Nitric Oxide 41 (2014) 120–130

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

# AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells *in vitro*

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ARTICLE INFO

*Article history:*
Available online 19 April 2014

*Keywords:*
Mitochondria
Bioenergetics
DNA repair
Oxidative stress
Cytoprotection

ABSTRACT

The purpose of the current study was to investigate the effect of the recently synthesized mitochondrially-targeted $H_2S$ donor, AP39 [(10-oxo-10-(4-(3-thioxo-3*H*-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide], on bioenergetics, viability, and mitochondrial DNA integrity in bEnd.3 murine microvascular endothelial cells *in vitro*, under normal conditions, and during oxidative stress. Intracellular $H_2S$ was assessed by the fluorescent dye 7-azido-4-methylcoumarin. For the measurement of bioenergetic function, the XF24 Extracellular Flux Analyzer was used. Cell viability was estimated by the combination of the MTT and LDH methods. Oxidative protein modifications were measured by the Oxyblot method. Reactive oxygen species production was monitored by the MitoSOX method. Mitochondrial and nuclear DNA integrity were assayed by the Long Amplicon PCR method. Oxidative stress was induced by addition of glucose oxidase. Addition of AP39 (30–300 nM) to bEnd.3 cells increased intracellular $H_2S$ levels, with a preferential response in the mitochondrial regions. AP39 exerted a concentration-dependent effect on mitochondrial activity, which consisted of a stimulation of mitochondrial electron transport and cellular bioenergetic function at lower concentrations (30–100 nM) and an inhibitory effect at the higher concentration of 300 nM. Under oxidative stress conditions induced by glucose oxidase, an increase in oxidative protein modification and an enhancement in MitoSOX oxidation was noted, coupled with an inhibition of cellular bioenergetic function and a reduction in cell viability. AP39 pretreatment attenuated these responses. Glucose oxidase induced a preferential damage to the mitochondrial DNA; AP39 (100 nM) pretreatment protected against it. In conclusion, the current paper documents antioxidant and cytoprotective effects of AP39 under oxidative stress conditions, including a protection against oxidative mitochondrial DNA damage.



## 1. Introduction

Emerging mitochondrial roles of hydrogen sulfide ($H_2S$) include antioxidant effects, modulation of mitochondrial cell death pathways and the regulation of cellular bioenergetics reviewed in [1–4]. With respect to antioxidant/cell death modulating responses, multiple studies have demonstrated that $H_2S$ donors can maintain mitochondrial integrity, reduce the release of mitochondrial death signals and attenuate mitochondrially-regulated cell death responses of various types [5–13]. With respect to the regulatory role of $H_2S$ on cellular bioenergetic responses, recent data show that $H_2S$, in lower concentrations, serves as a physiological electron donor and as an inorganic source of energy in mammalian cells; via these pathways, $H_2S$ acts as an alternative supporter of mitochondrial electron transport and ATP generation [4,14–17]. However, the mitochondrial effects of $H_2S$ typically follow bell-shaped curves, whereby elevation of $H_2S$ concentrations

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http://dx.doi.org/10.1016/j.niox.2014.04.008
1089-8603/

beyond a certain concentration becomes cytotoxic, genotoxic, pro-oxidant, and suppresses mitochondrial electron transport (reviewed in [17]). Closely related to the concentration-dependency of biological $H_2S$ responses, the rate of $H_2S$ generation is also critical: fast-releasing and slow-releasing $H_2S$ donors can affect different biochemical pathways and can exert different, even opposing, cellular responses [18,3].

The goal of the current study was to characterize the $H_2S$ donor AP39. AP39 consists of a mitochondria-targeting motif, triphenylphosphonium ($TPP^+$), coupled to a $H_2S$-donating moiety (dithiolethione) by an aliphatic linker (Fig. 1). The purpose of the synthesis of this structure is to target the $H_2S$ to the mitochondria, by exploiting the well-known tendency of $TPP^+$ to accumulate in mitochondria [19]. We have also compared the effects of AP39 with those of AP219, a structurally related molecule, which does not have the $H_2S$ donating moiety, as well as with ADT-OH (5-phydroxyphenyl-1,2-dithione-3-thione), the $H_2S$ donor moiety that is incorporated into the AP39 structure (Fig. 1). The effect of AP39 in endothelial cells was studied both under basal conditions and under conditions of oxidative stress. In addition to detecting overall cell viability, we have also assessed changes in mitochondrial electron transport/cellular bioenergetics and in mitochondrial DNA damage.

## 2. Materials and methods

### 2.1. Materials

AP39, ADT-OH and AP219 were synthesized in-house as described [20–22]. Antimycin A, 7-azido-4-methylcoumarin (AzMC), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 2-deoxyglucose, oligomycin and rotenone were obtained from Sigma–Aldrich (St. Louis, MO, USA).

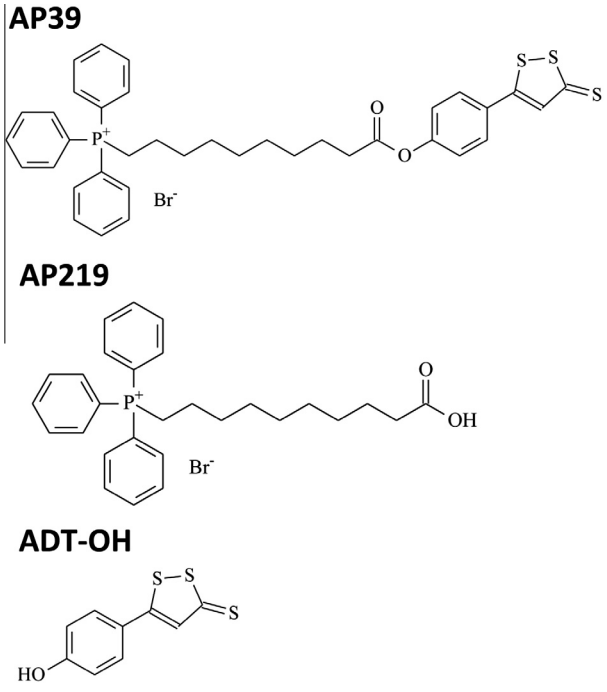


**Fig. 1.** Chemical structures of AP39 and the two control molecules AP219 and ADT-OH. AP219 is an AP39-like scaffold that does not have the $H_2S$ donor group and is the predicted product of AP39, if the compound undergoes hydrolysis by intracellular esterases. ADT-OH is the $H_2S$ donor moiety used in AP39, without the mitochondrially targeted $TPP^+$ group.

### 2.2. Cell culture

The murine brain microvascular endothelial cell line, bEnd.3 (ATTC #CRL-2299, Manassas, VA) was maintained in DMEM containing 1 g/l glucose supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 10% $CO_2$.

### 2.3. Detection and cellular localization of $H_2S$

40,000 of bEnd.3 cells were seeded in Lab-Tek II chamber coverglass system (Nalgen Nunc International) and incubated at 37 °C and 10% $CO_2$ humidified incubator overnight. The $H_2S$-sensitive fluorescent dye 7-azido-4-methylcoumarin (AzMC) [23] was incorporated into a cell-based assay [24] to detect $H_2S$ production. The cells were loaded with AzMC and MitoTracker Red CMXRos (Invitrogen M7512) fluorogenic dyes at 10 μM and 50 nM final concentrations, respectively, for 30 min. Various concentrations of AP39, or the control compounds AP219 or ADT-OH, were added with fresh media and cells were further incubated for 1 h. In another set of the experiments, cells were pretreated with the mitochondrial uncoupling agent FCCP (0.5 μM) for 1 h; then AP39 was added and $H_2S$ imaging was performed. Cells were washed three times with PBS and the specific fluorescence of the various dyes was visualized using a Nikon eclipse 80i inverted microscope with a Photometric CoolSNAP HQ2 camera and the NIS-Elements BR 3.10 software.

### 2.4. Bioenergetic analysis in cultured cells

The XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) was used to measure bioenergetic function, as originally described [25,26], and as employed by us in prior studies [13,16]. The measurement of oxygen consumption rate (OCR) after oligomycin (1.5 μg/ml) was used to assess ATP production rate and the measurement of OCR after FCCP (0.5 μM) was used to assess maximal mitochondrial respiratory capacity. 2-deoxyglucose (100 mM) was used to estimate cellular glycolytic dependency and antimycin A (2 μg/ml) and rotenone (2 μM) were used to inhibit the flux of electrons through Complex III and I, to detect residual non-mitochondrial oxygen consumption rate, which is considered to be due to cytosolic oxidase enzymes. Simultaneously with the OCR measurements, extracellular acidification rate (ECAR), an index of glycolytic function, was also measured.

### 2.5. MTT assay

The MTT method was performed as described [27]. Briefly, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the cells at a final concentration of 0.5 mg/ml and cells were cultured at 37 °C for 1 h. The cells were washed with PBS and the formazan dye was dissolved in DMSO. The amount of converted formazan dye was measured at 570 nm with a background measurement at 690 nm on a Powerwave reader (Biotek). The method detects Complex II-dependent mitochondrial activity, and is often used to estimate mitochondrial function and/or cell viability.

### 2.6. LDH assay

Lactate dehydrogenase (LDH) release was determined as a cytotoxicity assay, a secondary measurement for determination of cell death, as described [27]. Briefly, 30 μl of supernatant was saved before addition of MTT and mixed with 100 μl freshly prepared LDH assay reagent containing 85 mM lactic acid, 1 mM nicotinamide adenine dinucleotide ($NAD^+$), 0.27 mM N-methylphenazonium