**Supporting Information**

# Title: FDA approved L-type channel blocker Nifedipine reduces cell death in hypoxic A549 cells through modulation of mitochondrial calcium and superoxide generation

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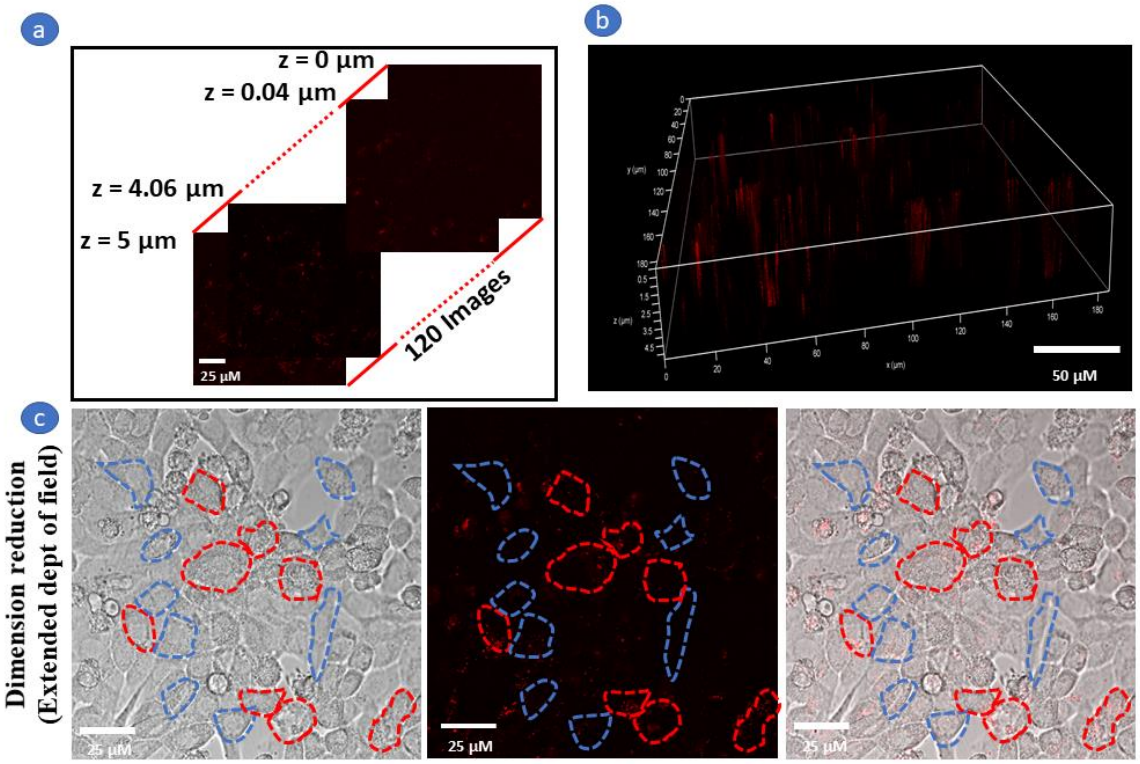
**Supplementary Table S1**: Imaging parameters used for 3D imaging of mitochondrial calcium, ROS, and nuclear morphology

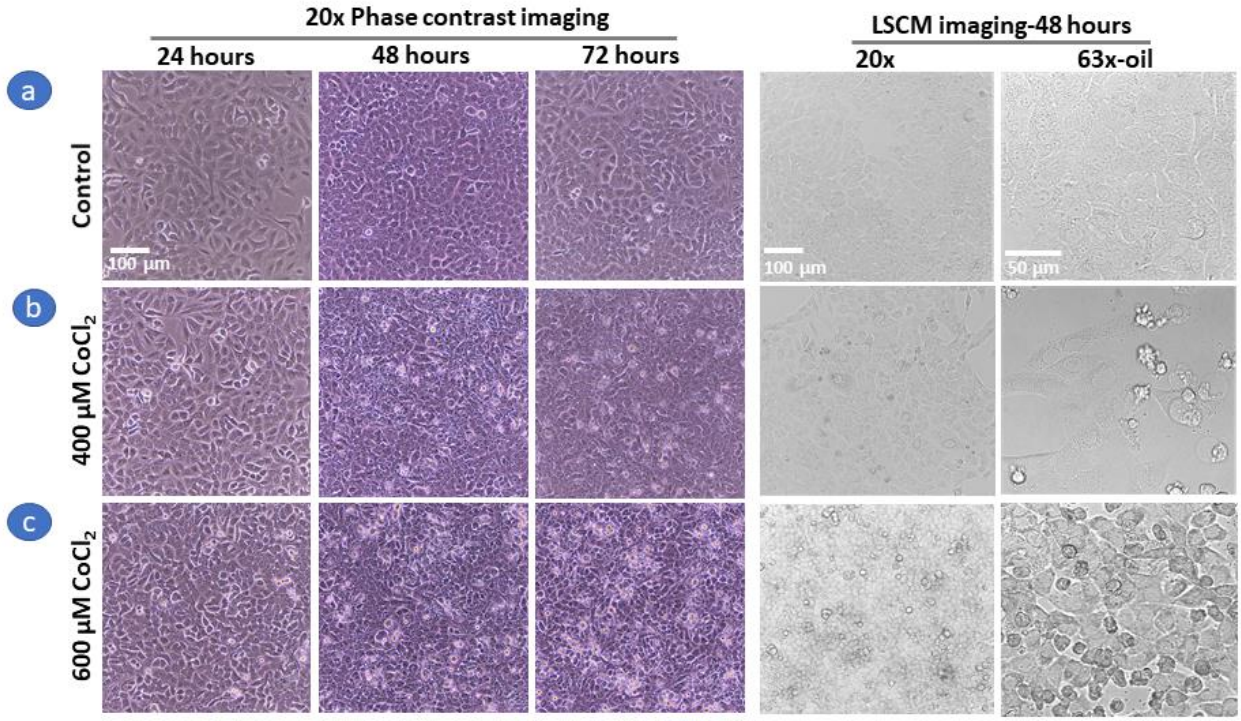
| S.No | Rhod-2 | Mitosox | Hoechst 33342 |
|---|---|---|---|
| z-axis thickness | 5 µm | 5 µm | 25 µm |
| z- axial resolution<br>(Distance between two z-stacks) | 0.04 µm | 0.04 µm | 0.5 µm |
| No of Images | 120 | 120 | 50 |

**Supplementary Figure S1**:



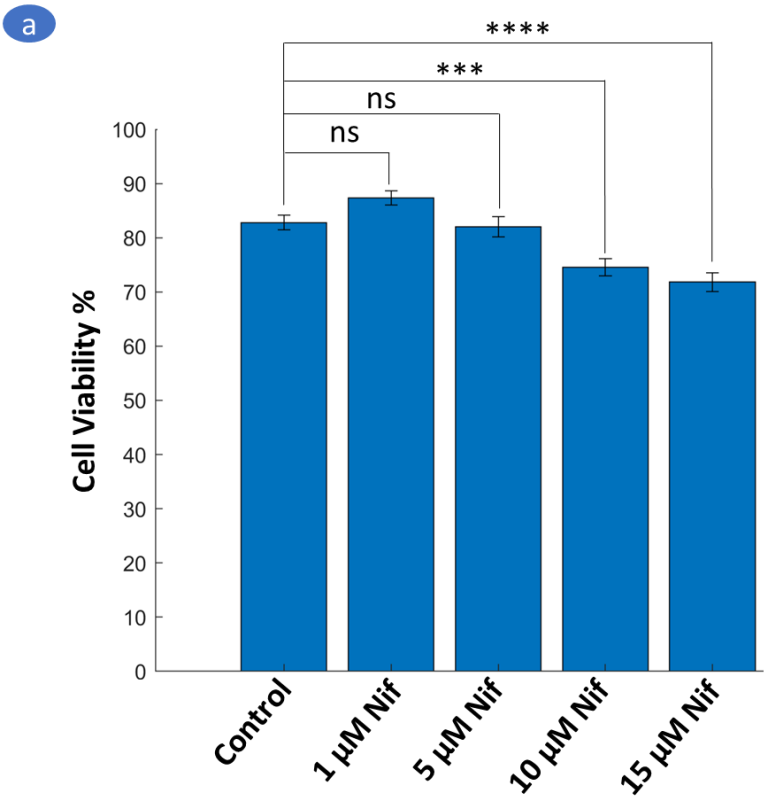

**Supplementary Figure S1**: Z-stack imaging, 3D reconstruction from z-stacks and 2D compression from z-stack imaging of Rhod-2 (a) Representative z-stack images spanning 5 µm (b) 3D reconstruction from 120 z-stacks of Rhod-2 fluorescence intensity, (distance between z-stack images =0.04 µm and thickness of the slice imaged = 5 µm. (c) 2D compressed image obtained by dimension reduction of 120 z-stacks (120) manual ROI (Blue dotted lines- live cells having no/less mitochondrial $Ca^{2+}$ and Red dotted lines- Stressed/Dead cells having high mitochondrial $Ca^{2+}$) was drawn along the periphery of the cell, Left: BF images, Middle: Rhod-2 fluorescence intensity using excitation at 561 nm, Right: BF + 561nm Exc Merge.

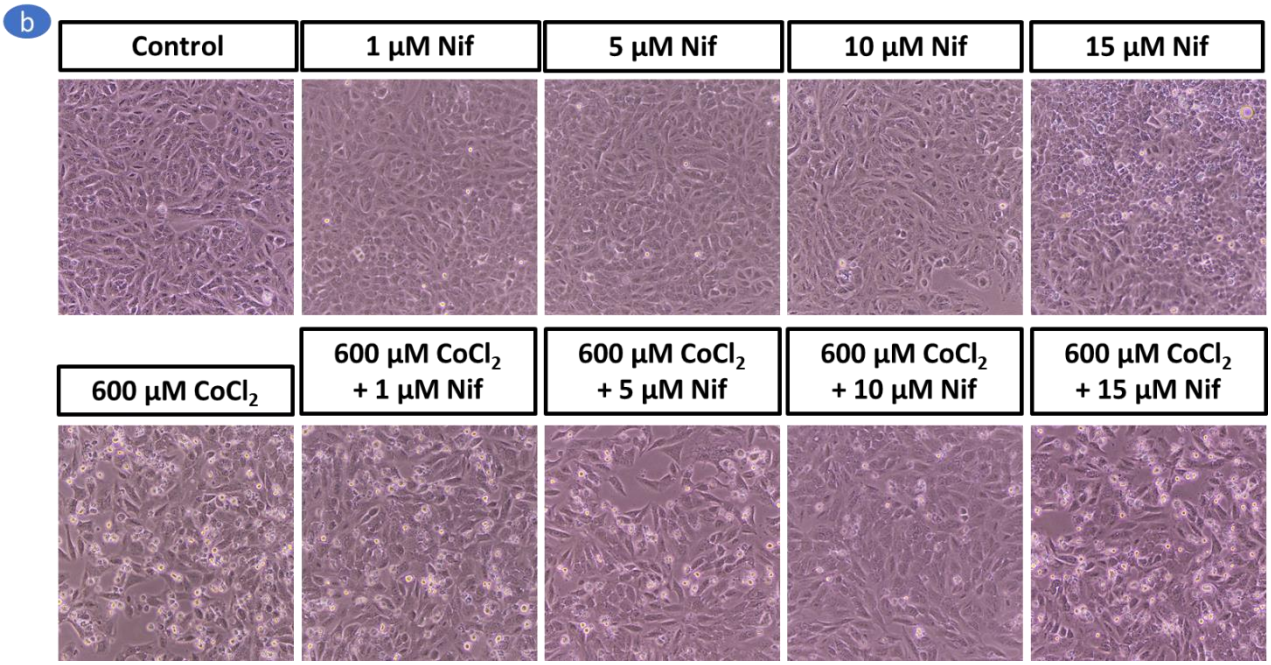
**Supplementary Figure S2**:

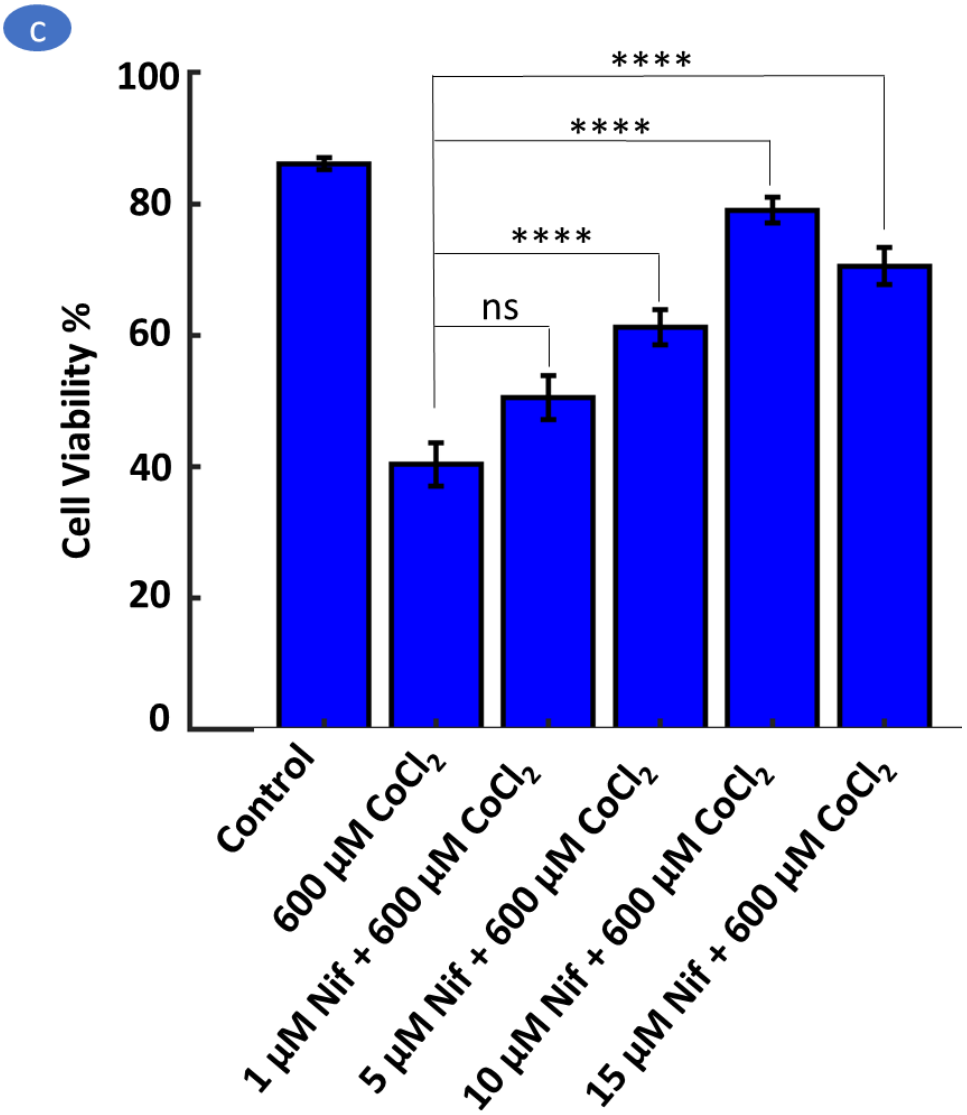


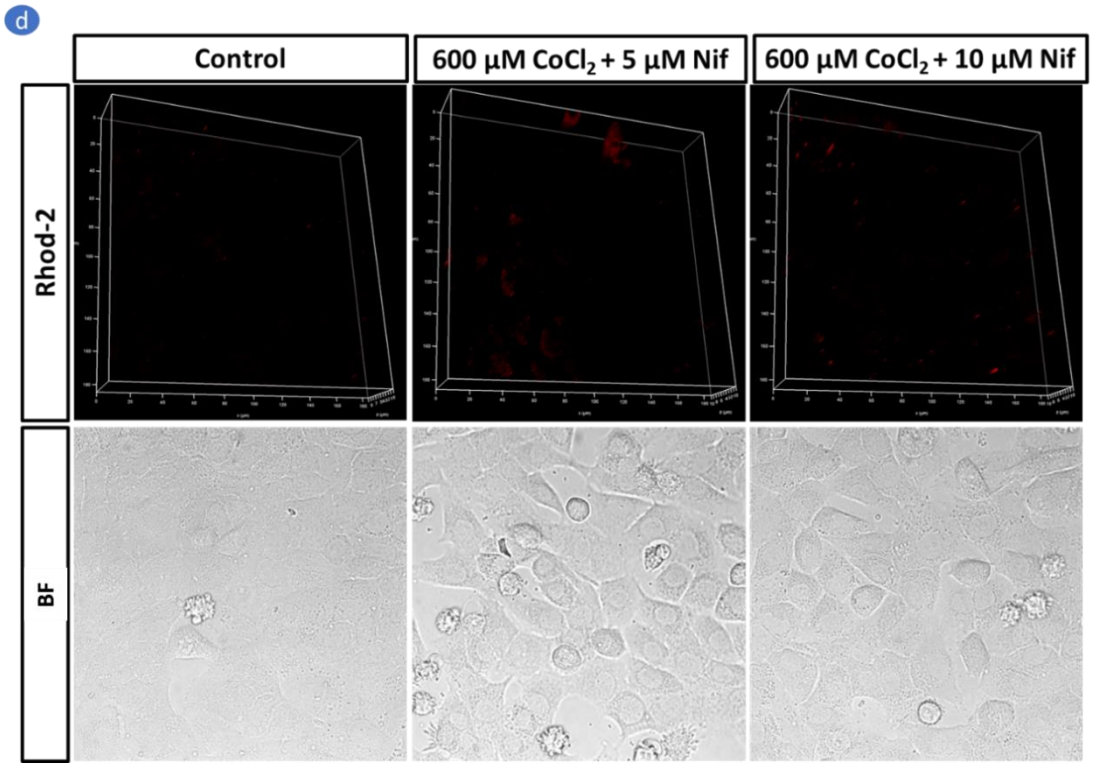

**Supplementary figure 2**: A549 cell morphology at various concentrations of $CoCl_2$. a) Control b) 400 µM $CoCl_2$ c) 600 µM $CoCl_2$, A549 cells were analyzed under phase-contrast microscopy in a time-dependent manner, after inducing chemical hypoxia with $CoCl_2$ for 24, 48, and 72 hours. Cell morphology was also analyzed using 63X oil objective in confocal microscope where it is observed that there is a significant modification in morphologies and presence of dead cells in case of treatment with 600 µM $CoCl_2$ for 48 hours.

**Supplementary Figure S3**:



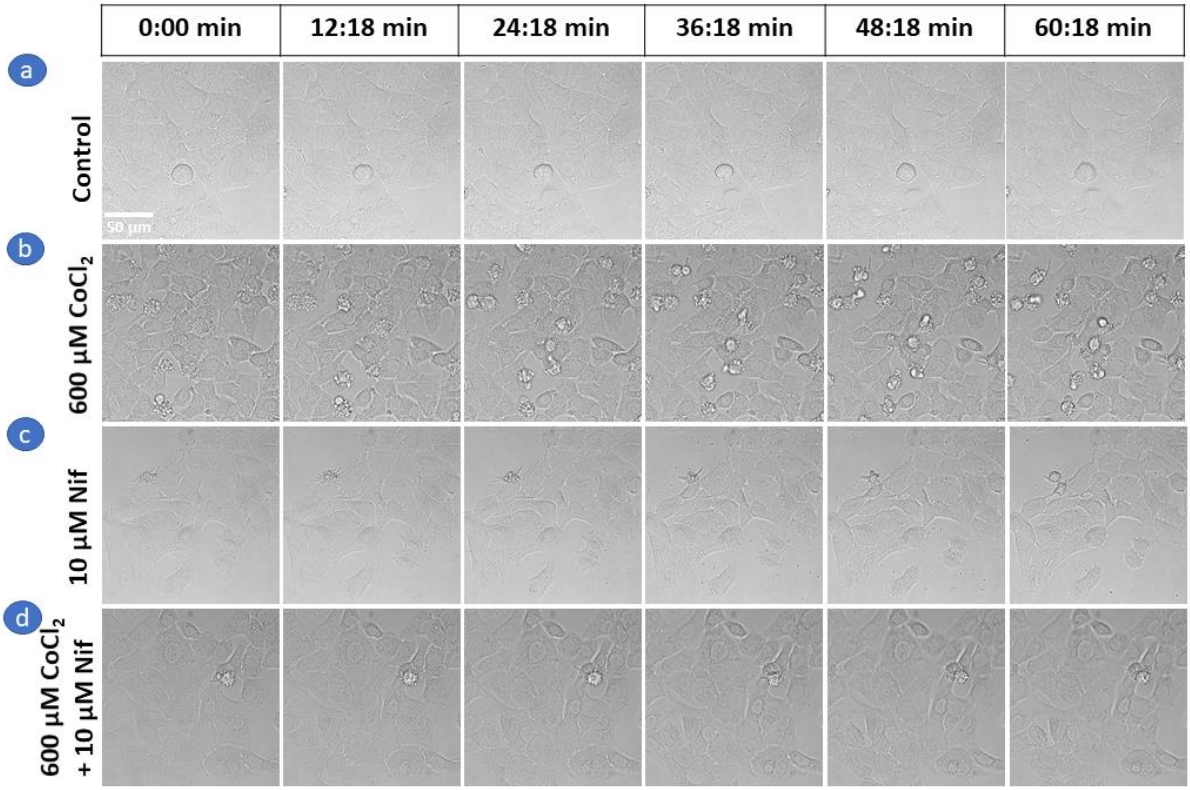




c
Cell Viability %
100
80
60
40
20
0
****
****
****
ns
Control
600 µM CoCl2
1 µM Nif + 600 µM CoCl2
5 µM Nif + 600 µM CoCl2
10 µM Nif + 600 µM CoCl2
15 µM Nif + 600 µM CoCl2



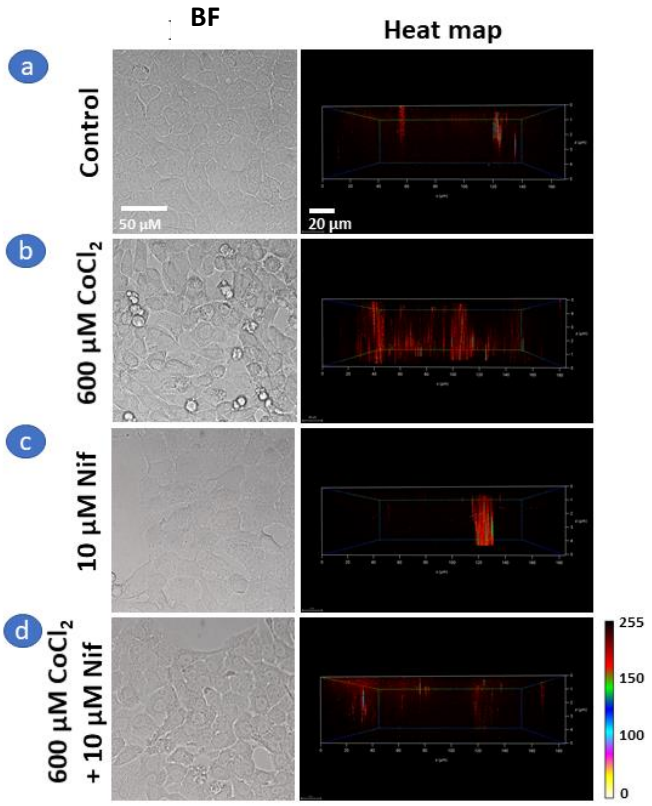

**Supplementary figure 3**: (a) Bar diagram shows nifedipine dose-dependent toxicity on A549 cells. A549 cells treated with 1 µM, 5 µM, 10 µM and 15 µM of nifedipine and cell viability was measured using trypan blue assay at 24 hours and was represented in the. Data are indicated as mean ± SD (n =20). (b) Nifedipine shows a dose-dependent protective effect for $CoCl_2$ treated A549 cells. Phase-contrast images after treatment with $CoCl_2$ at 48 hours with different doses of nifedipine. Excess dead cells were observed in 600 µM $CoCl_2$ treatment. (c) Nifedipine shows dose-dependent protective effect for $CoCl_2$ treated A549 cells. A549 cells after various combinations of treatment control and different doses- 1 µM, 5 µM, 10 µM and 15 µM of nifedipine (Blue), and 600 µM $CoCl_2$, 600 µM $CoCl_2$ + 1 µM Nifedipine, 600 µM $CoCl_2$ + 5 µM Nifedipine, 600 µM $CoCl_2$ + 10 µM Nifedipine and 600 µM $CoCl_2$ + 15 µM Nifedipine (orange). Cell viability was measured using trypan blue assay at 24 hours of nifedipine treatment was represented in the bar diagram. Data are indicated as mean ± SD (n =20). (d) Hypoxia-induced ROS production in A549 cell population and subsequent reduction of mitochondrial ROS through treatment with Nifedipine. Representation of MitoSOX from 3D images corresponding to control, 600 µM $CoCl_2$ + 5 µM Nifedipine, and 600 µM CoCl2 + 10 µM Nifedipine. BF Imaging was performed using a 63X oil objective. Additionally, 3D imaging was performed to measure the intensity of MitoSOX in single cells.

**Supplementary Figure S4**:



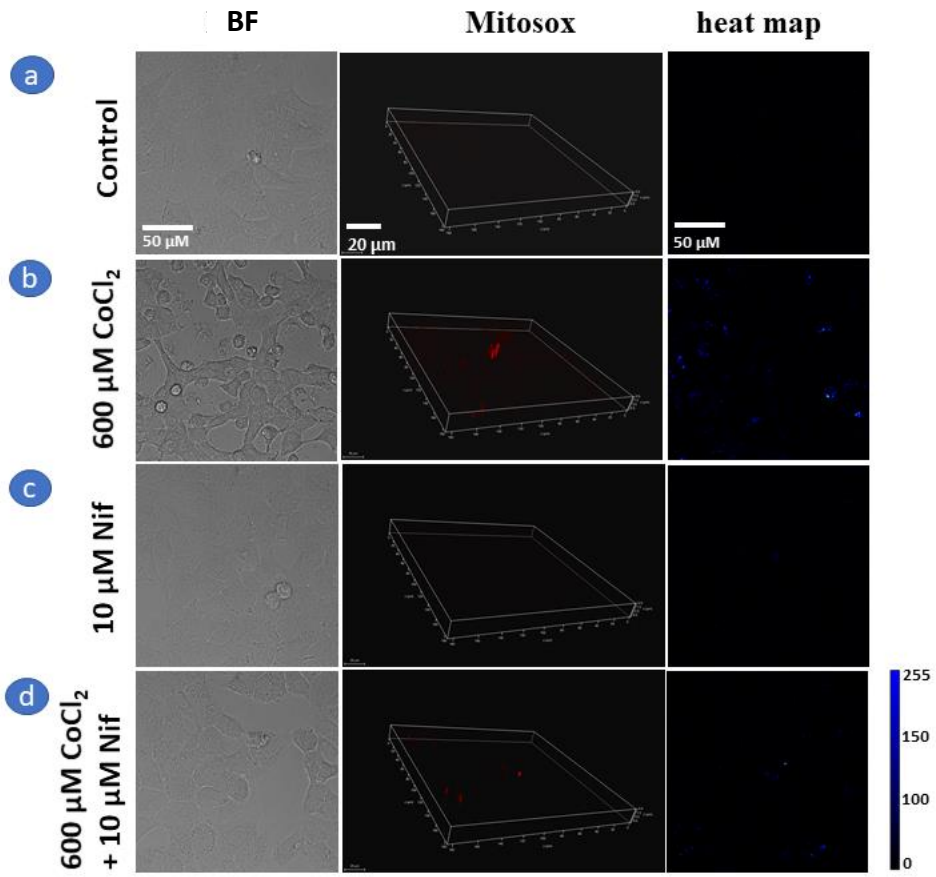

**Supplementary figure 4**: Time-lapse imaging (1-hour time frame) of $CoCl_2$ induced cell death in A549 cells and revival with Nifedipine. Representative images of cell morphology captured by BF imaging in four conditions, including a) control, b) 600 µM $CoCl_2$, c) 10 µM Nifedipine, and d) 600 µM $CoCl_2$ + 10 µM Nifedipine. A549 cells were treated with $CoCl_2$ for 24 hours, followed by treatment with Nifedipine for 24 hours. 63X oil objective was used for time-lapse BF imaging for 1 hour (images were captured every 3 minutes).

**Supplementary Figure S5**:



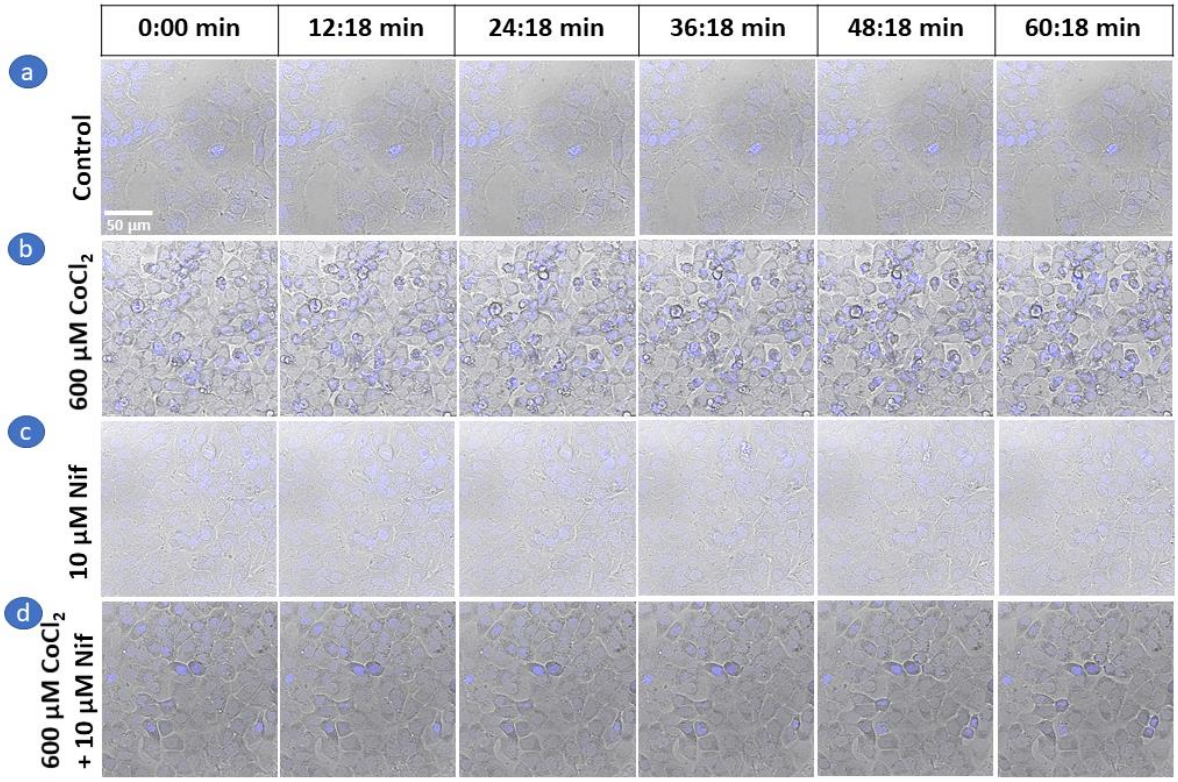

**Supplementary figure 5**: Assessment of nifedipine in reducing the mitochondrial calcium overload. Effect of Nifedipine on mitochondrial $Ca^{2+}$ in a cell population when cells are subjected to chemical hypoxia for 24 hours using 600 µM $CoCl_2$. Confocal microscopy was performed with a 63X oil objective. Heat map representation of Rhod-2 dye in x-z plane from 3D images (z-thickness = 5 µm) corresponding to various conditions including a) control, b) 600 µM $CoCl_2$, c) 10 µM Nifedipine and d) 600 µM $CoCl_2$ + 10 µM Nifedipine.

**Supplementary Figure S6**:



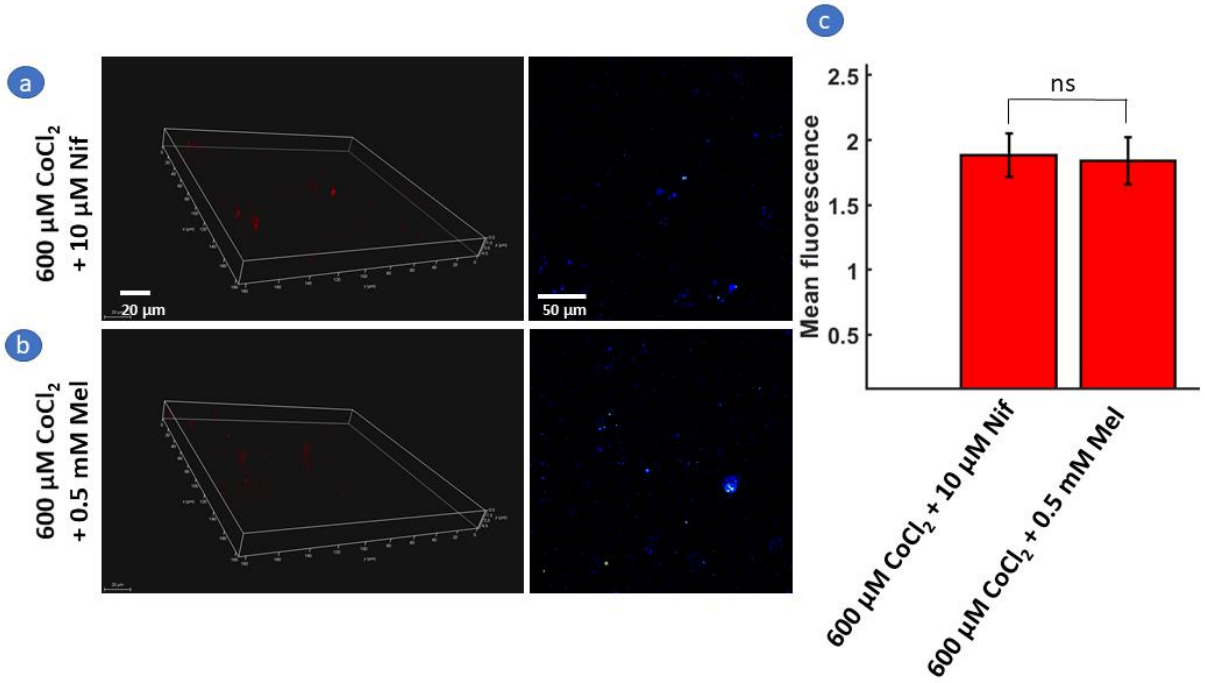

**Supplementary figure 6**: Hypoxia-induced ROS production in A549 cell population and subsequent reduction of mitochondrial ROS through treatment with Nifedipine. Heat map representation of MitoSOX from 3D images corresponding to various conditions including a) control, b) 600 µM $CoCl_2$, c) 10 µM Nifedipine and d) 600 µM $CoCl_2$ + 10 µM Nifedipine. BF Imaging was performed using a 63X oil objective. Additionally, 3D imaging was performed to measure the intensity of MitoSOX in single cells.

**Supplementary Figure S7**:



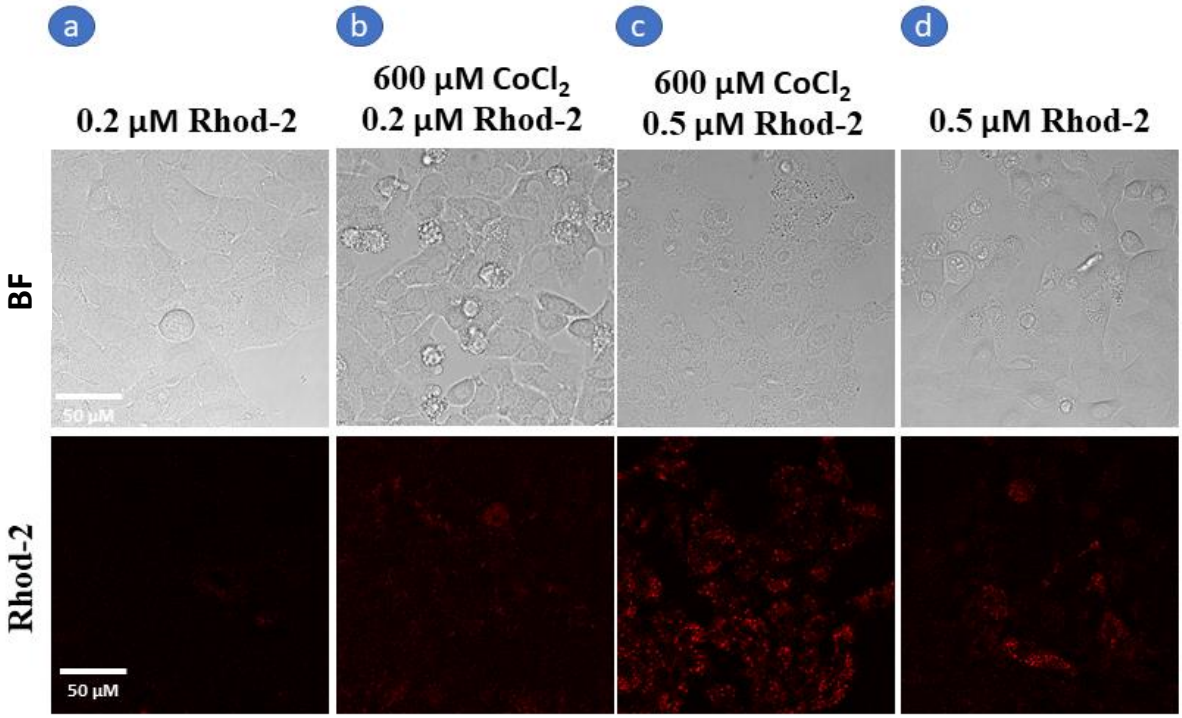

**Supplementary figure 7**: Time-lapse imaging (1-hour time frame) of CoCl2 induced cell death in A549 cells and a revival with Nifedipine. Representative images of cell morphology with nuclear staining (Hoechst 33342) in a) control, b) 600 µM $CoCl_2$, c) 10 µM Nifedipine, and d) 600 µM $CoCl_2$ + 10 µM Nifedipine. A549 cells were treated with $CoCl_2$ for 24 hours, followed by treatment with Nifedipine for 24 hours. 63X oil objective was used for BF imaging

**Supplementary Figure S8**:




**Supplementary figure 8:** Comparison of ROS levels in revival using nifedipine and melatonin. 3D images are presented in the first column, and a corresponding heatmap representation for 2D compression is presented in the second column. 3D imaging and representation of 2D compression (z-axis 5 µm) show ROS levels in A549 cells a) treated with 600 µM $CoCl_2$ + 10 µM Nifedipine and b) treated with 600 µM $CoCl_2$ + 0.5 mM of melatonin, c) comparison of ROS production for the treatment of hypoxic A549 cells by melatonin and nifedipine the effect of Nifedipine and melatonin are comparable in the protection of hypoxia-induced A549 cells. Mel- melatonin, Data are indicated as mean ± SD (n-30 cells). $p > 0.5$ (ns - not significant), Kruskal-Wallis test.

**Supplementary Figure S9**:




**Supplementary figure 9**: Measurement of mitochondrial calcium at various concentrations of Rhod-2 dye. a) control cells + 0.2 µM Rhod-2, b) 600 µM $CoCl_2$+ 0.2 µM Rhod-2, c) 600 µM $CoCl_2$+ 0.5 µM Rhod-2, d) control cells + 0.5 µM Rhod-2. The result shows that a higher concentration of Rhod-2 may induce a change in cell morphology and cell death when cells are already subjected to hypoxia. Mitochondrial $Ca^{2+}$ was measured using monitoring of Rhod-2 intensity after 24 hours treatment with $CoCl_2$. BF and Rhod-2 fluorescence imaging was performed simultaneously in confocal microscopy using 63X objective. A lower concentration of Rhod-2 helps in retaining the shape and morphology of A549 cells under control and hypoxic condition.

**Supplementary video 1:** Time-lapse video for monitoring the cell and nucleus morphology in A549 cells treated with 600 μM $CoCl_2$**.** A549 cells were treated with 600 μM $CoCl_2$ for 48 hours, and the nucleus was stained with Hoechst 33342. Time-lapse images were acquired every 3-minute interval for one hour using a 63X oil objective.

**Supplementary video 2:** Time-lapse video for monitoring the cell and nucleus morphology in cells treated with 600 μM $CoCl_2$ and 10 μM Nifedipine. A549 cells were first treated with 600 μM $CoCl_2$ for 24 hours. Then these cells were treated with 10 μM Nifedipine for 24 hours, and the nucleus was stained with Hoechst 33342. Time-lapse images were acquired every 3-minute interval for one hour using a 63X oil objective.

**Supplementary video 3:** Time-lapse video for Rhod-2 intensity for measuring mitochondrial $Ca^{2+}$ in cells treated with 600 μM $CoCl_2$ using 4D imaging A549 cells were treated with 600 μM $CoCl_2$ for 48 hours and was stained with Rhod-2. Time-lapse images were acquired every 3-minute interval for one hour using 63X oil objective (z-thickness = 5 μm).

**Supplementary video 4:** Time-lapse video for Rhod-2 intensity for measuring mitochondrial $Ca^{2+}$ in cells treated with 600 μM $CoCl_2$ using 4D imaging. A549 cells were first treated with 600 μM $CoCl_2$ for 24 hours. Then these cells were treated with 10 μM Nifedipine for 24 hours and were stained with Rhod-2. Time-lapse images were acquired every 3-minute interval for one hour using 63X oil objective (z-thickness = 5 μm).

**Supplementary video 5:** Time-lapse video for monitoring the cell and nucleus morphology in control A549 cells (without any treatment). A549 cells were incubated for 48 hours, and the nucleus was stained with Hoechst 33342. Time-lapse images were acquired every 3-minute interval for one hour using a 63X oil objective.

**Supplementary video 6:** Time-lapse video for monitoring the cell and nucleus morphology in cells treated with 10 μM Nifedipine. A549 cells were first incubated for 24 hours. Then these cells were treated with 10 μM Nifedipine for 24 hours, and the nucleus was stained with Hoechst 33342. Time-lapse images were acquired every 3-minute interval for one hour using a 63X oil objective.