

# The Effect of the Classical TSP0 Ligand PK 11195 On in Vitro Cobalt Chloride Model of Hypoxia in Non-Neuronal and Brain Cell Lines

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## Research Article

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# Abstract

The mitochondrial translocator protein (TSPO) is a modulator of the apoptotic pathway involving reactive oxygen species (ROS) generation, mitochondrial membrane potential ( $\Delta\psi_m$ ) collapse, activation of caspases and eventually initiation of the apoptotic process.

In this *in vitro* study, H1299 lung cells and BV-2 microglial cells were exposed to the hypoxic effect of  $\text{CoCl}_2$  with or without PK 11195. Exposing the H1299 cells to 0.5 mM  $\text{CoCl}_2$  for 24 hours resulted in decreases in cell viability (63%,  $p < 0.05$ ), elevation of cardiolipin peroxidation levels (38%,  $p < 0.05$ ), mitochondrial membrane potential depolarization (13%,  $p < 0.001$ ), and apoptotic cell death (117%,  $p < 0.05$ ). Pretreatment with PK 11195 (25  $\mu\text{M}$ ) exhibited significant protective capacity on  $\text{CoCl}_2$ -induced alterations in the mentioned processes.

Exposure of BV-2 cells to increasing concentrations of  $\text{CoCl}_2$  (0.3, 0.5, 0.7 mM) for 4 hours resulted in alterations in the same cellular processes. These alterations were obtained in a dose-dependent manner, except the changes in caspases 3 and 9. The novel ligands as well as PK 1195 attenuated the *in vitro* hypoxic effects of  $\text{CoCl}_2$ .

It appears that the TSPO ligand PK 11195 can prevent  $\text{CoCl}_2$ -induced cellular damage in both non-neuronal and brain cell lines, and they may offer new therapeutic options in hypoxia-related lung and brain diseases which fail to respond to conventional therapies.

## 1. Introduction

Hypoxia refers to an abnormally exposure of cells or tissues to low level of oxygen. The cellular response to hypoxia has been of great interest to researchers since hypoxia is relevant to essential biological processes including angiogenesis, cellular survival/proliferation, energy metabolism, erythropoiesis, extra-cellular matrix function, invasion/metastasis, iron metabolism, pH regulation, multi-drug resistance and stem cell survival <sup>1,2</sup>.

Neurodegenerative diseases are defined by the progressive loss of neurons, synapses, and protein misfolding, and aggregation of proteins <sup>3</sup>. Reduced oxygen supply has been suggested as an important contributor to pathogenesis of neurodegenerative diseases. Hypoxia was found to induce oxidative stress, inflammation and apoptosis, among other cellular processes, contributing to the pathophysiology of neurodegeneration <sup>3</sup>.

$\text{CoCl}_2$  is a hypoxia-mimicking agent <sup>4</sup>. It inhibits prolyl hydroxylase-domain enzymes (the oxygen sensors) through replacement of  $\text{Fe}^{2+}$  with  $\text{Co}^{2+}$  making these enzymes unable to label hypoxia-inducible factor (HIF)-alpha for degradation <sup>5</sup>. It should be noted that  $\text{CoCl}_2$  mimics HIF-1 alpha accumulation, but no other effects of hypoxia. In addition to its effects on HIF-1 alpha accumulation, exposure to  $\text{CoCl}_2$  can

also modulate processes and pathways, such as apoptosis and reactive oxygen species (ROS) generation <sup>6,7</sup>.

The mitochondrial translocator protein (TSPO) was shown to regulate ROS generation, proliferation, angiogenesis and apoptosis, cellular processes that are relevant to the toxic effect of CoCl<sub>2</sub> <sup>8,9</sup>.

TSPO is highly expressed during inflammation and in various tumor types, such as glioblastomas <sup>10</sup>. In the U118MG glioblastoma cell line, exposed to CoCl<sub>2</sub> at various concentrations was shown to increase ROS formation, cause the collapse of the mitochondrial membrane potential and cause cell death <sup>11-14</sup>. TSPO knockdown using siRNA, or its blockade using the TSPO antagonist PK11195, significantly counteracted the CoCl<sub>2</sub>-induced effects <sup>9</sup>. Several studies investigated the role of TSPO in CoCl<sub>2</sub>-induced hypoxia <sup>7,9,15</sup>. Our research group evaluated the effects of TSPO ligands on CoCl<sub>2</sub>-induced cytotoxicity, on the human H1299 lung cancer and glial cell line. Further investigations on this topic may produce implications on the role of TSPO as a therapeutic target in hypoxic conditions.

In the present study, we evaluated the protective effects of the TSPO ligand PK 11195 in non-neuronal (lung cancer cell line) and brain (microglial cell line) cells exposed to the hypoxic agent CoCl<sub>2</sub> <sup>7</sup> in an attempt to identify the cellular mechanisms that are involved in such putative beneficial effects.

## 2. Methods

### 2.1. Cell lines

In this study, we used two cell lines: **1)** human H1299 lung cancer cell line from the American Type Culture Collection (ATCC). Culture medium used consisted of RPMI (high glucose, with no L-Glutamine and no Sodium Pyruvate), supplemented with Fetal Bovine Serum (10%), 2 mM L-Glutamine and gentamycin (50 µg/ml). **2)** Murine BV-2 microglial cell line (generously provided by Zvi Vogel's laboratory, Weizmann Institute of Science, Rehovot, Israel): The BV-2 cells were cultured in Dulbecco's modified Eagle's medium high glucose containing 4.5 g/l glucose, 4 mM L-glutamine and supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/mL) <sup>16</sup>. These two cell types were cultured at 37 °C in 5% CO<sub>2</sub> and 90% relative humidity.

### 2.2. TSPO ligands pretreatment

The *in vitro* experiments included the following groups: vehicle control group pretreated with 1% ethanol-containing 0.5% Fetal Calf Serum (FCS, biological industries, Beit Ha'Emek, Israel); a group pretreated with 1% ethanol-containing serum starvation medium.

The two cell lines were seeded in 96-well plates (5x10<sup>3</sup> cells per well) or 6-well plates (2.5x10<sup>5</sup> cells per well) (depending upon the type of experiment) and grown in complete medium for 48 hours at 37°C and

5% CO<sub>2</sub> until the desired confluency 80% was reached. Then, pretreatment with the TSPO ligand PK 11195 (25 µM) in serum-starvation medium was applied for another 24 hours.

### 2.3. Cobalt Chloride (CoCl<sub>2</sub>) exposure

CoCl<sub>2</sub> (Sigma-Aldrich, Rehovot, Israel) was prepared at the concentrations required for each specific experiment and applied to the CoCl<sub>2</sub>-treated groups for 24 hours<sup>7,9</sup>.

### 2.4. Cell counting

Cells were grown until 80% confluency was reached, the medium was discarded, cells were washed with phosphate buffer saline (PBS) and collected following trypsinization. For cell counting, 100 µl of the cells was placed in an Eppendorf tube then mixed with 100 µl of trypan blue (ratio 1:1). Under the light microscope, the cells were counted by hemocytometer (Neubauer slide, Sigma Aldrich, Rehovot).

### 2.5. XTT assay

The two cell lines (H1299 and BV-2) were seeded in 96-well plates (5,000 cells/well) and grown for 48 hours in complete medium. Then pretreated with the required TSPO ligand for another 24 hours, followed by exposure of the cells to the desired concentrations of CoCl<sub>2</sub> for 30 minutes, 4 hours, or 24 hours. Assessment of cellular viability was performed using XTT cell viability kit (Biological Industries, Bait Ha'Emek, Isreal), following the manufacturer's protocol: 150 µl medium from each well was removed followed by adding 50 µl from the XTT mixture to the remaining 50 µl medium within the plate, then the plates were incubated in dark for one hour and a half. Reduction of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) depends on mitochondrial dehydrogenases and reductases, which results in orange formazan dye production, a process that occurs only in viable cells. The amount of the orange dye indicates the cellular viability and the optic density (O.D.) was measured using Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) with absorbance with endpoint photometric at 492 nm wavelength and reference wavelength of 620 nm.

### 2.6. JC-1 assay

The mitochondrial membrane potential ( $\Delta\psi_m$ ) depolarization was assessed using the JC-1 assay, which based on cationic, lipophilic tetra-ethyl-benzimidazolyl-carbocyanine iodide JC-1 dye. After seeding the H1299 cells in 6-well plates (250,000 cells/well) for 48 hours and pretreated with 25 µM PK 11195 for another 24 hours, cells were exposed to 0.5 mM CoCl<sub>2</sub> for 24 hours, then cells were trypsinized (600 µl trypsin), collected and centrifuged (660 *g* for 5 minutes at room temperature) followed by removing the supernatant and resuspended in 600 µl of PBS and cells were centrifuged again. Dilution of JC-1 with PBS (1:500), 600 µl were applied and incubated for 30 minutes in dark. Cells were centrifuged again and 400 µl of PBS were added followed by cells filtration and transfer to FACS tubes. In case of intact cells with high  $\Delta\psi_m$ , JC-1 enters the mitochondria and forms J-aggregates emitting red fluorescence at 590

nm. In contrast, cells exposed to  $\text{CoCl}_2$ , with low  $\Delta\psi_m$ , the JC-1 dye remains in cytosol compartment in a monomer form emitting green fluorescence at 527 nm indicating  $\Delta\psi_m$  depolarization (Zeno et al., 2009). The median fluorescence intensity (MFI) indicates  $\Delta\psi_m$  depolarization that was calculated by red/green ratio using FACS, and the results were analyzed using FlowJo (FlowJo LLC, Ashland, Oregon)

### 2.7. Nonyl Acridine Orange (NAO) assay- cardiolipin peroxidation indicator

H1299 Cells were seeded in 6-well plates (250,000 cells/well), after 48 hours, 25  $\mu\text{M}$  of PK 11195 was applied for 24 hours, then cells were exposed to 0.5 mM  $\text{CoCl}_2$  for another 24 hours. Afterwards, cells were trypsinized and centrifuged (660 *g* for 5 minutes at room temperature) followed by neutralizing trypsin with complete medium. The cells were washed with PBS and centrifuged again the supernatant was aspirated and 400  $\mu\text{l}$  of Nonyl Acridine Orange (NAO) stain (diluted with PBS at a ratio of 1:1000) was added. The cells were incubated in dark for 30 minutes, and centrifuged again, then 400  $\mu\text{l}$  of PBS were added and transferred to FACS tubes.

NAO stain was used to assess the cardiolipin peroxidation level. Cardiolipin, a polyunsaturated acidic phospholipid, biosynthesized and localized in the inner mitochondrial membrane. Cardiolipin is known to have high content of unsaturated fatty acid, which makes it more susceptible to ROS-related cardiolipin peroxidation that results in cytochrome c translocation to the cytosolic compartment, where it initiates the mitochondrial apoptotic cascade (Caballero et al., 2013). NAO staining of the cardiolipin content was performed as described previously (Kluza et al., 2002). Elevated MFI of NAO staining indicates lower cardiolipin content due to increased cardiolipin peroxidation. The MFI was measured using CyAN ADP FACS machine (Beckman Coulter, Brea, CA), and the results were analyzed using FlowJo (10<sup>th</sup> version, FlowJo LLC, Ashland, OR).

### 2.8. Necrosis/apoptosis assay

Necrosis/apoptosis assay kit (Abcam, Cambridge, UK) was used to detect apoptosis and necrosis levels in H1299 cells according to the manufacturer's instructions. Seeding of H1299 cells was performed in 6 well-plates (250,000 cells/well). After 48 hours, PK 11195 at a concentration of 25  $\mu\text{M}$  was applied for 24 hours then followed by 24 hours' exposure to 0.5 mM of  $\text{CoCl}_2$ . Cells were trypsinized using 600  $\mu\text{L}$  trypsin [Trypsin EDTA Solution B (0.25%), EDTA (0.05%)] (Biological industries, Beit Ha'Emek, Israel) centrifuged (660 *g* for 5 minutes at room temperature) then the supernatant was discarded, and the cells were resuspended in 200  $\mu\text{l}$  assay buffer (provided in the kit) and transferred to Eppendorf tubes. Staining was conducted by adding 2  $\mu\text{l}$  of Apopoxin to 100  $\mu\text{l}$  of sample for apoptotic cells detection, 1  $\mu\text{l}$  of 7-AAD to 100  $\mu\text{l}$  of sample for necrotic cells detection with subsequent incubation in dark for 1 hour. Before reading the samples, 300  $\mu\text{l}$  of assay buffer were added to each sample. Aria FACS machine (BD bioscience, San Jose, CA, USA) was used with Ex/Em = 490/525 nm for detection of apoptosis, Ex/Em = 550/650 nm for detection of necrosis. The 10th version of FlowJo (LLC, Ashland, OR, USA) was used for calculation.

### 2.9. Cellular ROS/superoxide assay

Cellular ROS/superoxide assay kit (Abcam, Cambridge, UK) was used to detect ROS levels in BV-2 cells upon exposure to 0.3, 0.5, and 0.7 mM CoCl<sub>2</sub> for 4 hours and 24 hours, according to the manufacturer's instructions. On the day of experiment, the medium was removed from wells and washed with 1X washing buffer (provided in the kit in 10X concentration). 100 µl/well of ROS detection dye were applied on the treatment groups for 1 hour in the dark at 37°C. Bottom reading of the plates using Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) was performed with Ex/Em= 488/520 nm.

#### 2.10. Caspases multiplex activity assay

Assessment of the apoptotic markers caspase 3 and caspase 9 was performed in BV-2 cells using the caspase multiplex activity assay kit (Abcam, Cambridge, UK). BV-2 cells were seeded in 96-well plates (10<sup>4</sup> cells/90 µl) and pretreated as required. Following the cells exposure to the desired concentrations of CoCl<sub>2</sub> (0.5 and 0.7 mM) for 4 hours. The diluted caspases 3 and 9 substrates (diluted in assay buffer at ratio of 1:200) were added to the cells and incubated for one hour. Measurements of fluorescence intensity at Ex/Em of 535/620 nm for caspase 3, and at Ex/Em of 370/450 for caspase 9 were conducted using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland).

#### 2.11. TSPO protein expression levels

Measurement of TSPO protein levels in BV-2 cells was performed using FACS upon 4 hours' exposure to 0.3, 0.5, and 0.7 mM CoCl<sub>2</sub>. Medium with the cells were collected from each sample and centrifuged (660 *g* for 5 minutes at room temperature), then supernatant was removed. Cells were washed using PBS and incubated with PBS containing 0.2% Tween 20 (PBS-T) for 10 minutes and centrifuged again at 660 *g* for 5 minutes at room temperature. Then, the cells were incubated overnight with primary anti-TSPO antibody (Abcam, Cambridge, UK) diluted with ratio 1:100 in 3% BSA in PBS-T. On the following morning, the cells were washed with PBS, incubated with goat anti-rabbit AlexaFluor488 secondary antibody for 1 hour, then washed and transferred to FACS tubes. MFI was measured using FACS machine. The results were analyzed using FlowJo 10<sup>th</sup> version (LLC, Ashland, OR, USA).

## 3. Results

The protective ability of PK 11195 to counteract the effect of CoCl<sub>2</sub>-induced mitochondrial damage via TSPO-related processes in non-neuronal (lung cancer) and central (microglial) cell lines included the assessment of: cell viability, oxidative stress, mitochondrial membrane potential depolarization, TSPO protein expression levels, and apoptotic markers.

### 3.1. H1299 lung cancer cell line

#### 3.1.1. The impact of CoCl<sub>2</sub> on cellular viability using XTT assay

Dose-response analysis of cellular viability was measured in H1299 cells after 24 hours' exposure to varying concentrations of CoCl<sub>2</sub> (0.1–1 mM). Significant decreases in cell viability were detected at 0.3 to

1 mM of CoCl<sub>2</sub>. Since a sufficient, but not complete suppressive effect of CoCl<sub>2</sub> on cellular viability was observed at 0.5 mM, this concentration was chosen for all the further experiments (p<0.001; Figure 1).

### 3.1.2. The impact of TSPO ligands on CoCl<sub>2</sub>-induced cellular death (using XTT assay)

Analysis of the protective capacity of the TSPO ligand PK 11195 at a concentration of 25 μM upon H1299 cells exposed to 0.5 mM of CoCl<sub>2</sub> is shown in Figure 2. H1299 cells were exposed to CoCl<sub>2</sub> (0.5 mM) with or without PK 11195. Following exposure to CoCl<sub>2</sub>, significant decreases by 54% (p<0.001; Figure 2) in cellular viability was demonstrated. Pretreatment with PK 11195 25 μM significantly prevented 63% (p<0.01; Figure 2).

### 3.1.3. The impact of PK 11195 on CoCl<sub>2</sub>-induced oxidative stress (using NAO assay)

Cardiolipin peroxidation levels were determined by FACS. Exposure of H1299 cells to 0.5 mM CoCl<sub>2</sub> induced a decrease of NAO fluorescent intensity by 38% (p<0.05 vs. control). Treatment with 25 μM PK 11195 24 hours prior to exposure to 0.5 mM CoCl<sub>2</sub>, prevented almost completely the CoCl<sub>2</sub>-induced decrease in NAO fluorescence intensity (Figures 3A and 3B).

### 3.1.4. The impact of PK 11195 on CoCl<sub>2</sub>-induced mitochondrial membrane potential depolarization (using JC-1 assay)

Exposure of H1299 cells to 0.5 mM CoCl<sub>2</sub> resulted in a significant mitochondrial membrane potential depolarization decrease by 13% (p<0.001) as compared to the control group. Pretreating the cells with PK 11195 at a concentration of 25 μM completely inhibited the CoCl<sub>2</sub>-induced membrane potential depolarization (Figures 4A and 4B).

### 3.1.5. The impact of PK 11195 on CoCl<sub>2</sub>-induced cellular damage: apoptotic and cell death

Exposing H1299 cells to 0.5 mM CoCl<sub>2</sub> for 24 hours led to significant rise in apoptotic cell death by 117% (p<0.05) as compared to the control group. Pretreatment with 25 μM of PK 11195 showed the capacity (p<0.05) to counteract the CoCl<sub>2</sub>-induced apoptosis (Figure 5). PK 11195 alone did not show any significant difference than control.

## 3.2. BV-2 glial cell line

### 3.2.1. The toxic effect of CoCl<sub>2</sub>: Cellular viability using XTT assay

Dose-response study of cellular viability of BV-2 microglial cells exposed to CoCl<sub>2</sub> (0.1, 0.3, 0.5, 0.7, 0.9 mM) for 30 minutes, 4 hours and 24 hours.

After 30 min of exposure to the various concentrations of CoCl<sub>2</sub>, significant decreases in cell viability were observed at 0.7 and 0.9 mM by 20% (p<0.05) and 29% (p<0.001), respectively. After 4 hours of exposure

to  $\text{CoCl}_2$ , significant reductions were observed at 0.5, 0.7, and 0.9 mM by 14% ( $p < 0.01$ ), 32% ( $p < 0.001$ ), and 44% ( $p < 0.001$ ), respectively. After 24 hours of  $\text{CoCl}_2$  exposure, a significant concentration-dependent decrease in cell viability by 19% ( $p < 0.001$ ) at 0.1 mM and reached maximal reduction at 0.9 mM by 90% ( $p < 0.001$ ; Figure 6).

### 3.2.2. The impact of PK 11195 on $\text{CoCl}_2$ -induced oxidative stress using cellular ROS/superoxide detection assay kit

Measurements of oxidative stress levels in BV-2 cells following 4 hours and 24 hours' exposure to  $\text{CoCl}_2$  at 3 different concentrations (0.3, 0.5 and 0.7 mM) with and without the TSPO ligand PK11195 at a concentration of 25  $\mu\text{M}$ . Significant increase by 26% ( $p < 0.001$ ) in oxidative stress levels was detected following 4 hours' exposure of BV-2 cells to 0.7 mM  $\text{CoCl}_2$  as compared to control group. PK11195 (25  $\mu\text{M}$ ) did not prevent this increase in oxidative stress (Figure 7A). 24 hours' exposure of cells to  $\text{CoCl}_2$  (0.3, 0.5, and 0.7 mM) resulted in significant increases in oxidative stress levels by 117%, 198% and 210% ( $p < 0.001$  for all), respectively. Pretreatment with PK 11195 (25  $\mu\text{M}$ ) significantly attenuated this increase in oxidative stress induced by  $\text{CoCl}_2$  exposure for 24 hours ( $p < 0.001$ ; Figure 7B).

### 3.2.3. The impact of PK 11195 on $\text{CoCl}_2$ -induced changes in TSPO protein expression using FACS

A significant elevation by 42% ( $p < 0.05$ ) of TSPO protein expression levels was obtained following exposure to 0.7 mM  $\text{CoCl}_2$ , as compared to the control group. Pretreatment with 25  $\mu\text{M}$  PK 11195, totally prevented the  $\text{CoCl}_2$ -induced TSPO expression elevation following exposure to 0.7 mM  $\text{CoCl}_2$  ( $p < 0.001$ ; Figure 8).

### 3.2.4. The impact of PK 11195 pretreatment on $\text{CoCl}_2$ -induced cellular apoptosis: caspases 3 and 9 using caspases multiplex activity assay kit

Evaluation of caspases 3 and 9 levels after exposing BV-2 cells for 4 hours to various concentrations of  $\text{CoCl}_2$  with or without 25  $\mu\text{M}$  PK 11195 pretreatment. Only at 0.7 mM,  $\text{CoCl}_2$  induced significant increase in caspase 3 levels by 196% ( $p < 0.001$ ), PK 11195 prevented this elevation by 83% ( $p < 0.01$ ; Figure 9A). The levels of caspase 9 were not affected by  $\text{CoCl}_2$  or by the presence of PK 11195.

## 4. Discussion

In the present study the capacity of the TSPO ligand PK 11195 to counteract the  $\text{CoCl}_2$ -induced hypoxic cellular damage was evaluated in both non-neuronal (lung) and central (microglia) cell lines.

### 4.1. Lung cancer cell line (H1299)

We used an established *in vitro* model of pulmonary hypoxia by exposing H1299 lung cancer cell line to cobalt chloride and investigated the protective effects of TSPO ligands in this cellular model<sup>7,9</sup>. According to our data, lung derived cells exposed to various concentrations of  $\text{CoCl}_2$ , ranging from 0.1

mM to 1 mM, for 24 hours led to a dose-dependent reduction in cell viability with maximal toxicity at 1 mM as compared to a control group. Based on the current dose-dependent results, and similar to previous studies<sup>8,9</sup>, which showed sufficiently effective toxic damage that mimics hypoxia at a concentration of 0.5 mM of CoCl<sub>2</sub>, we assessed in our experiments the impact of this concentration on a variety of TSPO-related cellular processes, including: cell death, mitochondrial membrane potential depolarization, cardiolipin peroxidation and ROS generation. We evaluated the protective effects of the TSPO ligands PK 11195 at a concentration of 25 μM in the CoCl<sub>2</sub> hypoxic cellular model. PK 11195 exhibited significant inhibitory effect on CoCl<sub>2</sub>-induced cell viability reduction.

TSPO has been reported to be involved in several mitochondrial processes affected by CoCl<sub>2</sub> exposure, including apoptosis, ROS generation and collapse of mitochondrial membrane potential. One putative pathway for the CoCl<sub>2</sub>-induced cytotoxicity is through an harmful impact on mitochondrial functions mediated by TSPO, including CoCl<sub>2</sub>-induced apoptosis mediated by ROS generation, cardiolipin peroxidation, mitochondrial membrane potential depolarization, and decreased cellular metabolism and viability<sup>8,9</sup>.

In our present study, using ROS generation as an indicator of oxidation, PK 11195 exhibited a potent inhibitory effect on CoCl<sub>2</sub>-induced cardiolipin peroxidation. These findings further strengthen the previously published data on U118MG cells as a model for glioma<sup>9</sup>.

It is likely that the CoCl<sub>2</sub>-induced accumulation of ROS interfered with the mitochondrial homeostasis of the mitochondrial membrane potential. Indeed, a significant increase was seen in depolarization of the mitochondrial membrane potential in CoCl<sub>2</sub>-treated cells as compared to unexposed control group. It appears that CoCl<sub>2</sub> led to ROS generation and disruption of mitochondrial potential which eventually led to cytochrome c release and subsequent initiation of apoptotic pathway<sup>8</sup>. A previous microscopic study revealed morphological, nuclear, and cytological changes as features of apoptosis including condensed chromatin, DNA fragmentation, cell shrinkage and cell surface blabbing<sup>17</sup>. In this study, necrosis/apoptosis assay was used to assess the harmful effects of CoCl<sub>2</sub> on cell viability (apoptotic or necrotic cell death). Following exposure to CoCl<sub>2</sub>, H1299 cells showed significant elevation in apoptosis levels, but not necrosis, as was reported previously<sup>8,9</sup>. Notably, another study performed by our group using cigarette smoke as a hypoxia-causing agent leading damage to cellular hypoxia, also resulted in apoptotic cell death, rather than necrotic cell death<sup>8</sup>.

In the current study, PK 11195 exhibited significant inhibitory effect on CoCl<sub>2</sub>-induced cell viability reduction. A previous study demonstrated the efficacy of the classical TSPO ligand PK 11195 in counteracting the effect of the CoCl<sub>2</sub>-induced damages in astrocytic cell line (U118MG)<sup>9</sup>. In another study, in the same H1299 lung derived cells, the classical high affinity TSPO ligand PK 11195 exhibited a significant protective activity<sup>8</sup>. However, the relationship between the affinity of the ligand to TSPO and the pharmacological activity in the various cell lines and the various cellular functions/pathways and

models for specific pathological damage is yet unclear. Moreover, the anti-hypoxic effects of PK 11195 are relevant to long-term hypoxia (24 hours), but the relevance to shorter period of hypoxia is yet unclear.

#### 4.2. Microglial BV-2 cells

BV-2 microglial cells were chosen to investigate the effect of  $\text{CoCl}_2$  in a cell line from the central nervous system which will enable the differentiation of the effects of  $\text{CoCl}_2$  in cell lines from non-neuronal (lung) and central (brain) origin.

$\text{CoCl}_2$  was used to establish the hypoxic model in BV2 cells. Similar to the H1299 Cells, the impact of  $\text{CoCl}_2$  was concentration-dependent (as assessed at 0.1, 0.3, 0.5, 0.7, 0.9 mM for 30 minutes, 4 hours and 24 hours). Such concentration-dependent alterations in cellular processes were reported also in U118MG glioma cell line<sup>9</sup>. Our findings are consistent with a previous study conducted in our lab on human glioma cells (U118MG cell line), which showed a dose-dependent toxicity of  $\text{CoCl}_2$ <sup>9</sup>. Our data showed that microglial cells derived from the central nervous system may differ from lung cells in their sensitivity to  $\text{CoCl}_2$ . After 4 hours and 24 hours of  $\text{CoCl}_2$  exposure, BV-2 cells exhibited significant reductions in viability in a dose and time-dependent manner.

In the present study, the  $\text{CoCl}_2$ -induced dose-dependent decrease in cellular viability of BV-2 cell line was demonstrated. Comparable effects were obtained between the alterations which occurred using 3 concentrations of  $\text{CoCl}_2$  (0.3, 0.5, 0.7 mM) with exposure times of 24 hours. The higher concentration of  $\text{CoCl}_2$ , namely 0.7 mM, was used in order to obtain more severe hypoxic conditions for the assessment of the protective effects of PK 11195.

In our study using BV-2 cells, we demonstrated an impact of  $\text{CoCl}_2$  exposure on TSPO protein expression levels after 4 hours at the chosen concentration. A significant reduction in TSPO levels was detected at concentration of 0.7 mM as compared to the control group. Furthermore, PK 11195 inhibited that reduction in TSPO levels, indicating the possible involvement of TSPO in the hypoxic pathway initiated by *in vitro*  $\text{CoCl}_2$  exposure.

Similarly, our evaluation of oxidative stress in this cellular model showed a significant elevation after 4 hours of exposure to  $\text{CoCl}_2$  at 0.7 mM, however the pretreatment with PK11195 did not show any protective activity in  $\text{CoCl}_2$ -induced oxidative stress. This inconsistency might indicate the lack of involvement of TSPO in pathways leading to oxidative stress (elevation of superoxide and ROS generation) in BV-2 cells. This observation in microglial cell line is in contrast to what was demonstrated in H1299 cell line, where ROS generation measured by cardiolipin content was prevented by the TSPO ligand PK 11195. The inhibitory effect of PK 11195 on  $\text{CoCl}_2$ -induced oxidative stress in H1299 cells was achieved at 0.3 and 0.5 mM  $\text{CoCl}_2$  but not at 0.7 mM, suggesting limited protective capacity of the ligand in preventing excessive oxidative stress.

Additionally, the finding of apoptotic cell death occurrence in H1299 cells and BV2 cells supports further our previous observations<sup>9,18</sup>. Apoptosis is a form of programmed cell death, in which caspases are strongly involved. Caspases divided into initiator caspases and executioner caspases. In the present study, caspase 3 (initiator) and 9 (executioner) were assessed as apoptotic markers after exposure to the required duration and concentration of CoCl<sub>2</sub>. The elevation in apoptotic markers was inhibited by PK11195. Interestingly no significant effect was shown regarding caspase 9 levels.

In conclusion, CoCl<sub>2</sub> as mimicking agent of hypoxia leads to alterations in several apoptosis-associated processes which occur in parallel to a reduction in the levels of TSPO protein levels. Such processes involve essential mitochondrial functions that after a certain time point and at specific CoCl<sub>2</sub> concentration (0.7 mM) may reach an irreversible damage. The high affinity classical TSPO ligand PK 11195 ligand at a concentration of 25 μM, can prevent some cellular damages caused by exposure to CoCl<sub>2</sub>, however, there are cytotoxic cellular pathways that are insensitive to TSPO ligands and the beneficial effects at present are relevant to only long-term hypoxia (24 hours). Such pathways, mainly relevant to generation of oxidative stress, might occur in a non TSPO-related fashion, and thus no inhibitory/protective impact of TSPO ligands can be obtained.

## Declarations

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## Figures

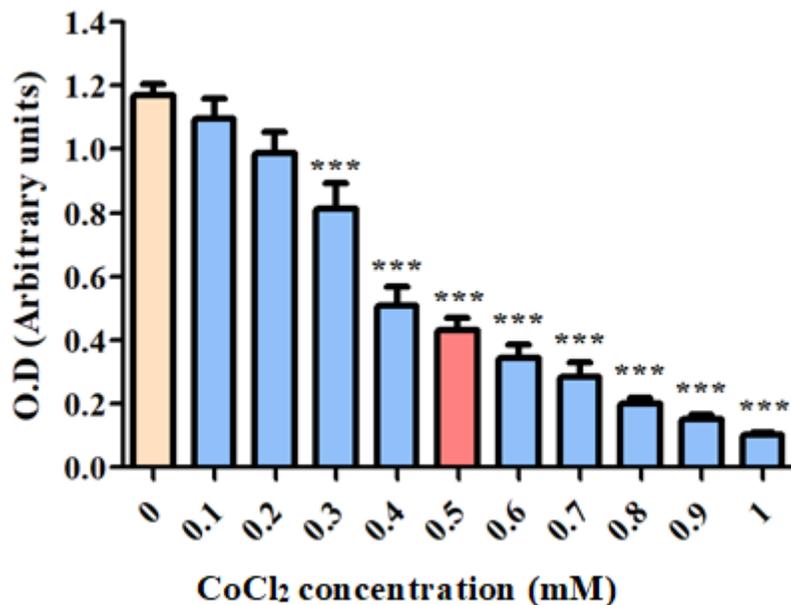


Figure 1

Dose-response analysis of H1299 cell viability (using XTT assay kit) after 24 hours' exposure to CoCl<sub>2</sub> (0.1 - 1 mM). Results are expressed as mean ± SEM (8 replicates for each group). \*\*\*p<0.001 compared to control.

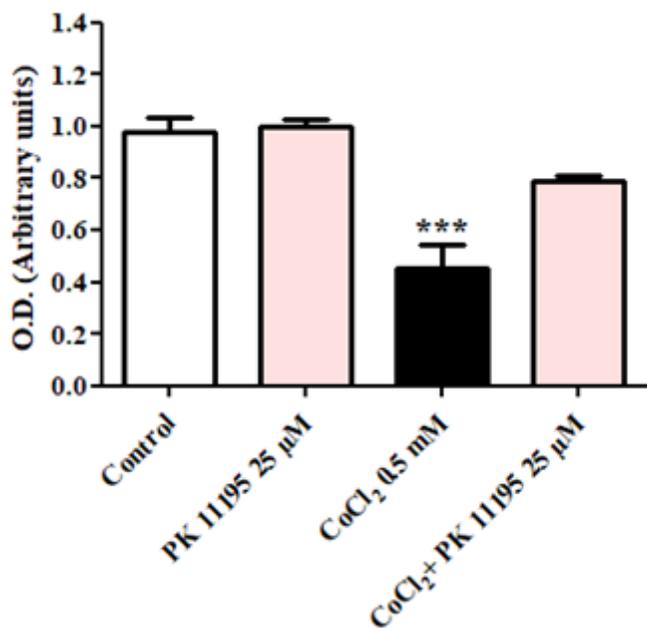
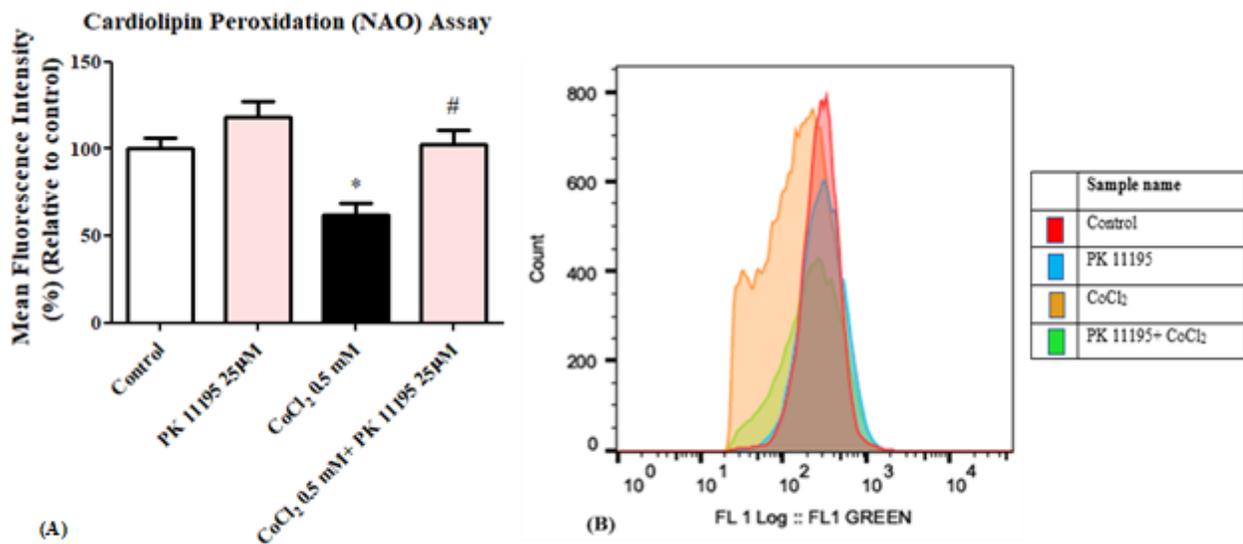


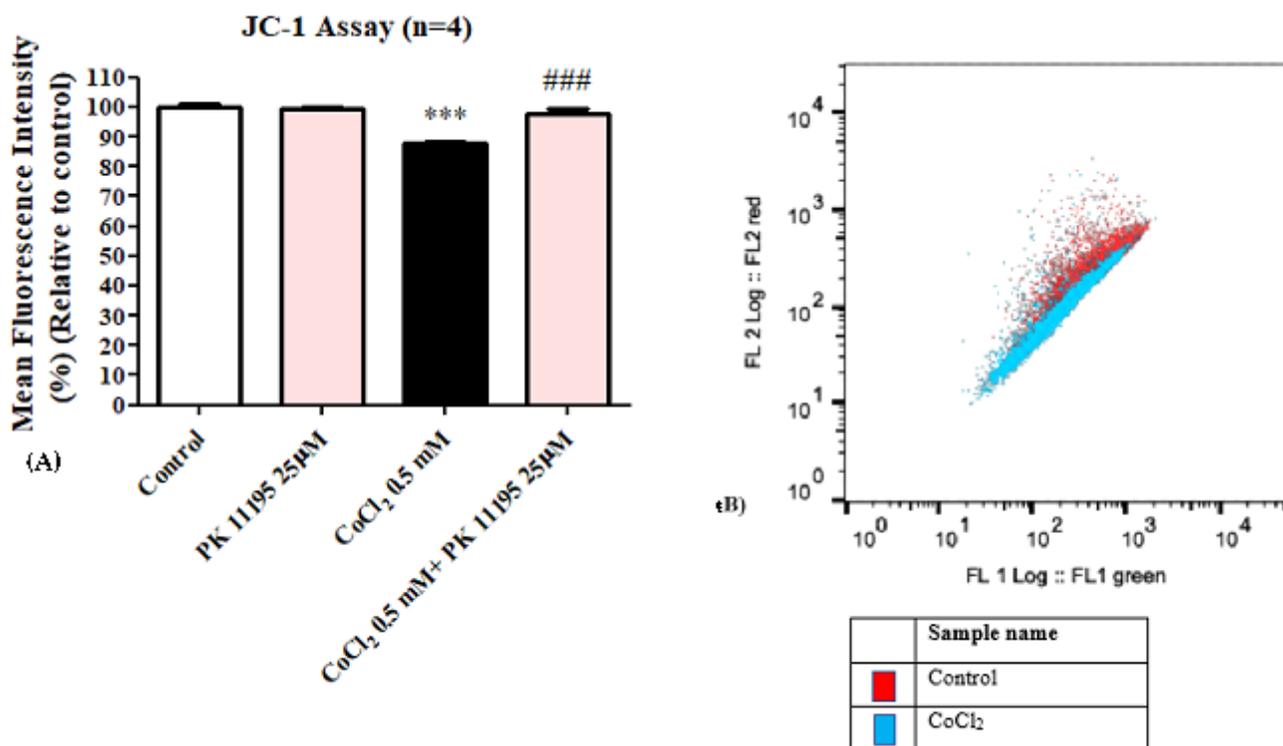
Figure 2

Evaluation of the protective ability of the TSPO ligand PK 11195 to counteract the effect of CoCl<sub>2</sub>-induced cell death. The protective ability of PK 11195 (n=5 replicates) to counteract the effect of 0.5 mM of CoCl<sub>2</sub> was measured using XTT analysis. Results are expressed as means ± SEM \*\*\*p<0.001 vs. control and vs. CoCl<sub>2</sub> 0.5 mM+ PK11195 25 µM.



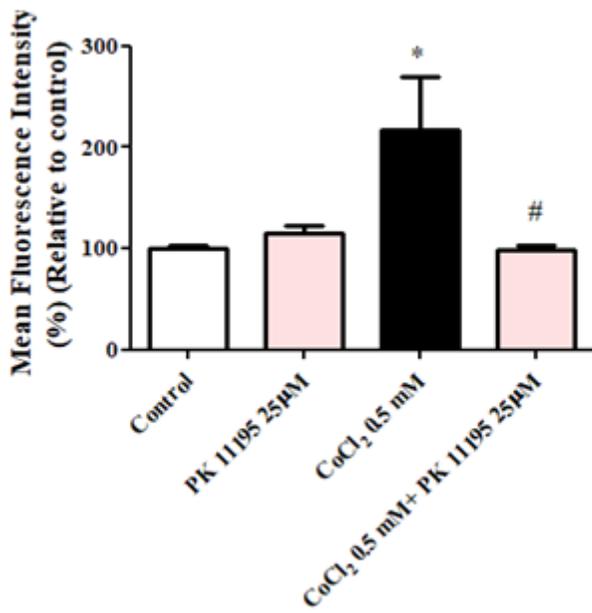
**Figure 3**

Determination of the efficacy of 25 µM PK 11195 to counteract the effect of 0.5 mM CoCl<sub>2</sub>-induced cardiolipin peroxidation in H1299 cells. The protective efficacy of PK 11195 is shown as (A) a bar graph: results are expressed by mean ± SEM (n=5 replicates in each group), and (B) a histogram. \* = p<0.05 compared to control and # = p<0.05 compared to CoCl<sub>2</sub>.



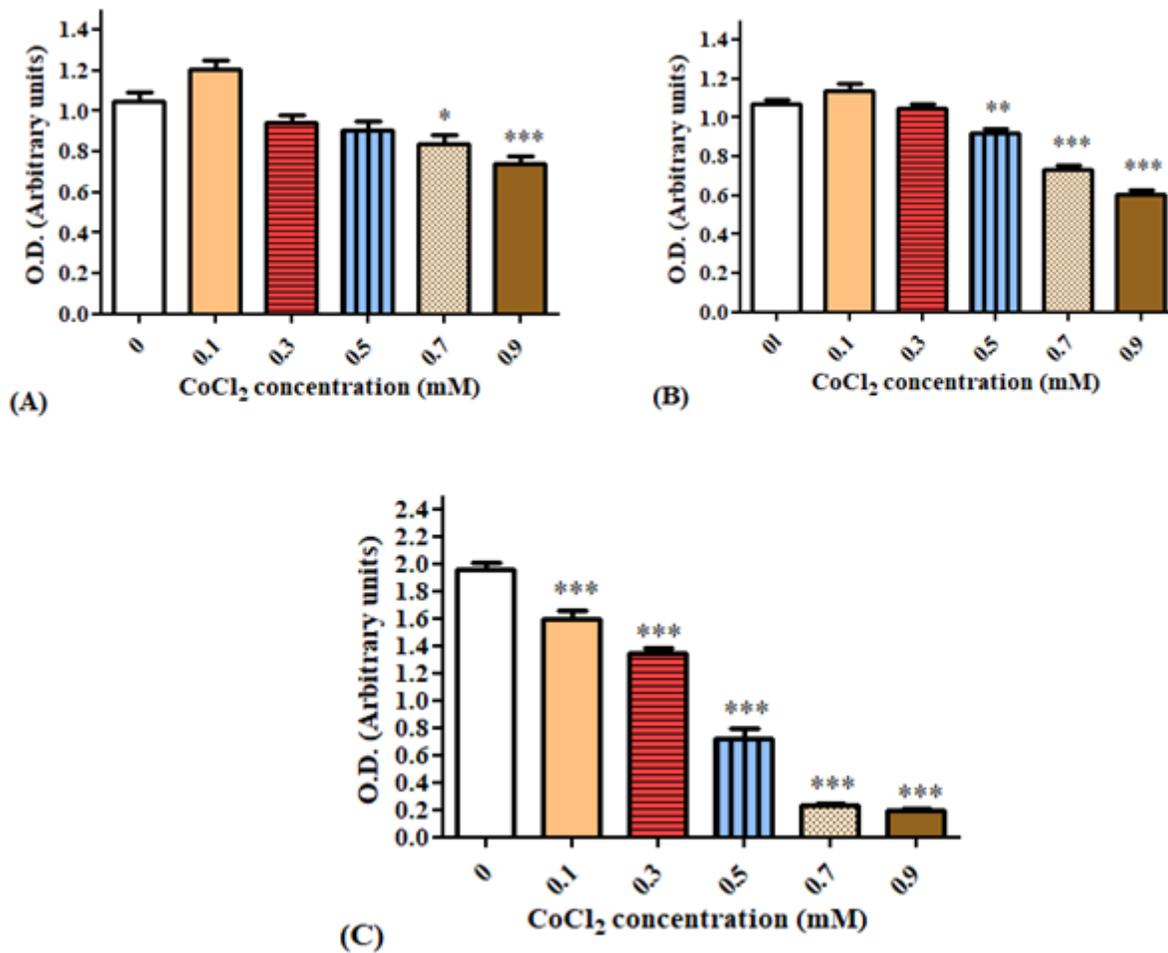
**Figure 4**

assessment of mitochondrial membrane potential (JC-1) following 24 hours exposure of H1299 to 0.5 mM CoCl<sub>2</sub>. Inhibition of CoCl<sub>2</sub>-induced mitochondrial membrane potential depolarization by 25 μM PK 11195 expressed as (A) a bar graph, and (B) a histogram. Results are expressed by means ± SEM (n=4 replicates in each group). ### and \*\*\*p<0.001.



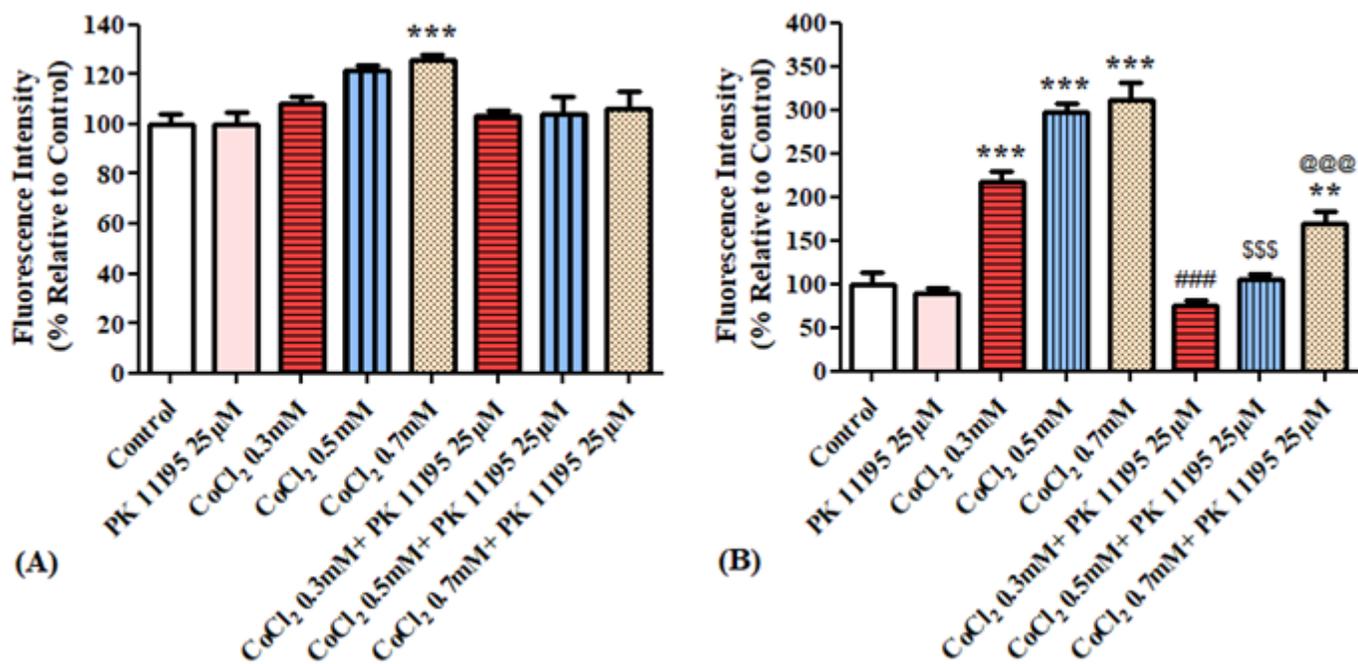
**Figure 5**

Evaluation of apoptotic cell death levels assessed by apoxin dye following 24 hours' exposure of H1299 to 0.5 mM CoCl<sub>2</sub> with or without pretreatment with 25 μM PK 11195. Results are expressed as mean ± SEM (n=4 replicates in each group). # and \*p<0.05.



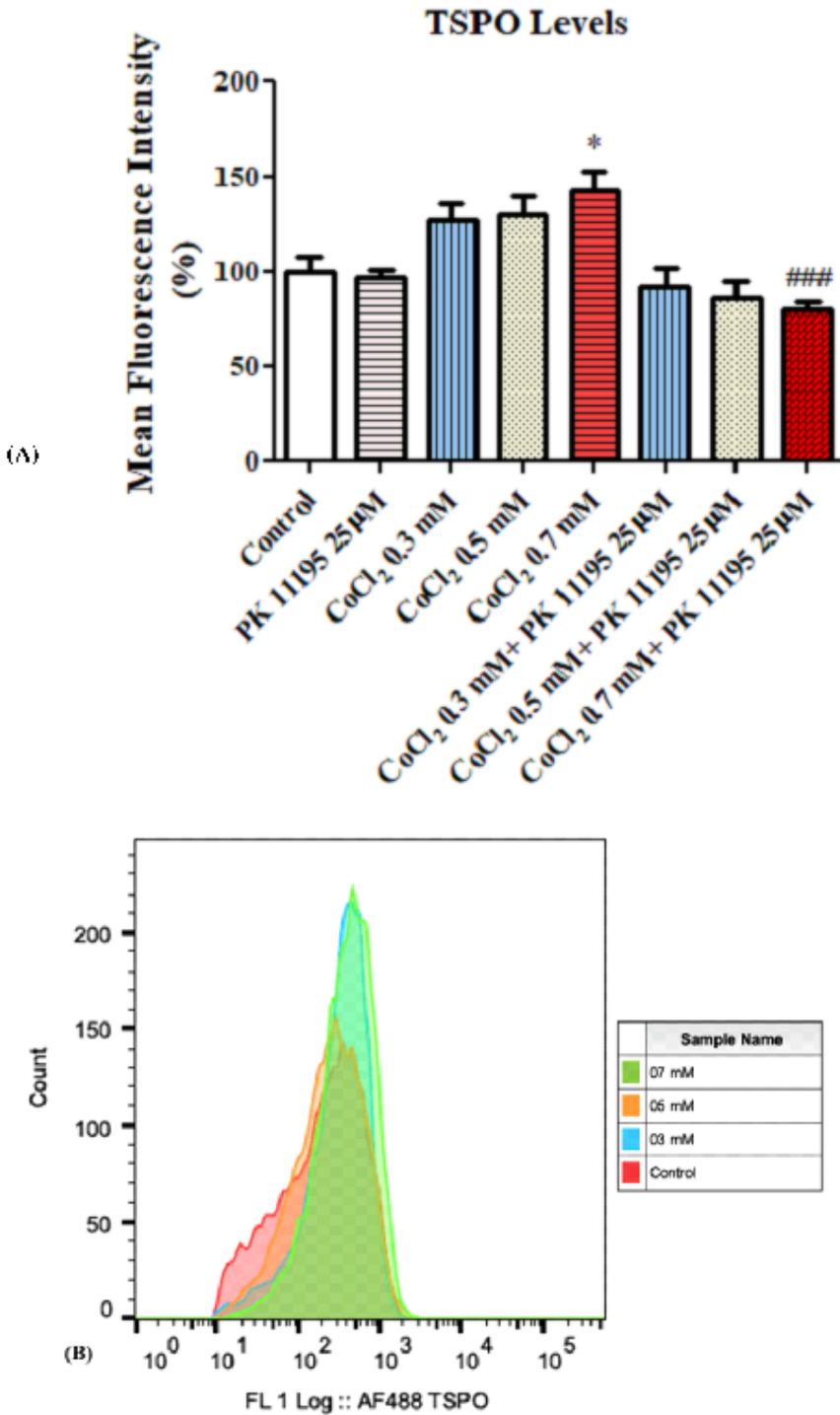
**Figure 6**

Cellular viability, as measured by XTT, of BV-2 cells exposed to increasing concentrations of CoCl<sub>2</sub>. XTT assay was performed after (A) 30 minutes, (B) 4 hours, and (C) 24 hours of CoCl<sub>2</sub> exposure at concentrations of 0.1, 0.3, 0.5, 0.7 and 0.9 mM. Results are expressed as mean  $\pm$  SEM (n=8 replicates for each group). \*p<0.05, \*\*p<0.01, and \*\*\*p<0.001 vs. control.



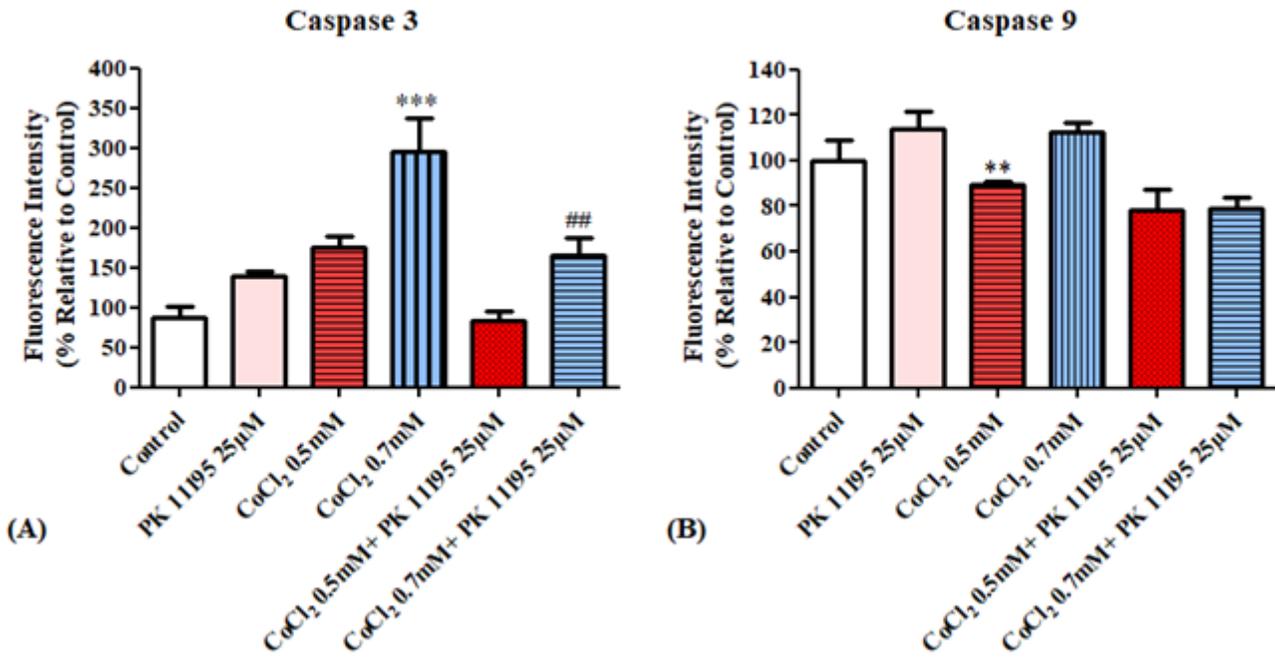
**Figure 7**

Oxidative stress levels as assessed by ELISA (ROS/superoxide detection as-say) following exposure of BV-2 microglial cells to various concentration of CoCl<sub>2</sub> for 4 and 24 hours with and without pretreatment. Application of PK 11195 (25  $\mu$ M) as a pretreatment, differentially prevented the elevation of oxidative stress levels caused by the exposure to (A) 4 hours (n=4 replicates in each group) and (B) 24 hours (n=5 replicates in each group), CoCl<sub>2</sub>. Results are expressed as mean  $\pm$  SEM. \*\*\*p<0.001 vs. control; @@@, \$\$\$, ### vs. corresponding CoCl<sub>2</sub> concentrations without PK11195.



**Figure 8**

TSPO protein expression levels as assessed by FACS following 4 hours' exposure of BV-2 cells to CoCl<sub>2</sub> at various concentrations with and without PK 11195 pretreatment (25  $\mu$ M). Alterations in TSPO level following exposure of CoCl<sub>2</sub> for 4 hours (A) and representative FACS determination of TSPO levels (B). Results are expressed as mean  $\pm$  SEM (n=5 replicates for each group). \*p<0.05 vs. control and ###p<0.001 vs. CoCl<sub>2</sub> 0.7 mM.



**Figure 9**

Alterations of caspase 3 and 9 levels following 4 hours' exposure of BV-2 microglial cells to various concentrations of CoCl<sub>2</sub>. (A) caspase 3 levels, (B) caspase 9 levels. Results are expressed as mean ± SEM (n=5 replicates for each group). \*\*\*p<0.001 vs. control, ## vs. CoCl<sub>2</sub> 0.7 mM.