in MCF-7, NIH3T3 and HEK293 cells as substantial cell death were seen within four hours. At 20 µM dose, while mitoapocynin was more toxic for NIH3T3 and H9c2 cells, PhC11TPP was more noxious for the HEK293 cells. Unlike for other cells, in Neuro2A and H9c2 cells, the death rate was progressive, irrespective of the concentration of mitoapocynin or PhC11TPP. Noticeably, among all these cell lines tested, the Neuro2A cells were least sensitive to both mitoapocynin and PhC11TPP as more than 60% of the cells remained viable with the highest dose (50 µM) even after 12 h. To confirm that the cell death were due to apoptosis, we treated cells with mitoapocynin/PhC11TPP, stained with propidium iodide and measured the DNA content in sub-G1 fraction by flow cytometry (Fig. 4b). We also examined whether the generation of superoxide by mitoapocynin is a transient burst as it is seen in cells treated with growth factors, cytokines and hormones [39, 40]. As shown in Fig. 4c, when MCF-7 cells were treated with 10 µM mitoapocynin or PhC11TPP, superoxide generation continued till 2 h, the last time point we had tested. Also, to ensure that the cell death is due to oxidative stress, we treated MCF-7 cells with 0.5–1 mM NAC followed by treatment with mitoapocynin or PhC11TPP (20 µM). As shown in Fig. 4d, NAC partially protected the cells from apoptosis induced by either mitoapocynin or PhC11TPP.

In mitoapocynin treated cells H_2O_2 does not accumulate in the cytosol

Superoxide generated by the mitochondrial electron transport chain, is immediately converted into hydrogen peroxide by the mitochondrial superoxide dismutases. The hydrogen peroxide thus generated often diffuse to the other cellular compartment, especially the cytosol, to elicit physiological responses [41]. We thus examined if mitoapocynin causes any increase in cytosolic hydrogen peroxide. MCF-7 cells were transfected with the plasmid

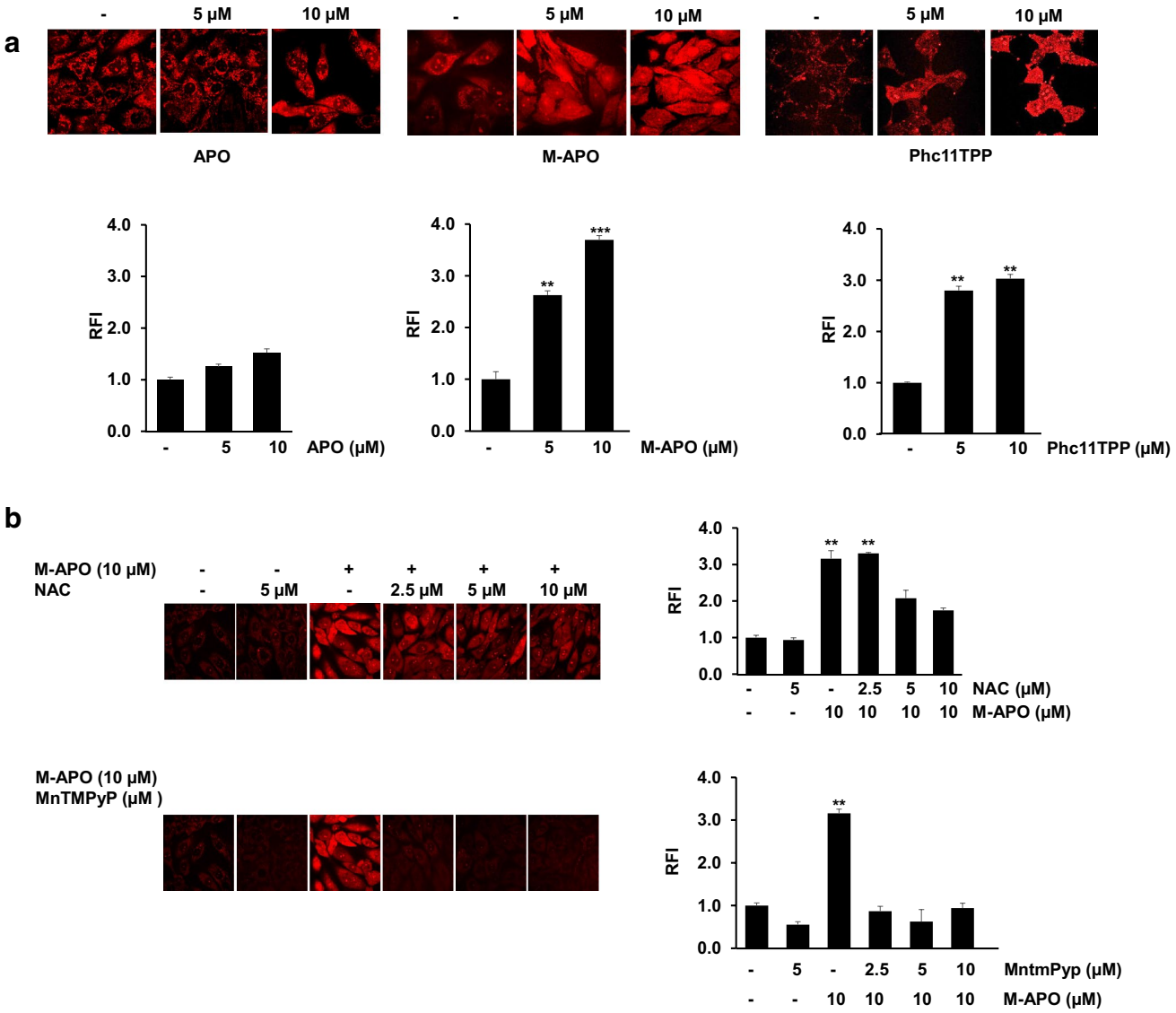


Fig. 3 The triphenylphosphonium tag used for delivering apocynin to the mitochondria induces robust ROS generation. **a** H9c2 cells were labelled with MitoSoxRed together with APO/M-APO/PhC11TPP for 15 min. Excess MitoSoxRed was washed off and cells were re-incubated with APO/M-APO/Phc11TPP for 10 min. Live cells images were captured in a chamber with 5% CO_2 at 37 °C using A1R HD confocal microscope (Nikon) with excitation maxima of 510 nm at 60X. **b** H9c2 cells were treated with the antioxidant N-acetyl cysteine (NAC) or the SOD mimetic MnTMPyP along with MitoSoxRed for 20 min. Post wash, cells were again incubated with M-APO (10 µM) for 15 min and images were captured with excitation maxima of 510 nm laser line by A1R HD confocal microscope (Nikon). Relative florescence intensity (fold change) was expressed as the Mean ± SEM of three independent experiments performed in duplicate. ****$P \leq 0.0001$ versus control; ***$P \leq 0.001$ versus control, **$P \leq 0.01$ versus control, *$P \leq 0.05$ versus control. *RFI* Relative florescence intensity (fold change)

Hyper-Cyto that encodes a cytosolic hydrogen peroxide sensing fluorescent protein. Cells were then treated with mitoapocynin and MitoSoxRed. As shown in Fig. 5, there were no appreciable increase in fluorescence in the cytosolic Hyper-Cyto in mitoapocynin or PhC11TPP treated cells. This suggest that the superoxide that is generated by mitoapocynin largely remain in the mitochondria and does not increase the level of cytosolic H_2O_2.

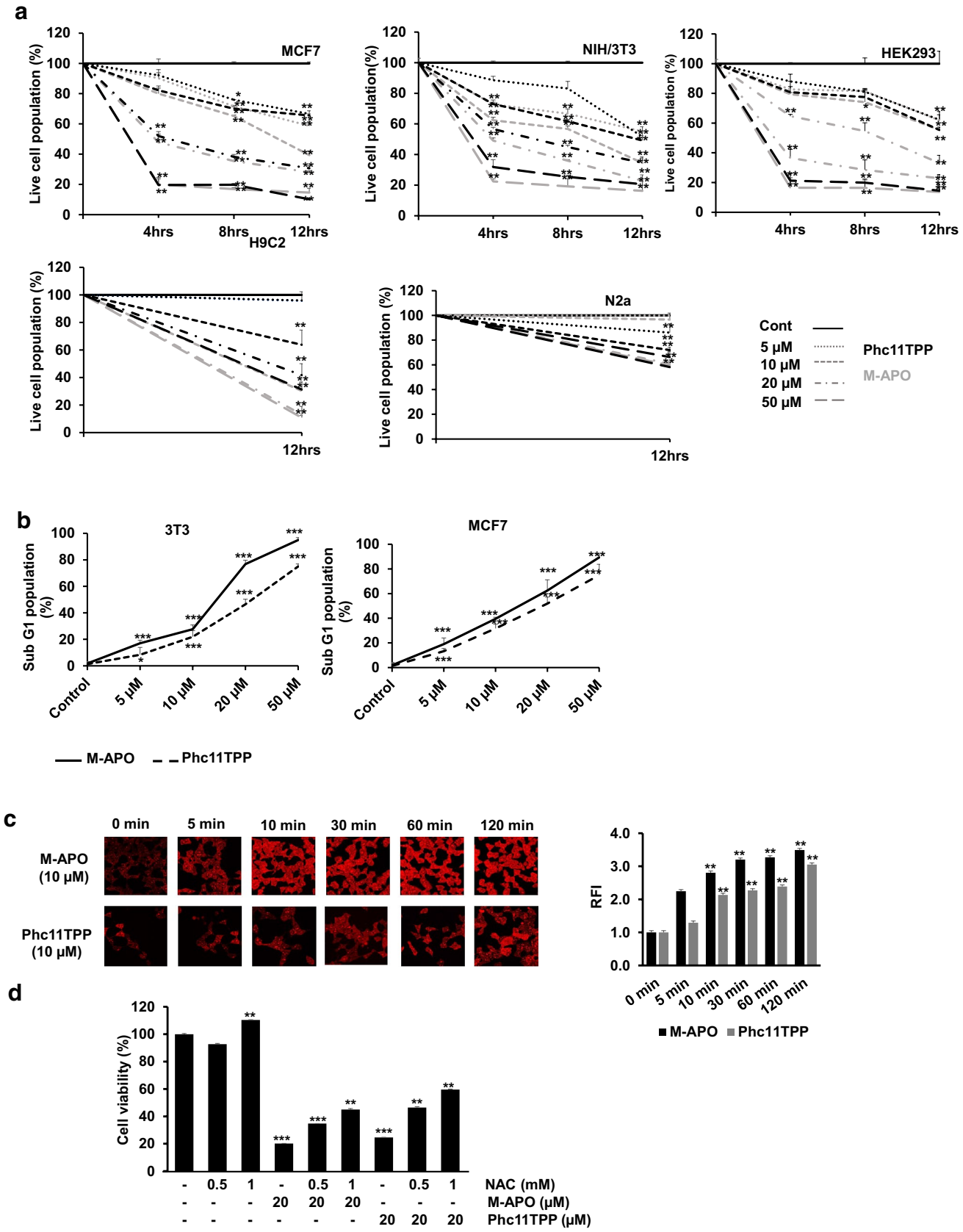
Mitoapocynin causes disassembly of the mitochondrial electron transport complexes

In a study using *C elegans*, it has been demonstrated that mitochondrial SOD-2 & 3 remains associated with the electron transport complexes, protecting those from oxidative damage. In worms deficient in *sod-2*, supercomplexes are damaged due to oxidative stress [42]. Considering that mitoapocynin generates ROS followed by cell death, we

looked into the possible damage to the mitochondrial electron transport complexes. MCF-7 cells were treated with mitoapocynin or PhC11TPP for 2 and 4 h, mitochondrial fractions were isolated and resolved on a Blue Native gradient PAGE. Immunoblotting was done with the antibody cocktail recognizing electron transport complexes. As shown in Fig. 6, Complex I and V are sharply decreased in mitoapocynin treated cells and the decrease was sharp for the Complex I at 4 h. In PhC11TPP-treated cells, there were also a decrease in Complex I and V, but unlike in mitoapocynin treated cells, even after 4 h, some of the Complex V was still remaining. Also, in PhC11TPP treated cells, Complex IV was not visible at 4 h. These data suggest that upon treatment with mitoapocynin and PhC11TPP, mitochondrial electron transport complexes, particularly Complex I and V are damaged and this is likely to be the cause of cell death induced by these two reagents.

Discussion

Mitochondria is second only to the nucleus for its structural and functional complexity. Besides carbon metabolism and ATP generation, it also play key roles in calcium homeostasis, redox signalling, apoptosis etc. It is thus a common target for the antioxidants; diagnostic probes; drugs for cancer, cardiovascular, and neurodegenerative diseases [43]. The lipophilic triphenylphosphonium cation has widely been used for delivering anticancer compounds, antioxidants, micoRNAs, and photosensitizers into the mitochondria [44, 45]. Delivery of several natural antioxidants viz., co-enzyme Q [Mitoquinone], vitamin E [Mitochromanol], SOD mimetic [MitoTEMPOL] and apocynin [mitoapocynin] have shown promising results [44]. The objective of our study was to attenuate the mitochondrial ROS generated in H9c2 cells upon treatment with NE and to follow its physiological consequences. Since our earlier study have clearly demonstrated that apocynin can mitigate the hypertrophic effects of NE on rat heart, we wanted to have a mechanistic insight by selectively targeting it to the mitochondria, the primary source of ROS in NE treated cells [35]. Although mitoapocynin have been used for mitigating neuronal dysfunctions [25–28], its efficacy on the cardiac muscle cells have not been examined. However, alleviation of the adverse effects of the β-adrenergic agonist isoprenaline by delivering Puerarin, an isoflavone; to the mitochondria has been reported [46]. Our observation that apocynin was moderately effective in quenching the DCFH-DA sensitive ROS was not surprising as there are certain inherent limitations in the assay. Firstly, the chemistry of oxidation of DCFH-DA is quite complex and under certain condition this probe itself could be a source of secondary ROS generation [36]. Secondly, upon treatment with NE, H9c2 cells generate multiple ROS, some of which have not been characterized as yet and a few of those might not be quenched by apocynin [33]. Thirdly, although numerous studies have shown that apocynin is both an antioxidant and an inhibitor of Nox2; its ability to inhibit Nox in non-leukocytes have been contested [47]. It has been shown that apocynin even generates ROS in mouse embryonic stem cells [48]. Therefore, depending upon the cell type, it might have multiple mode of actions [49]. Rather, what was perplexing is, mitoapocynin not only failed to neutralize the mitochondrial superoxide generated by NE, it increased the ROS level on its own in an additive manner. This was contrary to the prevailing understanding of the therapeutic value of delivering apocynin to the mitochondria. Like DCFH-DA, the accuracy of MitoSoxRed in measuring superoxide in the mitochondria has also been debated. These probes are prone to generation of secondary free radicals and might have an additional oxidation product [50]. Nevertheless, despite such limitations, if there is any in our assays, the generation of mitochondrial superoxide by mitoapocynin, either alone or in presence of NE was convincing as it is substantial and dose dependent. Further perplexing was the observation that slightly lower but still substantially high level of ROS was also generated by PhC11TPP, the triphenylphosphonium cationic moiety used for targeting apocynin to the mitochondria. Anticipating that these unexpected results could be due to certain specific characteristics of the H9c2 cells, we extended our study to several other cell lines. Five other cell lines of diverse tissue origins also showed ROS generation followed by cell death with both mitoapocynin and PhC11TPP. Since tools and techniques for measuring ROS generation is tissues are not available, we could not extend our study in vivo. Nevertheless, considering least commonality between the cell lines we have tested, we believe that the in vivo effects of mitoapocynin would not be very different from the ex vivo results. To be especially noted that we also observed subtle differences in the kinetics and the extent of toxicity of PhC11TPP and mitoapocynin between themselves for a given cell line; as well as between the cell lines. The killing effects of mitoapocynin or PhC11TPP was rather less on Neuro2a cells as compared to the other cell lines tested. It is thus likely that in the previous studies on the protecting effects of mitoapocynin neurodegeneration in experimental animals, the non-specific cytotoxicity of mitoapocynin might have gone unnoticed [26–28]. Although these results suggest that the primary source of ROS generation is PhC11TPP, it needs to be answered why apocynin could not mitigate it? Available literature suggests that upon oxidation by the ROS, apocynin is converted into its di- and trimers [51]. So, it is possible that, due to its covalent linking to the triphenylphosphonium group, its di- or trimerization is prevented. To be noted that in mouse embryonic stem cells, apocynin induces ROS rather than quenching it [49]. So, it is also possible that


a
MCF7
NIH/3T3
HEK293
H9C2
N2a
Live cell population (%)
4hrs
8hrs
12hrs
Cont
5 μM
10 μM
20 μM
50 μM
Phc11TPP
M-APO
b
3T3
MCF7
Sub G1 population (%)
Control
5 μM
10 μM
20 μM
50 μM
M-APO
Phc11TPP
c
0 min
5 min
10 min
30 min
60 min
120 min
M-APO (10 μM)
Phc11TPP (10 μM)
RFI
d
Cell viability (%)
NAC (mM)
M-APO (μM)
Phc11TPP (μM)

◂**Fig. 4** Mitoapocynin and PhC11TPP induces cell death. **a** MCF7, NIH3T3, H9c2, Neuro2A, and HEK 293 cells were incubated with M-APO or PhC11TPP (5–50 μM) for different durations till 12 h in a tissue culture incubator. Upon completion of incubation for the desired durations, cell viability was measured by the MTT reagent as described in the Methods. The purple formazan crystals were solubilized in DMSO and the absorbance was measured at 570 nm in a microplate reader (Thermo Scientific Varioskan Flash). **b** Cells were incubated with M-APO or PhC11TPP (5–50 μM) for 8 h, followed by trypsinisation and centrifugation to make single cell suspensions. The cells were then stained with Propidium iodide and analysed at 535 nm Ex max. The graph represents Sub G1 population as apoptotic cells. Cells were captured by BD FACS Aria Fusion (the raw data is given in supplementary Fig S1). **c** MCF7 cells were labelled with MitoSoxRed along with 10 μM of M-APO/PhC11TPP for 15 min. The unbound dye was then washed off and cells were re-treated with M-APO/PhC11TPP. At different time points as marked, live cell images were captured by A1R HD confocal microscope (Nikon) with excitation maxima of 510 nm laser line. Relative florescence intensity (fold change), was expressed as the Mean ± SEM of three independent experiments performed in duplicate (**d**). MCF7 cells were seeded in a 96 well plate and a subset were pre-treated with NAC (0.5 and 1 mM) of for 1 hr followed by a treatment with M-APO/PhC11TPP (20 μM) for 4 h. Cell viability was assayed by the MTT reagent. Data expressed as the Mean ± SEM of three independent experiments performed in duplicate. ***$P \leq 0.001$ versus control; **$P \leq 0.01$ versus control, *$P \leq 0.05$ versus control. *RFI* Relative florescence intensity (fold change)

in some cell lines, while PhC11TPP acts as a strong inducer of ROS, apocynin group attached to it adds to the ROS generation instead of quenching. This possibility is in tune with our consistent observation that the lower dose of apocynin (5 μM) is a better quencher of ROS than the higher dose i.e., 10 μM. Taken together, these results do not necessarily contradict what is established about apocynin, i.e., it is an antioxidant both in vivo, and ex vivo. It rather suggest that a cautionary approach is required while assessing its beneficial effects of mitoapocynin in quenching mitochondrial ROS in various tissues. Since metabolic conversion of apocynin is necessary for its antioxidant function in vivo, any structural constrains imposed upon it while conjugating it to a vector molecule ought to be carefully assessed.

Although the details analyses of the structure–function relationship triphenylposphonium conjugated apocynin is beyond the scope of this study, to reiterate the novelty of our observations, we have done another independent assay that shows both PhC11TPP and mitoapocynin damages the electron transport complexes in the mitochondria. This observation is highly novel and future study would be required to test whether the ROS generation by mitoapocynin and PhC11TPP cause damage to the electron transport complexes or it is due to the damage of the electron transport

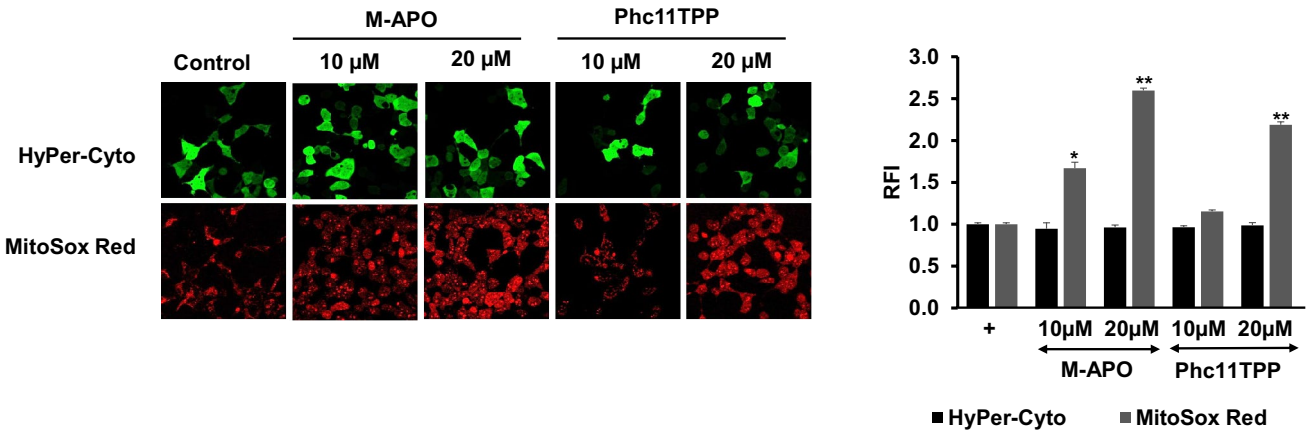


Fig. 5 Mitoapocynin or Phc11TPP does not induce cytosolic hydrogen peroxide MCF 7 cells were transiently transfected with the plasmid pHyPer-Cyto that expresses hydrogen peroxide sensitive fluorescence protein in the cytosol. Cells were labelled with MitoSoxRed (2.5 μM) together with M-APO/PhC11TPP for 15 min. Unbound dye was then washed off, followed by incubation with M-APO/PhC11TPP for 10 more minutes. Live cell images were captured at 60X with an Ex max at 488 nm and 510 nm for Hyper-Cyto and MitoSoxRed respectively. Quantification of the fluorescence intensity normalized to control was performed (right panel). Data expressed as the Mean ± SEM of three independent experiments performed in duplicate. **$P \leq 0.01$ versus control, *$P \leq 0.05$ versus control. *RFI* Relative florescence intensity (fold change)

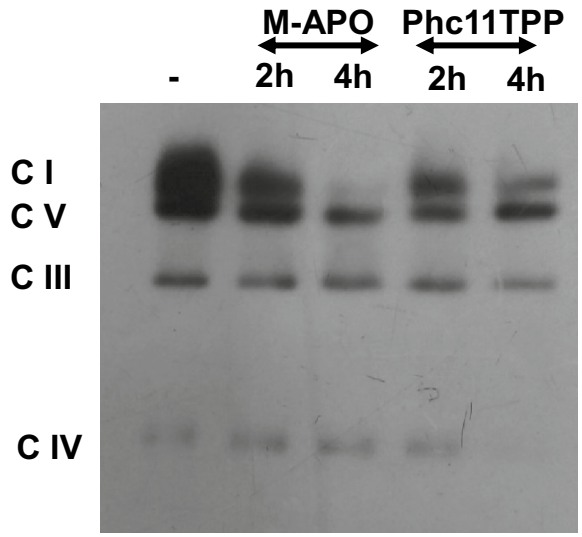


Fig. 6 Mitoapocynin or PhC11TPP damages mitochondrial electron transport complexes MCF-7 cells were treated with M-APO or PhC11TPP (20 μM) for 2–4 h and the mitochondrial fractions were isolated. Eighty micrograms of mitochondrial preparations were resolved on a gradient Blue Native Polyacrylamide Gel followed by immunoblotting using an antibody cocktail of antibodies for the mitochondrial electron transport complexes (NDUFB8 subunit of Complex I, the 30 kDa subunit of Complex II, the Core protein two of Complex IV, and the α subunit of Complex V)

complexes by these compounds, ROS is generated as a by-product. We thus would like to conclude our study with a cautionary note on the limitations of using triphenylphosphonium group for delivering therapeutics to the mitochondria and other organelles.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11010-020-04039-4.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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