Free Radical Research

ISSN: 1071-5762 (Print) 1029-2470 (Online) Journal homepage: https://www.tandfonline.com/loi/ifra20

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To cite this article: Samantha Scheinok, Benoit Driesschaert, Donatienne d'Hose, Pierre Sonveaux, Raphaël Robiette & Bernard Gallez (2019): Synthesis and characterization of a 5-membered ring cyclic hydroxylamine coupled to triphenylphosphonium to detect mitochondrial superoxide by EPR spectrometry, Free Radical Research, DOI: 10.1080/10715762.2019.1692137

To link to this article: https://doi.org/10.1080/10715762.2019.1692137

Published online: 19 Nov 2019.

FREE RADICAL RESEARCH
https://doi.org/10.1080/10715762.2019.1692137

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ORIGINAL ARTICLE

Synthesis and characterization of a 5-membered ring cyclic hydroxylamine coupled to triphenylphosphonium to detect mitochondrial superoxide by EPR spectrometry

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ABSTRACT

As mitochondrial superoxide is becoming an attractive metabolic and pharmacological target, there is an important need for developing analytical tools able to detect superoxide with high sensitivity and specificity. Among EPR-based methods, it has been recently reported that cyclic hydroxylamines offer a high sensitivity to measure superoxide production. Here, we report the synthesis and evaluation of mitoCPH, in which a 5-membered ring hydroxylamine was coupled to a triphenylphosphonium moiety to allow mitochondrial accumulation. MitoCPH efficiently reacted with superoxide with a bimolecular rate constant of $1.5 \times 10^4\ M^{-1}\ s^{-1}$. We assessed the ability of this compound to detect superoxide in PBS buffer, lysates, and in paraquat-stimulated cells. We compared its performance with CMH, a nontargeted 5-membered ring hydroxylamine, and mitoTEMPO-H, a classically used 6-membered ring hydroxylamine targeted to mitochondria. MitoCPH presented a higher sensitivity for superoxide anion detection than commonly used mitoTEMPO-H, both in buffer and in cell lysates. While we have described the ability of mitoCPH to detect superoxide in different cellular media, we cannot exclude other potential contributors to the nitroxide production from this probe. Therefore, mitoCPH should be considered as a mitochondria-targeted probe and its use as selective superoxide probe should be used cautiously.

ARTICLE HISTORY
Received 16 April 2019
Revised 30 October 2019
Accepted 4 November 2019

KEYWORDS
Cyclic hydroxylamine; EPR; ESR; mitochondria; paraquat; superoxide

The superoxide radical is a reactive oxygen species (ROS) formed by the one-electron reduction of oxygen. It is mainly produced by an electron leak from the mitochondria electron transport chain (ETC) and by the NADPH oxidase (NOX) family [1]. Although its concentration is tightly regulated by the antioxidant system and enzymes such as superoxide dismutase (SOD) in normal conditions, its amount sometimes exceeds the physiological level, leading to various pathologies [2–4]. Consequently, superoxide production in mitochondria has become an attractive pharmacological target and, in the recent years, a wide range of mitochondria-targeted antioxidants (MTA) have been developed [5].

The mitochondrial accumulation of MTA is based on the driving force of the negative potential of cellular and mitochondrial membranes that facilitates lipophilic cations to accumulate inside the compartment. The triphenylphosphonium cation (TPP^+) is the most classical structure used for mitochondria targeting. The Nernst equation predicts that accumulation of this type of structure in mitochondria is 100–1000 times higher than extracellular concentrations [6,7]. Diverse TPP^+-based structures have already been synthesised and tested *in vivo* as pharmacological agents [5] (Figure 1). For example, MitoQ demonstrated a potent mitochondrial antioxidant activity, low toxicity, and is presently tested in preclinical and clinical studies [8,9]. MitoTEMPO, known as a mitochondrial superoxide scavenger, was shown to inhibit cancer metastasis *in vitro* and *in vivo* [2]. In addition, Dikalova, *et al.* showed that mitoTEMPO diminished hypertension *in vivo* [4]. Recently, the group of Cheng demonstrated that a 3-undecyltriphenylphosphonium-carboxyproxyl nitroxide (mitoCP, Figure 1) inhibited the proliferation in different cancer cell lines probably by interfering with the ETC [10]. Furthermore, Dikalova, *et al* synthesised two mitoCP structures

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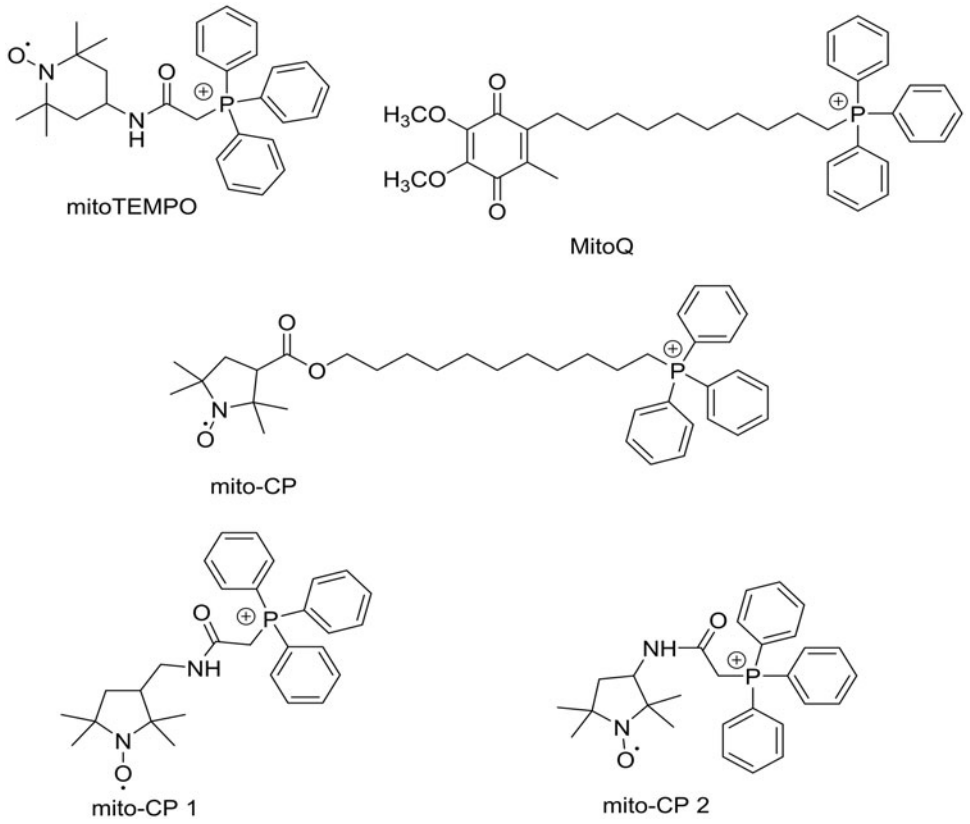

Figure 1. Examples of existing mitochondria-targeted antioxidants (MTA).

(mitoCP1 and mitoCP2, Figure 1) with a shorter alkyl chain as therapeutic agents in hypertension [11]. Other mitochondria-targeted structures are still investigated in different pathologies, including cancer [12].

Mitochondria-targeted compounds can also be used as analytical tools to detect mitochondrial superoxide. Electron paramagnetic resonance (EPR) is the method of choice to detect free radicals. As superoxide is a too short-lived species to be directly detected by EPR, there is a need to add a probe in the media for its detection. The most commonly used probes are spin traps (mitoDIPPMPO and mitoDEPMPO) and cyclic hydroxylamines (mitoTEMPO-H) [13–15]. Spin traps are diamagnetic species that produce paramagnetic compounds after their reaction with short-lived radicals. The stable spin adduct presents an EPR spectrum that is a fingerprint of the trapped free radical. Indeed, Abbas, *et al.* succeeded in measuring superoxide adducts by using mitoDIPPMPO in stimulated macrophages [16]. Cyclic hydroxylamines form paramagnetic species (nitroxides) after their reaction with ROS, leading to a three-line EPR spectrum. Because this reaction may occur with different oxidants, appropriate controls should be used to confirm the involvement of superoxide in the formation of the EPR spectrum.

Recently, a comparative study of different EPR approaches to detect the superoxide anion has been performed [17]. This previous study concluded that CMH (a 5-membered ring hydroxylamine) provided the best performances to measure superoxide production in PBS, lysates and cells (intra- and extracellular compartments). Among cyclic hydroxylamines, the paper also highlighted that CMH was much more sensitive than the existing mitochondria-targeted 6-membered ring hydroxylamine mitoTEMPO-H (Figure 2). As the sensitivity of detection is directly linked to the instability of the nitroxide, we hypothesised that a 5-membered ring hydroxylamine coupled to a TPP^+ moiety could be a better tool to detect mitochondrial superoxide. Indeed, existing probes include CMH (a nontargeted 5-membered ring structure) that accumulates slowly in mitochondria [15] whereas mitoTEMPO-H is a mitochondria-targeted 6-membered structure believed to be less stable than 5-membered ring structure. Based on this rationale, we synthesised a new 5-membered ring cyclic hydroxylamine targeting mitochondria by linking CPH to a butyltriphenylphophonium moiety (mitoCPH) (Figure 2). We then compared the ability of this compound to detect superoxide in PBS buffer and cell lysates, and compared its performances to mitoTEMPO-H and CMH. We also compared its sensitivity to detect the mitochondrial superoxide anion produced by paraquat-stimulated versus unstimulated cells.

Materials and methods

Reagents

CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) and mitoTEMPO-H (1-hydroxy-4-[2-(triphenylphosphonio)-acetamido]-2,2,6,6-tetramethylpiperidine) were purchased from Enzo Life Science (Antwerpen, Belgium). Superoxide dismutase (SOD), xanthine oxidase (XO), hypoxanthine (HX), diethylene triamine pentaacetic acid (DTPA), paraquat dichloride, and cytochrome c oxidase were obtained from Sigma-Aldrich (Overijse, Belgium). PBS was from ThermoFisher (Merelbeke, Belgium, Catalogue 10010023). All chemicals and solvents used for synthesis were purchased from Sigma-Aldrich (Overijse, Belgium). SiHa human cervix adenocarcinoma cells were from the American Type Culture Collection (ATCC, Manassas, USA).

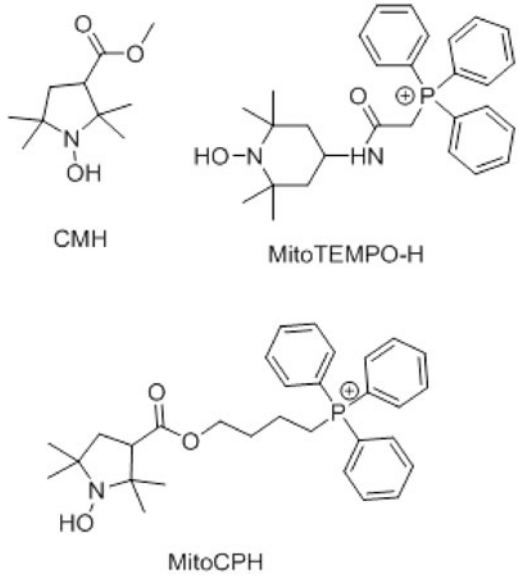

Figure 2. Chemical structures of cyclic hydroxylamines used for superoxide detection: CMH is a non-targeted agent; mitochondria-targeted compounds include the 6-membered ring mitoTEMPO-H, and the new 5-membered ring mitoCPH.

Chemistry

The chemical synthesis of mitoCPH is described in Figure 3(A).

Synthesis of 1-hydroxy-3-(((carbonyl)oxy)butyl-triphenylphosphonium)-2,2,5,5-tetramethylpyrrolidine bromide (1)

3-carboxy-proxyl (200 mg, 1.074 mmol) was stirred in dimethylformamide (DMF, 9 mL) in the presence of KI (24 mg, 0.143 mmol), Na_2CO_3 (136.8 mg, 1.29 mmol) and (4-Bromobutyl)triphenylphosphonium bromide (343 mg, 0.72 mmol) at 53 °C during 2 h. The yellow

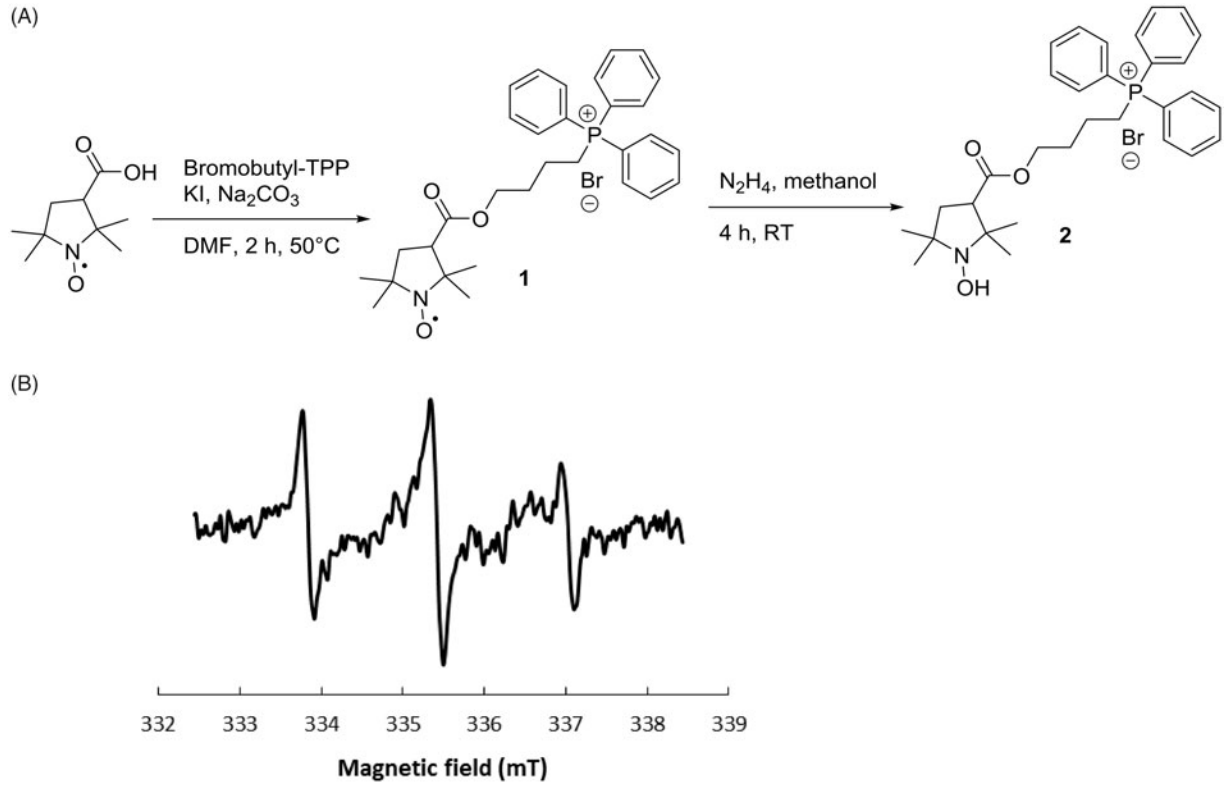

Figure 3. (A) Chemical synthesis of mitoCPH (2). (B) MitoCP° EPR signal recorded in the mitochondrial fraction of SiHa cells. This spectrum was recorded after incubation of SiHa cells in the presence of 160 μM of mitoCPH.

solution was diluted in dichloromethane and washed with 1 M sulphuric acid, then water and finally a saturated $NaHCO_3$ solution. The organic layer was extracted and dried with $MgSO_4$ and the solvent was evaporated under reduced pressure to about 2 mL. Diethyl ether was added in large excess to the yellow oil. The precipitated product was freeze-dried. The yield was 250 mg (60%). HRMS (ESI^+): calculated for $C_{31}H_{38}NO_3P^+$, 503.25838; found, 503.25818.

Synthesis of 3-(((carbonyl)oxy)butyl-triphenylphosphonium)-2,2,5,5-tetramethylpyrolidinooxy bromide or mitoCPH (2)

To an anhydrous methanol solution (2.44 mL) of nitroxide **1** (100 mg, 0.17 mmol), 1 M hydrazine in THF (7.84 mmol) was added dropwise. The mixture was stirred for 4 h at room temperature. The solvent was evaporated to dryness under reduced pressure at room temperature. The residue was washed with methanol and evaporated to dryness twice. The final product was dried and kept under argon (Figure 3(A)). The yield was 98 mg (98%). This protocol was adapted from Yordanov AT *et al.* [18].

HRMS (ESI^+): calculated for $C_{31}H_{39}NO_3P^+$,504.2662; found, 504.2648.

^1H NMR ($CDCl_3$, 300 MHz) δ: 0.85 (s,3H), 1.11 (s,3H), 1.12 (s,3H), 1.125 (s,3H) 1.6–1.8 (massif,3H), 1.9–2.1 (massif,1H), 2.14 (s, 1 H), 2.62 (m,1H), 3.66 (m, 1 H), 3.94 (s,2H), 4.14 (m, 2 H), 7.66–7.9 (m, 15 H).

^{13}C NMR ($CDCl_3$, 75 MHz): δ 19.6, 22.2, 22.7, 26.6, 27.4, 27.8, 29.4, 37.7, 49.0, 61.6, 63.4, 65.5, 117.5, 118.7, 130.8, 133.8, 135.3, 172.8.

Superoxide production in PBS and in lysates

The concentrations of the different reagents and probes were selected according to the literature [19]. PBS concentration was 0.01 M, pH 7.4. SiHa cells were cultured to obtain 5×10^6 cells/mL and were then lysed by sonication (20 s, Labsonic U, B. Braun). Superoxide was produced using a XO/HX system (XO final concentration 5, 2.5, 1, 0.25 mU/mL) in a PBS solution and in cell lysates (XO final concentration 5 mU/mL), both containing hypoxanthine (1 mM) and DTPA (1 mM). Concentration of the probe was 0.5 mM. To assess the specificity of the reaction, SOD (300 U/mL) was added to experimental media. The superoxide production rate was determined by the cytochrome c assay (50 μM). Briefly, ferricytochrome c was added to the media containing HX and XO and its reduction to form ferrocytochrome c was monitored at 550 nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Wokingham, UK). The concentration of cytochrome c was calculated using a $\Delta_{\varepsilon 550\,nm}$ value of 21,000 $M^{-1}\,cm^{-1}$. The slope of cytochrome c reduced per minute provided the rate of superoxide production in our conditions, which was 1.97 μM/min for 5 mU/mL of XO.

Measurement of mitochondrial superoxide produced in SiHa cells

Subconfluent SiHa cells were harvested and resuspended in Krebs Hepes buffer (pH 7.4) [15] at 20×10^6 cells/mL. The experimental mixture was prepared with cells, DTPA 1 mM, and the probe (50 μM). Paraquat (final concentration 900 μM) or PBS (for controls) was added just before the probe. The sample was then transferred into a gas-permeable polytetrafluoroethylene (PTFE) tubing that was placed in a quartz tube opened at both ends. The tube was then put in the heated cavity (310 K). Increase in signal amplitude was monitored until 10 min after the addition of the probe. Data are expressed as the difference in signal amplitude recorded at 10 min and the initial signal amplitude.

Measurement of nitroxide in mitochondrial fraction

SiHa cells were treated with 160 μM of mitoCPH during 10 min at 310 K. Then, the mitochondria were isolated according to Frezza's protocol [20] and transferred in the EPR cavity. The recorded spectrum represents "basal" probe oxidation of mitoCPH in mitochondria.

EPR settings

EPR measurements were performed using a Bruker EMX-Plus spectrometer (Bruker, Rheinstetten, Germany), operating in X-band (9.85 GHz) and equipped with a PremiumX ultra low noise microwave bridge and a SHQ high sensitivity resonator. Typical settings were as follows: microwave power: 2.518 mW (or 20 mW for cells); modulation frequency: 100 kHz; modulation amplitude: 0.1 mT; time constant: 20.48 ms; conversion time: 20.00 ms; data points: 1325; sweep width: 5.3 mT.

EPR experiments

Superoxide production was initiated in PBS or cell lysates as described above and was further transferred into a gas-permeable polytetrafluoroethylene (PTFE) tubing that was placed in a quartz tube opened at both ends. The tube was directly inserted in the cavity. The EPR cavity was heated at 310 K with air during all

experiments. A first EPR measurement was performed 2 min after the reaction started. All experiments were done in triplicates. Data are expressed in flux (concentration/min) of the nitroxide.

Reaction rate constant determination

The bimolecular constant of the reaction between mitoCPH and superoxide was determined by a standard competition approach using SOD as a competitor, as previously described [21]. The experiment was performed in an opened quartz tube at 310 K, and superoxide was generated using HX (1 mM) and XO (3 mU/mL). We monitored the kinetics of the reaction of mitoCPH (1 mM) with superoxide without or with different SOD concentrations (0.3; 0.6; 1.0; 1.5 U/mL). The ratio of the initial rates without and with SOD were calculated and expressed in function of the SOD concentrations used. Each SOD experiment was performed once.

Statistics

Data are shown as means ± SEM. Calibration curves using mitoTEMPO or mitoCP or CP° were performed to present the results in μM nitroxide. All experiments were performed in triplicates. One-way ANOVA was performed to compare the performances of the probes between each other. Two-way ANOVA was performed to compare the probes in paraquat-stimulated cells.

Results

Characterisation of the reaction of mitoCPH with superoxide

We first tested the ability of mitoCPH to enter into mitochondria. Figure 3(B) confirmed the presence of the new probe in the mitochondrial fraction of SiHa cells. The third line of the EPR spectrum is consistent with a partial immobilisation of the nitroxide. Next, we detected superoxide in PBS (pH 7.4). A rapid increase in the EPR signal intensity was observed in the presence of superoxide. This increase in EPR signal intensity was completely prevented in the presence of SOD (Figure 4(A)). In cell lysates, we also observed an increase in EPR signal in the presence of superoxide, but to a smaller extent (four times lower after 10 min) than observed in PBS. This increase in EPR signal intensity was partially inhibited by SOD (Figure 4(B)). The flux of 1.97 μM/min was selected according Figure 4(C) showing the accumulation of the nitroxides formation as a function of different superoxide fluxes for each probe.

The rate constant of the reaction between mitoCPH and superoxide was determined by using SOD as a competitive inhibitor. The evolution of EPR signal intensity generated by the reaction of mitoCPH with superoxide, in the absence or increasing concentrations of SOD, is presented in Figure 5. The ratio of the initial rates without and with SOD was calculated and expressed as a function of the SOD concentrations used (Figure 5). Assuming the value of k_{SOD} as $2 \times 10^9\ M^{-1}\ s^{-1}$ [22] the slope of the curve

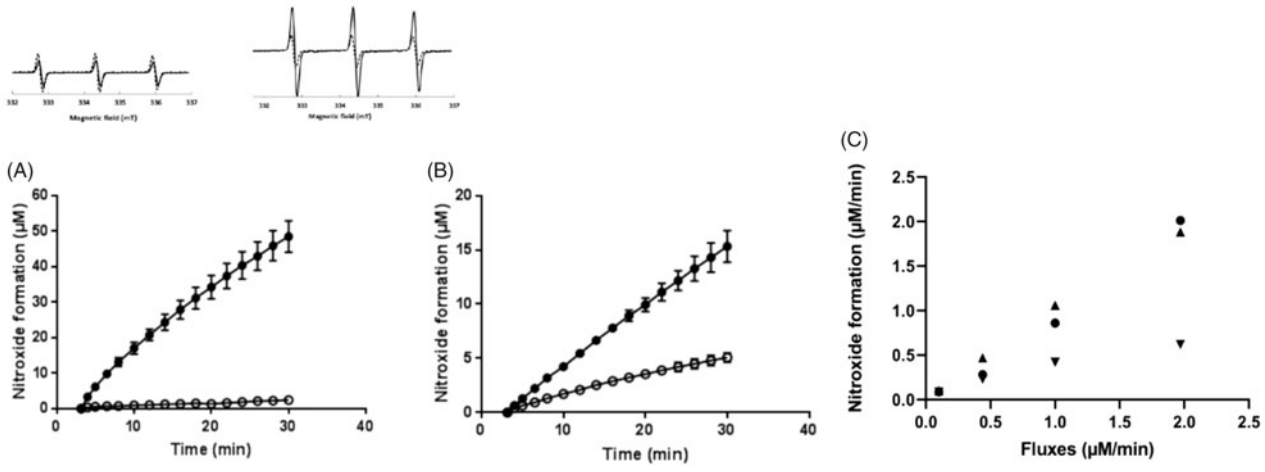

Figure 4. EPR monitoring of superoxide production in the presence of mitoCPH. Evolution of the EPR signal intensity over time in PBS buffer (A) and in cell lysates (B), in the absence (filled circle) or in the presence (open circles) of SOD (300 U/mL). Insert: EPR spectrum of mitoCPH in PBS after 10-min incubation in the presence of superoxide with (dashed line) or without SOD (black line). Superoxide was produced in PBS or cell lysates with HX 1 mM, DTPA 1 mM and XO 5 mU/mL. Concentration of the probe was 0.5 mM. (C) Nitroxides formation per minute at different superoxide generated fluxes (1.97, 1, 0.44 and 0.1 μM/min) using mitoTEMPO-H (reverse triangle), CMH (filled circle) or mitoCPH (triangle). Superoxide was produced in the presence of XO 5, 2.5, 1 or 0.25 mU/mL in addition of HX 1 mM and DTPA 1 mM in PBS. Concentration of the probes was 0.5 mM.

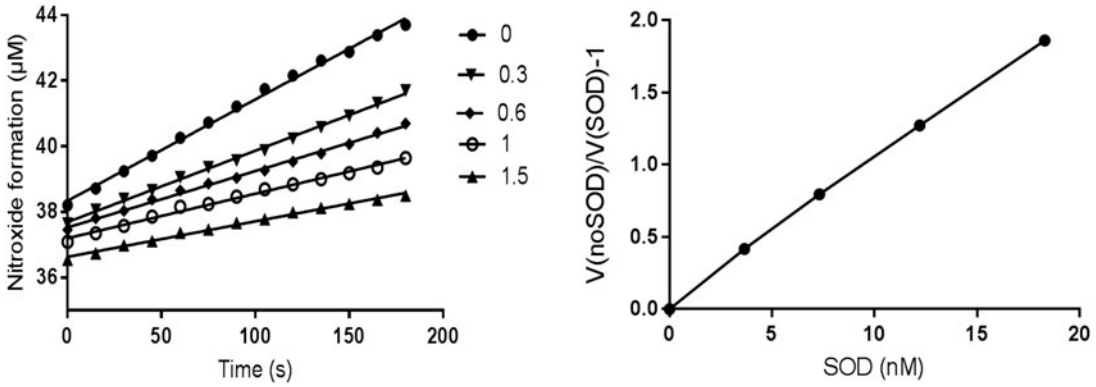

Figure 5. Determination of the bimolecular constant rate of mitoCPH oxidation by superoxide. On the left, evolution of the nitroxide concentration as a function of time upon superoxide generation in HX/XO system in the presence of different SOD concentrations. MitoCPH 1 mM; HX 1 mM; XO 3 mU/mL. On the left, dependence of relative efficiency of SOD-induced inhibition of nitroxide formation by superoxide on SOD concentration. Linear fit yields the bimolecular rate constant of mitoCPH oxidation by superoxide equal to $1.5 \times 10^4\ M^{-1}\ s^{-1}$.

provided a bimolecular constant rate of mitoCPH oxidation by superoxide anion of $1.5 \times 10^4\ M^{-1}\ s^{-1}$. This value is consistent with the constant rate calculated for other hydroxylamines [15].

Comparison of mitoCPH with other cyclic hydroxylamines

We compared mitoCPH to existing cyclic hydroxylamines, CMH, a permeable five-membered ring, and mitoTEMPO-H, a six-membered ring targeted to mitochondria. We first compared the sensitivity of mitoCPH in PBS using the HX/XO system producing superoxide at 1.97 μM/min at 310 K as determined by the cytochrome c reduction assay. In the experimental conditions used, the detection efficiency of the superoxide anion was 2.0 ± 0.07 μM/min, 0.6 ± 0.04 μM/min and 1.89 ± 0.11 μM/min for CMH, mitoTEMPO-H and mitoCPH, respectively (Figure 6(A)). The trapping efficiency measured was not significantly different between mitoCPH and CMH. However, we observed that mitoCPH trapped the superoxide anion significantly better than mitoTEMPO-H ($p < .001$). The measured trapping efficiency was also measured in cell lysates. CMH presented the best performance and was significantly better compared to the two other cyclic hydroxylamines ($p < .001$). In cell lysates (Figure 6(B)), mitoCPH still presented a significantly higher trapping efficiency (0.3 ± 0.01 μM/min) than mitoTEMPO-H (0.07 ± 0.007 μM/min) ($p < .05$).

Mitochondrial superoxide detection in paraquat-stimulated and unstimulated cells

We finally compared the two mitochondria-targeted molecules, mitoCPH and mitoTEMPO-H, in terms of sensitivity to detect mitochondrial superoxide. The accumulation of mitochondria-targeted-carboxyproxyl (mitoCP) [10], a structure similar to mitoCPH, was already assessed by others [23]. To this end, we stimulated the cells with paraquat, a pesticide known to produce mitochondrial superoxide as it targets mitochondrial ETC Complex I [24]. Figure 7 demonstrated that the EPR signal of the probes increased in cells stimulated by paraquat ($p < .001$ for mitoTEMPO-H and $p = .001$ for mitoCPH). Similar results were obtained using higher probes concentrations.

Discussion

As mitochondrial superoxide is becoming an attractive metabolic and pharmacological target, there is an increasing interest in developing strategies to modulate its concentration in tissues. There is also an important need for analytical tools able to detect superoxide with high sensitivity and specificity in order to assess the efficacy of these pharmacological modulators. EPR is potentially a method of choice for that purpose. In recent studies comparing different approaches for the detection of superoxide, it has been shown that superoxide-induced production of nitroxide from cyclic hydroxylamine CMH offered a high sensitivity, stability and specificity when using appropriate controls [15,17]. From the previous comparison [17], it also appeared that the yield of nitroxide formed was lower for mitochondria-targeted probe mitoTEMPO-H compared to CMH. As CMH accumulates in a small extent in mitochondria [15] we reasoned that by adding a TPP^+ moiety we would make an innovative mitochondrial tool with a high sensitivity and stability to detect mitochondrial superoxide. We hypothesised that the lower sensitivity of the mitoTEMPO-H could be linked to the

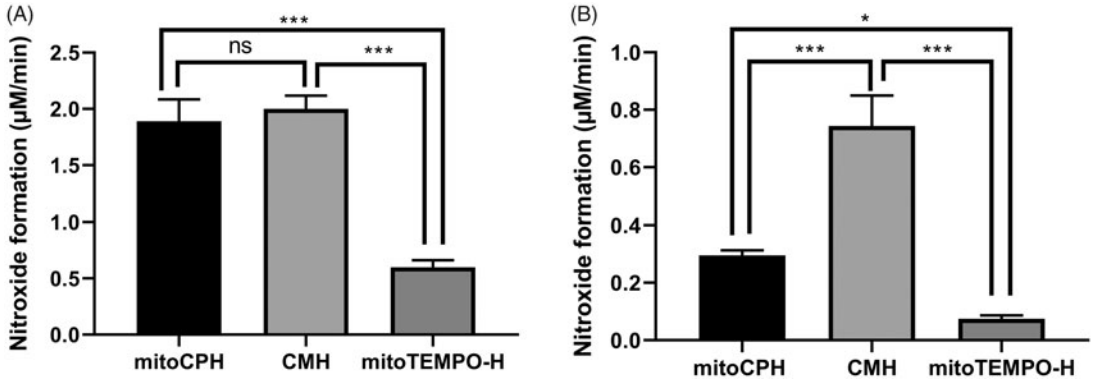

Figure 6. Comparison in trapping efficiency of the different cyclic hydroxylamines in PBS buffer (A) and in cell lysates (B). Rate of nitroxide apparition in 1 min for each probe. The generated superoxide flux was 1.97 µM/min. Superoxide was produced with HX 1 mM, DTPA 1 mM and XO 5 mU/mL ($n = 3$). Concentrations of the probes were 0.5 mM. $^{*}p < .05$, $^{***}p < .001$, ns $p > .05$ one-way ANOVA with Turkey test.

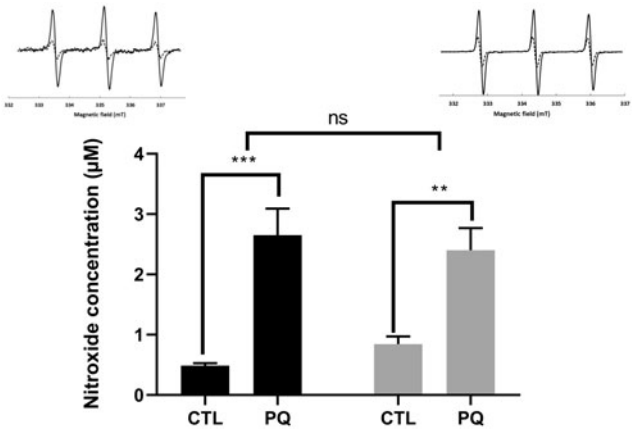

Figure 7. Detection of mitochondrial superoxide induced by paraquat (900 µM) during 10-min incubation at 310 K in EPR cavity in the presence of SiHa cells and mitoTEMPO-H (black) or mitoCPH (light grey). Data are expressed as the difference in EPR signal amplitude recorded at 10 min and the initial signal amplitude. Inserts: representative spectrum of mitoTEMPO-H (left) in unstimulated (dashed line) and stimulated cells (black line), and mitoCPH (right) in unstimulated (dashed line) and stimulated cells (black line). Experimental samples were obtained with 20×10^6 cells/mL, DTPA 1 mM, PQ (900 µM) or PBS and the probe (50 µM). $^{*}p < .05$, $^{***}p < .001$, ns > 0.05 by two-way ANOVA with Turkey test.

instability of the structure, as piperidinoxyl radicals are known to be more sensitive to bioreduction than pyrrolidinoxyl radicals [25]. Hence, in order to increase the sensitivity of detection of mitochondrial superoxide, we synthesised a 5-membered ring cyclic hydroxylamine because it could offer a higher stability of the nitroxide adduct and a resulting larger accumulation. The pyrrolidine structure was coupled to TPP^{+} as a mitochondria-targeting moiety. Of note, the corresponding nitroxide MitoCP has already been investigated as a pharmacological agent by others [10,23]. For analytical purpose, we decided to synthesise its reduced form, cyclic hydroxylamine mitoCPH because it is admitted that it is more sensitive to detect an EPR signal apparition than a signal decrease. Reily, *et al.* previously reported that the length of the linker may impact the mitochondrial accumulation of the compound. Here, we selected a butyl linker as it was reported that it does not interfere with the mitochondrial function and is lipophilic enough to accumulate easily to mitochondria [7]. The compound was successfully synthesised in two steps (Figure 3(A)). Of note, the compound should be kept under inert atmosphere (argon) because it may rapidly be oxidised.

As expected, mitoCPH efficiently reacted with superoxide (Figure 4). The bimolecular rate constant of the molecule was $1.5 \times 10^4\ M^{-1}\ s^{-1}$ (Figure 5). This value is consistent with the rate constant measured for other cyclic hydroxylamines [15]. Interestingly, this rate constant is faster than other EPR analytical tools including common spin traps, with a typical rate constant value of $10.9\ M^{-1}\ s^{-1}$ for EMPO at pH 7.2, as a typical example [26]. However, the rate of mitoCPH constant was lower than the one reported for antioxidant enzymes, such as SOD ($2 \times 10^9\ M^{-1}\ s^{-1}$). This means that, in biological samples, the concentration of mitoCPH should be large enough to compete with SOD. The performances of mitoCPH were benchmarked with CMH, an untargeted 5-membered ring hydroxylamine, and with mitoTEMPO-H, a commercially available mito-targeted 6-membered ring hydroxylamine (Figure 6(A,B). Our results highlighted that mitoCPH presents a higher sensitivity for superoxide anion detection than mitoTEMPO-H, both in buffer and in cell lysates. The efficiency of superoxide trapping was similar to CMH in buffer, but lower in cell lysates (Figure 6(A,B)). This may be explained by their low biostability. Indeed 5-membered ring are known to be more stable towards bioreduction than 6-membered ring which provide a theoretical background to explain the lower superoxide detection rate of mitoTEMPO-H

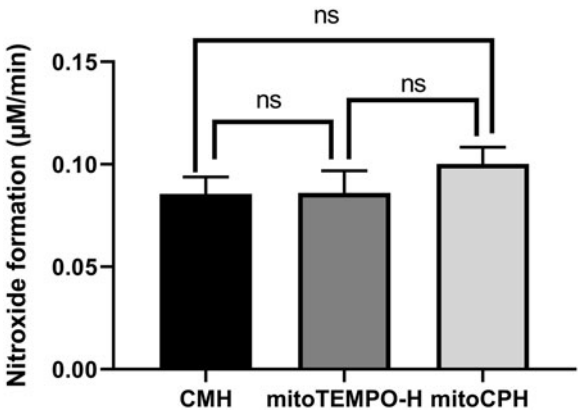

Figure 8. Nitroxides formation per minute at superoxide flux of 0.1 µM/min for each probe. Superoxide was generated using the XO/HX system in PBS and 1 mM DTPA was added. Concentration of the probes was 0.5 mM.

compared to the others. Furthermore, it is possible that the generated mitoTEMPO (and mitoCP° or CM°) is subjected to reduction (especially in the presence of NADH). Oxoammonium cation could also be produced in the cell lysates experiments leading to a smaller increase in EPR signal intensity. In general, mitoTEMPO-H appeared to have a slower rate constant which is responsible for its lower efficiency in detecting superoxide. Indeed, similar performance was observed for mitoTEMPO-H at lower superoxide fluxes (Figure 8). Of note, this low superoxide detection efficiency did not impact the feasibility of superoxide detection in PMA-stimulated cells [17]. In addition, NADH and cysteine have been shown to decrease nitroxide EPR signal in the presence of superoxide in PBS whereas no signal decrease was observed without these reagents [17,27,28]. Tempol was also demonstrated to prevent paraquat toxicity, most probably by several mechanisms including oxidoreduction reactions [29]. Furthermore, secondary ROS species such as peroxynitrite may also be responsible for a part of the signal increase. We can also suggest that the TPP^+ moiety plays a role on mitoCPH reactivity in complex media. Therefore, a control experiment (e.g. SOD for superoxide) should always be performed to confirm superoxide detection. Furthermore, as cells exhibit a lower superoxide flux and higher oxidising contents, intracellular superoxide detection may be challenging.

To test the possibility of mitochondrial superoxide detection with cyclic hydroxylamines, we used mitochondria-targeted probes mitoCPH and mitoTEMPO-H in cells stimulated by paraquat (Figure 7). MitoTEMPO-H seemed to exhibit a higher signal increase in paraquat-stimulated cells (compared to unstimulated cells) than did mitoCPH but the difference between the probes was not significant. It is important to note that the signal increase observed may also be due to other mechanisms than superoxide detection, including blocked reduction of the nitroxide by the electron transport chain in the presence of PQ or blocked reduction of the nitroxide by other cell components, due to a more oxidative environment in the presence of paraquat or a combination of all these processes. The confirmation of mitochondrial superoxide implication in the EPR signal increase should be done using intramitochondrial controls.

In conclusion, we have described the simple synthesis of a new mitochondria-targeted cyclic hydroxylamine that presents a high reactivity with superoxide. This compound offers a higher sensitivity than mitoTEMPO-H in PBS and lysates. While we have described the ability of mitoCPH to detect superoxide in different cellular media, we cannot exclude other potential contributors to the nitroxide production from this probe. Therefore, mitoCPH should be considered as a mitochondria-targeted probe and its use as selective superoxide probe should be used cautiously.

Acknowledgments

The authors thank Soumia El Aakchioui, Maximilien Richald, and Sébastien Clergue for their help with chemistry protocols, Laure Elens for help in statistical analysis and Thibaut Vazeille for the preparation of cells and lysates.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Samantha Scheinok is a Televie PhD fellow. Pierre Sonveaux and Raphaël Robiette are Senior Research Associates of the Belgian *Fonds National de la Recherche Scientifique* (FRS – FNRS). The work was supported by an Action de Recherche Concertée from the Communauté Française de Belgique (ARC 14/19-058) and by FRS – FNRS grant CDR J.0209.16. This research used the EPR facilities of the technological platform "Nuclear and Electron Spin Technologies" of the Louvain Drug Research Institute.

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