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**Highlights**

- The mitochondrial-targeted hydrogen sulphide donor, AP39 did not exhibit cytotoxic effects in SHSY5Y cells.
- AP39 generates $H_2S$ intracellularly and in co-localisation with the mitochondria.
- AP39 promotes the cellular bioenergetics in SHSY5Y.
- AP39 reduces mitochondrial reactive oxygen species.

# Neuroprotective effects of mitochondrial-targeted hydrogen sulphide donor, AP39 on $H_2O_2$-induced oxidative stress in human neuroblastoma SHSY5Y cell line

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**Abstract:**

Oxidative stress (OS) resulting from imbalance in the generation of reactive oxygen species (ROS) and/or the dysfunction of the antioxidant machinery, is a key mechanism associated with the onset of neurodegenerative disorders. Although the molecular mechanisms are still elusive, the onset of disorders such as Alzheimer's and Parkinson's disease have been associated with mitochondrial dysfunction. Recently, a mitochondrial-targeted hydrogen sulphide ($H_2S$) donor, AP39, has shown to promote cellular bioenergetics in OS related scenarios. The aim of this study was to explore the potential of AP39 to protect the mitochondrial function in an OS environment induced by hydrogen peroxide ($H_2O_2$). We assessed the potential effects of increasing concentrations of AP39 on cell viability, $H_2S$ availability and the mitochondrial bioenergetic response in resting (non-differentiated and differentiated) neuroblastoma SHSY5Y cell line. Further, we explored the role of AP39 in attenuating $H_2O_2$-induced mitochondrial dysfunction. Our results showed that nanomolar to micromolar concentrations of AP39 (0.1 μM – 3 μM) are not toxic to SHSY5Y cells, regardless of their differentiation status. Fluorescence detection of $H_2S$ observed AP39 co-localises within the mitochondria in a concentration dependent manner. Whilst a lower concentration of AP39 (0.3 μM) was required to improve the mitochondrial bioenergetics in resting non-differentiated cells, 1 μM produced this effect in their differentiated counterparts. In both, non-differentiated and differentiated cells, AP39 reduced $H_2O_2$-induced mitochondrial impairments by improving the parameters of the mitochondrial function and abrogating the generation of mitochondrial ROS. These suggest that mitochondrial targeted delivery of $H_2S$ may attenuate neuronal toxicity in neuronal disorders associated with OS-induced mitochondrial dysfunction.

**Keywords:** Oxidative stress, neurodegenerative disorders, mitochondrial dysfunction, hydrogen sulphide donors.

**Abbreviations:**

ADTOH: 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione.

6-OHDA: 6-hydroxydopamine.

ATP: Adenosine triphosphate.

AD: Alzheimer's disease.

AA: Antimycin.

FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone.

$H_2O_2$: Hydrogen Peroxide.

$H_2S$: Hydrogen sulphide.

MAP-2: Microtubule-associated protein 2.

mt-ROS: Mitochondrial reactive oxygen species.

OXPHOS: Oxidative phosphorylation.

OS: Oxidative stress.

OCR: Oxygen consumption rates.

PD: Parkinson's disease.

ROS: Reactive oxygen species.

RA: Retinoic acid.

Rot: Rotenone.

SEM: Standard error of mean.

SF7-AM: Sulphideflour-7 acetoxymethyl ester.

$TPP^+$: Triphenlyphosphonium.

**Introduction:**

Neurodegenerative disorders occur due to the progressive and irreversible degeneration of nerve cells [1]. These conditions are characterised by the loss of vulnerable neuron populations, leading to impaired movement (ataxias) and changes in cognitive functions. Some commonly known neurodegenerative disorders include Alzheimer's disease (AD) and Parkinson's disease (PD) [1]. AD is the most common cause of dementia, mainly affecting people over the age of 65 [2] while PD is a neurodegenerative movement disorder associated with selective loss of dopaminergic neurons in the substantia nigra of the brain [3]. The pathophysiology of both conditions has been linked to enhanced production of reactive oxygen species (ROS) [4,3] and impaired mitochondrial bioenergetics [5,6]. Mitochondria are the primary site of energy production within cells [7]. Mitochondrial oxidative phosphorylation (OXPHOS) provides energy for neural activity by way of adenosine triphosphate (ATP) production [8]. This process continuously produces ROS as a physiological by-product. However, dysregulation of cellular antioxidant activity and/or imbalance in ROS production may cause oxidative stress (OS) [9].

Hydrogen sulphide ($H_2S$) is a gaseous signalling molecule that can stimulate physiological responses in various tissues, including the central nervous system [10]. Within optimal concentrations, $H_2S$ promotes mitochondrial biogenesis and bioenergetics, thereby conferring protection against cellular stress [11]. $H_2S$ donors show potential therapeutic value against neurodegenerative disorders due to their ability to produce anti-oxidant, -inflammatory, and -apoptotic effects in pathological situations. In this regard, $H_2S$ donors have been shown to reduce cytotoxicity, intracellular protein oxidation, and lipid peroxidation in human neuroblastoma cell line SHSY5Y exposed to hypochlorous acid and 4-hydroxynonenal, which are increased in the temporal and frontal cortex of patients with AD [12,13]. Moreover, a rat model of PD, have shown a significant reduction of the endogenous $H_2S$ production in the substantia nigra of 6-hydroxydopamine (6-OHDA) exposed rats [14] whilst the administration of $H_2S$ donors have shown to protect against rotenone-induced apoptosis [15] and 6-OHDA-induced cytotoxicity [16] by preserving mitochondrial function [10].

Recently, a mitochondrial-targeted $H_2S$ donor, named AP39 was synthesised [17,18]. AP39 has shown potential for attenuating mitochondrial-associated OS by exerting cytoprotective effects and reducing mitochondrial DNA oxidative damage [17]. Administration of AP39 after cardiopulmonary resuscitation showed an improvement in neurological function and survival rate in mice by maintaining mitochondrial integrity and reducing ROS [19]. AP39 has also potential for modulating neuroinflammation as demonstrated in reducing cytokine release in brains areas affected by ischaemia in rats [20].

The aim of this study was to explore the potential of AP39 to protect neuronal mitochondrial function in an OS environment induced by hydrogen peroxide ($H_2O_2$). We assessed the effects of increasing concentrations of AP39 on cell viability, $H_2S$ availability and mitochondrial bioenergetics response in resting (non-differentiated and differentiated) neuroblastoma SHSY5Y cell line. Here, we report that AP39 targets delivery of $H_2S$ to the mitochondria and attenuate $H_2O_2$-induced mitochondrial dysfunction by improving the parameters of the mitochondrial function and abrogating the generation of mitochondrial ROS (mt-ROS).

**Materials and methods:**

**Reagents:**

AP39 (Cambridge Biosciences, UK) is a $H_2S$ donor consisting of the mitochondria targeting triphenylphosphonium ($TPP^+$) group ester-linked with the $H_2S$ donor ADTOH (5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione) by a ten-carbon alkyl chain (**Figure 1**). All-trans retinoic acid (RA) and $H_2O_2$ were obtained from Sigma Aldrich (Sigma Aldrich, USA).

**Cell culture and differentiation**

SHSY5Y neuroblastoma cells were maintained in RPMI 1640 (Gibco, UK) containing 5% FBS and 2 mM glutamine at 37°C in a humidified atmosphere of 5% $CO_2$. Cells were maintained up to passage 23. Differentiation to a more mature neuronal-like phenotype (d-SHSY5Y) was ascertained by culturing in low FBS (1%) RPMI 1640 supplemented with 10 μM of RA for 6 days. Briefly, cells were plated and left to attach overnight (day 0), at day 1, cell culture media was replaced to differentiation media (1% FBS and 10 μM RA). Medium was replaced with fresh differentiation media every 48h. Differentiation was measured by the extension of long neurites and detected by immunocytochemistry using antibodies against microtubule-associated protein 2 (MAP-2) (Sigma Aldrich, USA, catalogue # MA512826, 1:100). Cells were grown on glass coverslips (200K cells per coverslip). After differentiation, cells were washed in warm PBS and fixed in cooled ethanol for 5 minutes. Coverslips were permeabilised in 0.15% triton X-100, blocked in 10% goat serum blocking buffer for 1 h and incubated with first antibodies (overnight), followed by Alexa fluor (488) conjugated secondary antibodies (Abcam, UK, catalogue # ab175658, 1:500). Coverslips were mounted in glass slides using SlowFade™ Diamond Antifade Mountant (Thermo Fisher Scientific, UK). Staining was visualised using a Nikon Eclipse Ti-E inverted microscope using 60× objective lens. Images were recorded and quantification of immunofluorescence intensity performed in ≥50 cells at day 6, for each differentiation treatment.

**Cell treatments with AP39 and $H_2O_2$**

SHSY5Y and d-SHSY5Y were exposed to increasing concentrations of AP39 (0.1-10 μM) for 24 h in order to assess the effects of AP39 on cell viability. For other experimental protocols either resting cells or $H_2O_2$-stimulated neuroblastoma cells were exposed to (0.1-1 μM) AP39 for 1h as indicated in every figure legend. $H_2O_2$ (150-300 μM) was administered for 1 h alone or in combination with AP39 as indicated in the figure legends.

**Cell viability**

The cytotoxicity profile of AP39 towards SHSY5Y and d-SHSY5Y cells was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT is metabolised by viable cells to a purple coloured formazan. Briefly, SHSY5Y and d-SHSY5Y were plated at a cell density of $2.0 \times 10^4$ cells/well in 96-well plates and left to attach overnight. After cell attachment, cells were exposed to AP39 (0.1-10 μM) for 24 h. Next, cell media was removed and MTT reagent (0.5mg/mL) dissolved in RPMI 1640 was added to each well and cells incubated at 37°C in a humidified atmosphere of 5% $CO_2$ for 1.5 h. MTT reagent was then aspirated and formazan solubilised in DMSO. Absorbance was recorded using a Tecan plate reader at 570nm.

**Determination of intracellular $H_2S$ by fluorescence**

$H_2S$ availability was evaluated in cells using the specific fluorescent probe Sulphidefluor-7 acetoxymethyl ester (SF7-AM) (Sigma Aldrich, USA) as previously described [21]. Briefly, cells (SHSY5Y and d-SHSY5Y) were plated in 24 well plates at a cell density of $8.0 \times 10^4$ cells/well and left to attach overnight. Next, cells were exposed to AP39 (0.1-10 μM) for 1 h and then incubated with 2.5 μM SF7-AM in culture media for 30 min. Cells were washed twice with PBS and the fluorescence was measured at 495 nm/519 nm. Images were recorded at 20× using a Nikon Eclipse Ti-E inverted microscope. Analysis were performed using Image J.

**Detection of mitochondrial reactive oxygen species (mt-ROS)**

The generation of mt-ROS was ascertained using the fluorescent probe MitoSOX Red (Sigma Aldrich, USA) by fluorescent microscopy as previously reported [22]. Briefly, cells (SHSY5Y and d-SHSY5Y) were plated on coverslips ($2.0 \times 10^5$ cells/coverslip) and exposed to $H_2O_2$ and AP39 as described on the figure legends. Next, coverslips were washed in warm PBS and incubated with MitoSOX Red

(5μM) in PBS for 30 min, protected from light. Coverslips were carefully washed with PBS twice and fluorescence emitted at 580 nm was recorded and analysed using a Nikon Eclipse Ti-E inverted microscope using a 60× objective.

**Cellular bioenergetics**

Parameters of the mitochondrial function were assessed using an XF24 Extracellular Flux Analyser (Seahorse Biosciences/Agilent Technologies, UK) following protocols established in our lab [22,21,23]. Briefly, SHSY5Y and d-SHSY5Y were plated at 5.0 x $10^4$ cells/well using V7 24 well plates (Agilent Technologies, UK) and cells left to attach overnight at 37°C in a humidified atmosphere of 5% $CO_2$. Following this, cells were washed and media replaced with non-buffered DMEM (10 mM glucose, 1 mM pyruvate and 2 mM L-glutamine) to allow temperature and pH equilibrium. The addition of AP39 and/or $H_2O_2$ was performed using the available ports of the XF24 Flux Analyser, prior to the injections of drugs/inhibitors used to calculate parameters mitochondrial function. Oxygen consumption rates (OCR) were measured before and after AP39/ $H_2O_2$ injection (first injection), followed by sequential injections of oligomycin (1 μM) (Sigma Aldrich, USA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5 μM) (Sigma Aldrich, USA) and a mixture of rotenone and antimycin A (Rot/AA) (1 μM) (Sigma Aldrich, USA), to inhibit the ATP synthase, uncouple oxidative phosphorylation, and estimate non-mitochondrial respiration, respectively (75 μL per injection). As previously described [24], injections of these reagents allow parameters of the mitochondrial function, including: basal, maximal respiration, spare respiratory capacity, ATP-linked OCR and proton leak to be determined. Data was expressed as the rate of oxygen consumption ($pmolO_2$/min/μg protein) by time. The concentration of proteins per well was assessed using BCA protein assay (Bio-Rad) after the completion of Seahorse assays.

**ATP levels determination**

The ATP Determination Kit (Invitrogen, USA) was used to measure levels of ATP in SHSY5Y and d-SHSY5Y exposed to AP39. Briefly, cells 5.0 x$10^4$ cells/well were plated in 96-well plates, after overnight attachment, cells were exposed to AP39 for 1 h. After treatment, cells were processed as per manufacturer protocol and luminescence intensity was read using a Tecan microplate reader. Readings were expressed as percentage of control.

**Mitochondrial isolation and detection of mitochondrial oxidated proteins**

Isolation of mitochondria was performed using protocols available in our lab [23]. Briefly, SHSY5Y and d-SHSY5Y were grown in T125 flasks and exposure to $H_2O_2$ and AP39 was as described in the figure legend. Following this, mitochondria were isolated by cell disruption followed by differential centrifugation. Cells were scrapped in cold PBS and resuspended in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM, EGTA, pH 7.4). Cells were disrupted and homogenised using needle syringe. Homogenates were then centrifuged for 10 min at 11,000 x g, 4°C. The resulting supernatant was centrifuged for 10 min at 11,000 x g, 4°C and the pellet (containing the mitochondria) resuspended in RIPA buffer containing protease inhibitors. Mitochondria was disrupted by needle syringe and centrifuged for 10 min at 10,000 x g, 4°C. Finally, the supernatant was collected, and protein concentration was measured by BCA protein assay (Bio-Rad). A concentration of 10 μg of protein was used to evaluate the protein carbonyl formation, using the Protein Carbonyl Content Assay Kit (Abcam, catalogue # ab126287) as previously established in our lab and VDAC1 (porin), catalogue # ab34726 was used as the loading control.

**Statistical analysis**

Results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism software (version 8.1.0). A T-test was used to analyse the effects of differentiation conditions on MAP-2 expression between two groups (SHSY5Y and d-SHSY5Y) while

one-way analysis of variance (ANOVA) was employed to test the differences between more than two treatments. Post-hoc comparisons were made using the Tukey's multiple comparison's tests. All results are expressed as the mean of three independent experiments performed in triplicates. A $p < 0.05$ was considered statistically significant.

**Results:**

**SHSY5Y differentiation**

The differentiation of neuronal-like cells allows to obtain more mature neuron-like cell populations for *in vitro* testing [25]. To this end, using available protocols established in our lab, SHSY5Y were differentiated in low serum media containing RA (10 μM) for 6 days to obtain a more typical neuronal morphology [23]. As shown in **Figure 2A**, at days 0 cells display a characteristic undifferentiated phenotype with no particular neurite outgrow. At days 3-6, exposure to RA allowed the formation of dendrite-like projections. Successful differentiation was assessed by immunocytochemistry using antibodies against the neuronal marker, MAP-2 [26]. Differentiated SHSY5Y (d-SHSY5Y) showed an increased expression of MAP-2. By Day 6, the fluorescence intensity of MAP-2 increased approximately by 60% in comparison to SHSY5Y (**Figure 2B**). The differentiation was key for our next set of experiments in were we aimed to explore the effects of AP39 and to a non-toxic concentration of AP39 that would stimulate the mitochondrial bioenergetics while contrasting potential differences between non-differentiated and those displaying a more mature neuronal-like phenotype.

**High concentrations of AP39 reduce SHSY5Y viability**

The cytotoxicity profile of AP39 towards SHSY5Y and d-SHSY5Y cells was assessed with a MTT assay to establish suitable concentrations for following experiments. AP39 (0.1-3 μM) showed no statistical difference in the reduction of MTT reagent, representative of the viability of the cells. However, at 10 μM, AP39 demonstrated a reduction of both SHSY5Y and d-SHSY5Y cells' viability (**Figure 3A** and **3B**, respectively). Based on these observations and previous reports [21,19,27,28], we set the following experiments to a range of concentrations of AP39 between 0.1-1 μM.

**AP39 accumulates within the mitochondria and releases $H_2S$**

Once we established a range of AP39 concentrations not detrimental to cell viability, we aimed to explore whether these were sufficient to increase the intracellular availability of $H_2S$. Using the fluorescent probes; SF7-AM and Mitotracker Red, we observed that in both, SHSY5Y and d-SHSY5Y, non-treated cells (controls) displayed SF7-AM fluorescence with no mitochondrial preference. However, cells treated with AP39 (0.1-1 μM) induced a concentration-dependent increase in $H_2S$ generation and these increased signals of SF7AM-$H_2S$ detecting probe co-localised within the mitochondria (**Figure 4A** and **4B**, SHSY5Y and d-SHSY5Y, respectively). This suggests AP39 degrades within the mitochondrial compartment of cells to release $H_2S$.

**Effects of AP39 on cellular bioenergetics in resting cells**

The effects of AP39 on cellular bioenergetics was assessed in resting SHSY5Y and d-SHSY5Y (**Figure 5**) cells. We aimed to explore the effects of AP39 on human neuroblastoma cells' bioenergetics and to evidence whether the differentiation of SHSY5Y may have an impact on their response to AP39. In SHSY5Y we evidenced that AP39 (0.1-0.3 μM) induced a dose-dependent response in parameters of the mitochondrial function as evidenced by increased OCR, that resulted in increased basal respiration ($p<0.05$) vs control (**Figure 5A** and **5B**). However, at 1 μM, a non-significant increase in OCR, similar to those induced by 0.1 μM was observed, suggesting that AP39 may have a biphasic effect on non-differentiated human neuroblastoma cells. The OCR-linked to ATP production, although not statistically different to control, suggested that AP39 may promote OXPHOS and therefore the mitochondrial generation of ATP (**Figure 5B**). Therefore, we assessed ATP levels and observed AP39 (0.3-1 μM) significantly increased ATP generation ($p<0.05$ and $p<0.01$ for 0.3 μM

and 1 μM AP39 exposed cells, respectively) (**Figure 5C**). Based on these observations, we recognised 0.3 μM as the optimal concentration of AP39 for SHSY5Y that would generate mitochondrial-$H_2S$ while improving parameters of the mitochondrial function and selected this as the concentration to explore in further experiments.

The differentiation of SHSY5Y cells is accompanied by changes in mitochondrial metabolism and antioxidant defences in response to oxidative stress [26]. We aimed to explore whether these would impact the response to AP39 in resting conditions. Our experiments using the Seahorse-Agilent XF24 showed that AP39 (1 μM) significantly improved the basal and maximal respiration in comparison to non-treated cells (**Figure 5D** and **5E**). AP39 0.1-0.3 μM showed a dose-dependent response and a tendency to improve other parameters of the mitochondrial function such as the maximal respiration and ATP-linked OCR. Similar to our observations in SHSY5Y, we evidenced that AP39 improves the generation of ATP (**Figure 5F**). In contrast to our observations in SHSY5Y, we evidenced that for d-SHSY5Y, the optimum concentration of AP39 to stimulate the cellular bioenergetics was 1 μM. Therefore, further experiments using d-SHSY5Y were performed using an AP39 concentration of 1 μM.

**AP39 improves cellular bioenergetics in cells challenged with $H_2O_2$**

Next, we aimed to explore whether AP39 would protect against $H_2O_2$ -induced mitochondrial impairments and to contrast these effects between SHSY5Y and d-SHSY5Y. To verify our OS model, we exposed SHSY5Y to $H_2O_2$ for 1h (150-300 μM) and observed $H_2O_2$ (300 μM) led to a reduction in OCR levels, with significantly reduced basal and maximal respiration linked to a reduced generation of ATP (**Supplementary figure 1**). Then we exposed SHSY5Y and d-SHSY5Y to $H_2O_2$ and AP39 an evaluated the effects on the mitochondrial bioenergetics. To this end, we injected $H_2O_2$, AP39 or $H_2O_2$/AP39 directly to the wells using the first injection port available in the XF24 instrument. This approach allowed to evidence in real-time, the effects on OCR. $H_2O_2$ significantly reduced baseline OCR levels ($p<0.001$ vs control and AP39) resulting in reduced basal respiration ($p<0.05$ vs AP39 only) (**Figure 6A** and **6B**) and reduced maximal respiration ($p<0.05$ vs control and AP39 only) (**Figure 6B**). However, whilst co-exposure of $H_2O_2$ and AP39 resulted in reduced basal respiration, it was enough to improve the maximal respiration (**Figure 6B**).

**Figure 6C** and **6D** shows the effects of co-exposure of $H_2O_2$ and AP39 to d-SHSY5Y. In contrast to our observations in SHSY5Y, we evidenced that $H_2O_2$ does not significantly reduce the baseline OCR and basal respiration in d-SHSY5Y. We only observed a significant impairment of the maximal respiration in $H_2O_2$-exposed d-SHSY5Y ($p<0.05$ vs control). The co-administration of $H_2O_2$ and AP39 showed a tendency to improve the basal respiration and maximal respiration to levels similar to non-treated cells (**Figure 6C** and **6D**).

**AP39 reduces mitochondrial oxidative stress in cells exposed to $H_2O_2$**

In order to study the overall mitochondrial oxidative status, we investigated the effects of AP39 in the generation of mitochondrial specific reactive oxygen species (mt-ROS) using the fluorescent probe MitoSOX Red and analysed mitochondrial protein carbonyls in isolated mitochondria by western blot. We analysed the effects of $H_2O_2$ on mt-ROS generation in both SHSY5Y and d-SHSY5Y. As shown in **Figure 7A** and **7B**, $H_2O_2$ (300 μM) increased the MitoSOX Red fluorescence signal. Interestingly, $H_2O_2$-challenged SHSY5Y showed increased intensity of the signal when contrasted to d-SHSY5Y cells also exposed to $H_2O_2$. These observations correspond to our observation in **Figure 6** where we found d-SHSY5Y are less affected by $H_2O_2$ exposure. In both, SHSY5Y and d-SHSY5Y, co-administration of $H_2O_2$ with AP39 (0.3 μM for SHSY5Y and 1 μM for d-SHSY5Y, respectively) showed a reduction in the MitoSOX fluorescence intensity, suggesting that under OS, AP39 is able to reduce the generation of mt-ROS. In line with these findings, SHSY5Y showed higher mitochondrial protein carbonyl content when compared to d-SHSY5Y (**Figures 7A** and **7B**, respectively). In SHSY5Y, AP39 was observed to reduce the mitochondrial protein carbonyl content in comparison to $H_2O_2$-treated

SHSY5Y (**Figure 7A**). However, in d-SHSY5Y we evidenced that carbonyl content in AP39 co-treated with $H_2O_2$ was more evident in some molecular weight bands.

**Discussion:**

OS is a key component in the pathophysiology of several neurological conditions [29,30], including neurodegenerative disorders such as Parkinson's disease [3], and Alzheimer's disease [31,4]. In this study, we explored the potential of AP39, a novel mitochondrial-targeted $H_2S$ donor, to protect against OS *in vitro*, contrasting the effects on non-differentiated and differentiated human neuroblastoma cell line SHSY5Y exposed to $H_2O_2$. Our results suggest a broad range of AP39 concentrations are not cytotoxic to SHSY5Y while nanomolar concentrations of AP39 are sufficient to release therapeutic levels of $H_2S$ in an OS cellular environment, favouring accumulation within the mitochondria. In addition, our results demonstrated AP39 was able to promote mitochondrial bioenergetics in both SHSY5Y and d-SHSY5Y, however, higher concentrations of AP39 are necessary to promote the mitochondrial bioenergetics in d-SHSY5-Y in comparison to their non-differentiated counterparts. Linked to these observations, we observed AP39 stimulated the generation of ATP in resting cells. Finally, AP39 exerted protective effects in both SHSY5Y and d-SHSY5Y cells exposed to $H_2O_2$ by supporting the mitochondrial bioenergetics and reducing mitochondrial oxidative status.

There is an increased interest in the biology of $H_2S$ as a large number of scientific reports have unravelled many of its downstream physiological effects [30]. $H_2S$ not only supports neuronal functions but also has been implicated in the improvement of impaired learning and memory [30]. Treatments based on inorganic sulphide salts (including NaHS, Na2S, and CaS) have been probed as potential treatments to address neurological impairments [32-34]. However, these salts provide immediate $H_2S$ release which may be unsuitable in the treatment of chronic disease states. Furthermore, inorganic sulphide salt administration result in a non-specific delivery of $H_2S$. Exogenous $H_2S$ is able to impart neuroprotective effects acting as an anti-oxidant, -inflammatory and -apoptotic agent [33]. Since these $H_2S$ donors deliver $H_2S$ not specifically to cellular compartments, a novel mitochondrial targeting slow release $H_2S$ donor, AP39, consisting of the mitochondria targeting $TPP^+$ group ester-linked with the $H_2S$ donor ADTOH was recently developed [18,17]. The $TPP^+$ moiety is driven by the plasma membrane potential resulting in a fast uptake across the plasma membrane [35], resulting in a 500-fold accumulation of linked drugs in mitochondria [36]. In addition, the ADTOH moiety is a popular $H_2S$ donor observed to exert its pharmacological effects through a concomitant increase of both GSH and $H_2S$ [37-40] (**Figure 1**).

To compare the effects of AP39 on human neuroblastoma cells, we first differentiated SHSY5Y using RA protocols. This approach has previously been shown to induce important changes to the cell's phenotype and expression of antioxidant machinery while modulating the response of d-SHSY5Y to conditions generating OS [26]. Initially, our approach aimed to verify the appropriate concentration of AP39 that would be non-toxic to cells while improve the mitochondrial bioenergetics. We observed that the condition of differentiation did not alter the cell viability of human neuroblastoma cells to increasing concentrations of AP39 as assessed by MTT assay. Reports by Gero et al 2016, showed that sub-micromolar concentrations of AP39 are safe to use on endothelial cells [18], while our report suggest that human neuroblastoma cells are slightly more resistant to AP39 toxicity as we evidenced tolerability in a micromolar range. Interestingly, $H_2S$ generation from AP39 occurs through multiple steps each of which are affected by various metabolites in the mitochondria [18]. Further, it has been suggested that the $H_2S$ concentration within mitochondria remains below the toxic limit since normalisation of the mitochondrial potential will reduce drug accumulation [36], suggesting that an even at 10 μM, AP39 may not necessarily cause toxicity due to insufficient drug accumulation in mitochondria.

The role of mitochondrial dysfunction in the onset of neurological disorders has been linked to the increased oxygen demand, susceptibility to peroxidation of lipids and imbalance in antioxidant mechanisms [41]. In this regard, the genesis of PD has been implicated at the genetic and

environmental levels [42,43]. As described, AP39 has shown potential for protecting the mitochondrial function and mt-ROS generation by the targeted delivery of $H_2S$. Our observations evidenced that for both, SHSY5Y and d-SHSY5Y, sub-micromolar levels of AP39 caused accumulation of $H_2S$ in the mitochondria. However, we observed that d-SHSY5Y required a greater concentration of AP39 to significantly improve mitochondrial bioenergetics. Previously, it has been demonstrated that the differentiation of SHSY5Y leads to reduced mitochondrial membrane potential observed as an approximately 200% reduction in mitochondrial TMRM fluorescent probe intensity [44]. Consistent with these observations, we infer that higher concentrations of AP39, perhaps required to normalise the mitochondrial membrane potential, are necessary to exert its effects on the mitochondrial function. Following this rationale, to exert a similar effect in SHSY5Y, less AP39 might be required, resulting in a different response to AP39 in regards to the SHSY5Y differentiation status. However, more analysis and quantification of mitochondrial to cytoplasmic levels of $H_2S$ might be necessary to support this hypothesis.

ROS are emerging as key signalling molecules participating in a myriad of physiological functions [45]. Increased generation and accumulation of ROS, in particular at the mitochondrial level, may result in mitochondrial OS leading to a range of cellular concerns including inflammation and death [9]. The modulation of oxidative damage to the mitochondria may be a novel strategy to address OS-related neuronal disorders with AP39 demonstrating potential to reduce intracellular oxidant production, both *in vitro* and *in vivo*. Interestingly and in contrast to other research groups exploring the potential for AP39 to protect the neuronal functions we observed that human derived cells may have a different behaviour in response to AP39. As we stated before, a range of concentrations of AP39 up to 1 μM may produce beneficial effects against OS. However, other authors exploring mice-derived neurons have shown that up to 0.1 μM AP39 is sufficient to stimulate the cellular bioenergetics while concentrations of 0.250 μM resulted in a inhibition of the cellular bioenergetics [27]. These striking differences in terms of AP39 concentration suggest that human cells types may respond differently to other organisms' cells and stress the importance of exploring the effects of novel molecules using efficient human models. Moreover, similar to previous reports [26], our results regarding d-SHSY5Y, displayed a somewhat OS-resistant phenotype (**Figures 6** and **7**). These effects may be attributed to the variation in the expression of mitochondrial protein levels [26]. Consequently, these observations suggest that a mature neuronal-like cells may better resemble and test the response to $H_2S$-inhibitory effects. However, the exact molecular mechanisms implicated in these observations remain to be investigated in further experiments.

**Conclusions**

As far as we are aware, our results provide novel evidence of the potential of AP39 in human neuronal-like cells for the modulation of mitochondrial related-OS and describe the contrasting effects of AP39 on non-differentiated and differentiated human neuroblastoma cells SHSY5Y. These observations suggest that nanomolar to micromolar concentrations of AP39 may be sufficient to exert beneficial effects on the neuronal bioenergetics, including the modulation of the mitochondrial bioenergetics and ROS generation. As neurological disorders are associated with increased OS and defective mitochondria, our results suggest that $H_2S$-related compounds, in particular AP39, may offer new therapeutic opportunities that warrant further exploration.

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**Conflicts of interests:**

Authors declare no conflict of interest.

**Authors contributions:**

LSA conceived the study, secured funding, performed experiments, drafted and edited the manuscript, MM edited the manuscript and SN performed experiments and edited the manuscript.

**Figures legends:**

**Figure 1**

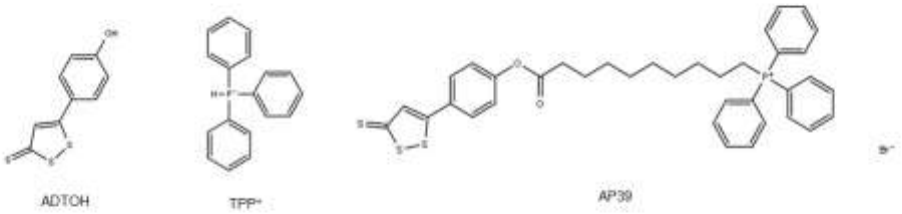


**Figure 1.** Chemical Structures depicting ADTOH (5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione), $TPP^+$ (triphenylphosphonium) and AP39 ($TPP^+$ linked to ADTOH).

**Figure 2**

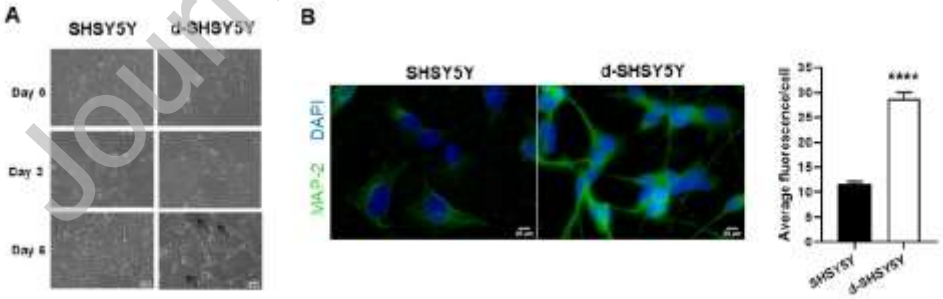


**Figure 2.** Differentiation of neuronal SHSY5Y cell line using retinoic acid (RA) in low FBS environment. (A) Bright field images depicting SHSY5Y differentiation with 10 μM RA and 1% FBS. Neurite extension was effective after 6 days (arrows). (B) Immunocytochemistry against MAP-2

(green) and DAPI (blue). Quantification was performed with ≥ 50 cells for each differentiation treatment at day 6. Data= mean ± SEM. ****p<0.0001 vs non-differentiated SHSY5Y.

Figure 3

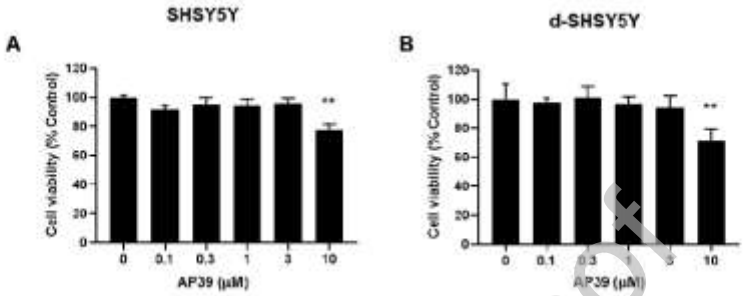


**Figure 3.** Effect of AP39 on SHSY5Y and d-SHSY5Y cell viability. (A) SHSY5Y and (B) d-SHSY5Y we exposed to 0.1-10 µM AP39 for 24 h and cell viability ascertain by MTT assay. Data= mean ± SEM. **p<0.01 vs control (no AP39).

Figure 4

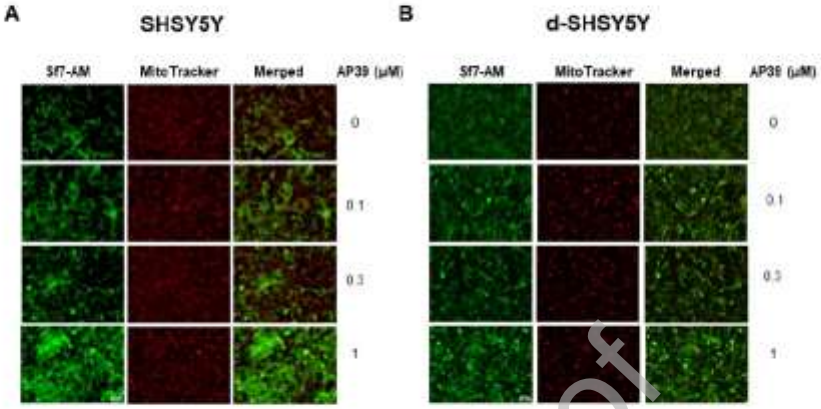


**Figure 4.** AP39 generates intracellular $H_2S$. (A) SHSY5Y and (B) d-SHSY5Y were exposed to various concentrations of AP39 for 1 h and intracellular $H_2S$ was detected using Sf7-AM fluorescent probe and mitochondrial localization was monitored by MitoTracker Red as described in Materials and Methods.

Figure 5

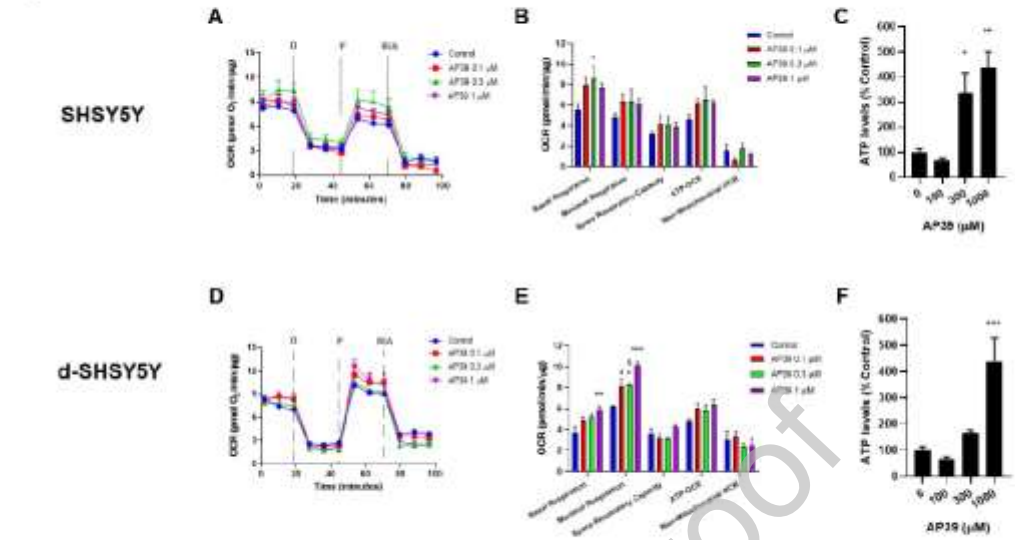


**Figure 5**. Effect of AP39 on resting non-differentiated and differentiated SHSY5Y mitochondrial bioenergetics exposed to increasing concentrations of AP39. (A-C) oxygen consumption expressed by time, their calculated mitochondrial bioenergetics parameters and ATP levels in SHSY5Y and (D-F) d-SHSY5Y. H/V: $H_2O_2$ (300 μM) and/or vehicle. O: Oligomycin. F: FCCP. R/A: mixture of antimycin A and rotenone. Data= mean ± SEM. *p<0.05, **p<0.01 vs control (no AP39). $ p<0.05 vs AP39.

Figure 6

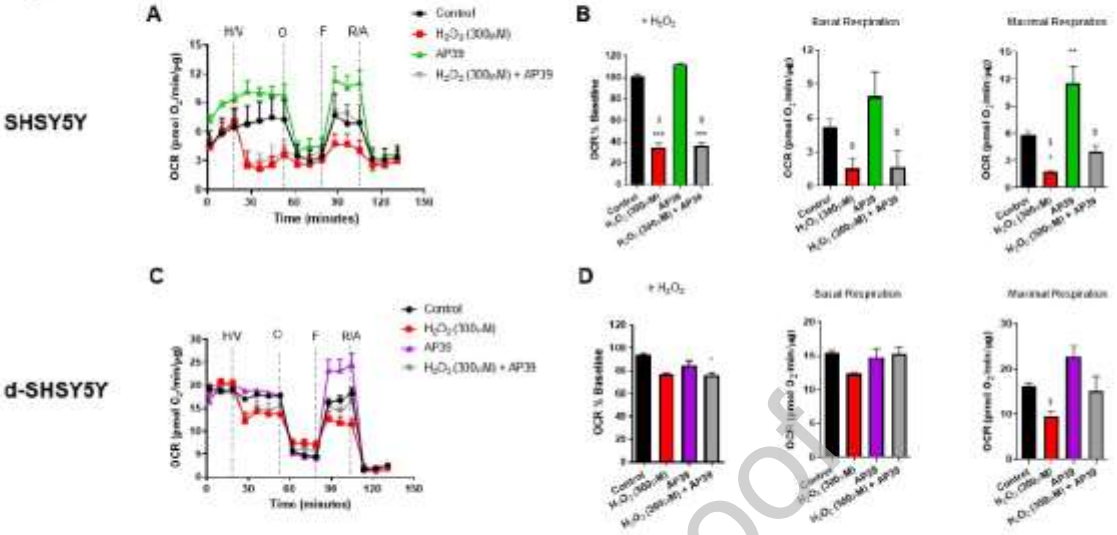


**Figure 6.** AP39 improve the mitochondrial respiration in $H_2O_2$-exposed neuroblastoma cells. Oxygen consumption traces expressed by time and their calculated mitochondrial bioenergetics parameters in non-differentiated (A-B) and differentiated (C-D) SHSY5Y cells. H/V: $H_2O_2$ (300 µM) and/or vehicle. O: Oligomycin. F: FCCP. R/A: mixture of antimycin A and rotenone. Data= mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs control (no AP39). $ p<0.05 vs AP39.

Figure 7

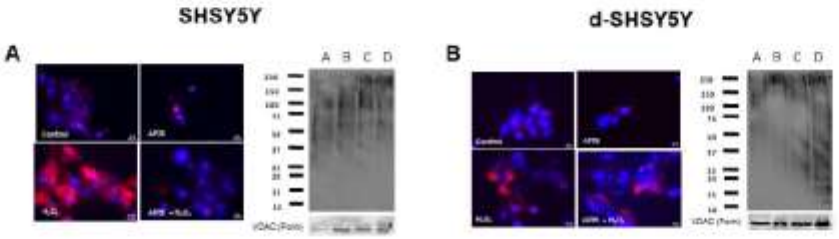


**Figure 7.** AP39 ameliorates $H_2O_2$-induced mt-ROS generation. The generation of mitochondrial specific ROS was detected by fluorescence microscopy using the fluorescent probe MitoSOX Red using 60X magnification. Protein carbonyl content was detected in isolated mitochondria by western blot in (A) SHSY5Y and (B) d-SHSY5Y. VDAC1 (porin) was used as mitochondrial protein loading control. A: Control. B: $H_2O_2$. C: AP39. D: $H_2O_2$ + AP39.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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