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Targeted fluorescent probes for detection of oxidative stress in the mitochondria

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ABSTRACT

Mitochondrial oxidative stress has been implicated in aging, neurodegenerative diseases, diabetes, stroke, ischemia/reperfusion injury, age-related macular degeneration (AMD) and cancer. Recently, we developed two new mitochondria-targeting fluorescent probes, MitoProbes **I**/**II**, which specifically localize in mitochondria and employed both in vivo and in vitro for detection of mitochondrial oxidative stress. Here, we report the design and synthesis of these agents, as well as their utility for real-time imaging of mitochondrial oxidative stress in cells.

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Mitochondrial oxidative stress is implicated in aging and neurodegeneration, diabetes, cardiovascular disease, ischemia/reperfusion injury, age-related macular degeneration (AMD) and cancer.[1–11] The generation of reactive oxygen species (ROS) during mitochondrial oxidative phosphorylation is a normal cellular process that can have damaging effects when uncontrolled. An often employed approach for ROS detection, electron spin resonance (ESR) spectroscopy is limited by low spatial resolution and a lack of applicability to real-time imaging of ROS at the single cell level. Conversely, fluorescent analysis is a powerful and convenient tool for monitoring the biological events in a living specimen. Unfortunately, few fluorescent probes for the detection of oxidative stress are currently available.[12,13] For example, fluorescent probes such as dihydrorhodamine are not preferentially localized intracellularly prior to oxidation.[12] Typical mitochondrial dyes are limited in their utility because of instability upon illumination. Rhodamine 123, for example, is susceptible to photobleaching and exhibits strong photoinduced toxicity.[14] As well, the JC-1 dye is not mitochondrial specific, rendering it unsuitable for evaluation of intracellular mitochondrial function.[14] Recently, Chang et al developed a fluorescent probe for mitochondrial specific H_2O_2 detection.[15,24] Here, we report the design and synthesis of two novel fluorescent probes that specifically target mitochondria and show convincing evidence for utility in monitoring mitochondrial oxidative stress in real time.

Design and Synthesis of Mitochondria-Targeting Fluorescent Probes (MitoProbes **I**/**II**) for Monitoring Mitochondrial Oxidative Stress. Our strategy for monitoring mitochondrial oxidative stress is to employ fluorogenic spin probes that can be detected by both fluorescence and ESR spectroscopy. The combination of both nitroxide and fluorescent moieties in one molecule yields a non-fluorescent compound. This likely results from quenching of the excited singlet state of aromatic fluorescent compounds by the nitroxide moiety.[16,17] Free radicals can react efficiently with the nitroxide moiety of the probes. This results in a diamagnetic compound, thereby eliminating intra-molecular quenching and enhancing fluorescence. The fluorescent probes we designed, which contain cationic residues, can pass through the outer mitochondrial membrane. The inner membrane is much more hydrophobic, and therefore it is important to preserve a high degree of lipophilicity in our molecular design. Thus, our newly synthesized fluorescent probes (MitoProbe **I**/**II**) contain both cationic and hydrophobic residues to provide electrostatic driving forces for uptake through both mitochondrial membranes (Fig. 1).

The synthesis of MitoProbe **I**/**II** was accomplished under mild conditions (Scheme S1). Starting from rhodamine B, a tertiary amide can be readily prepared by coupling with alkyne derivatized piperazine. Formation of a tertiary amide bond between rhodamine B and piperazine as a linker moiety prevents cyclization of the rhodamine derivative into a non-fluorescent lactam form. Thus, MitoProbe **I**/**II** could be readily prepared via "click reaction" via reaction of the tertiary amide with 4-azido-tempo, which was synthesized from 4-hydroxyl-2,2,6,6-tetramethyl-piperidine 1-oxyl according to a previously reported procedure.[17,18] In order

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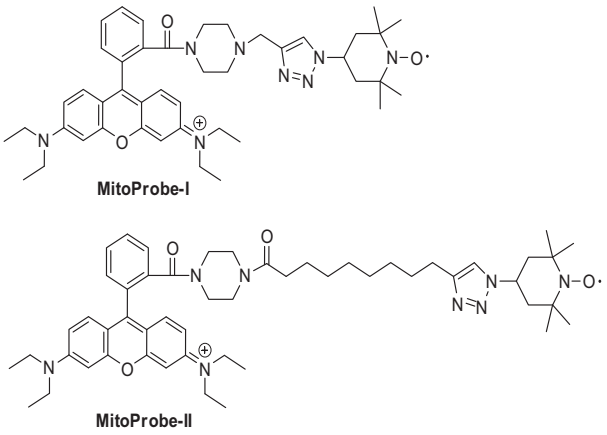

Figure 1. Chemical structure of MitoProbes **I**/**II**.

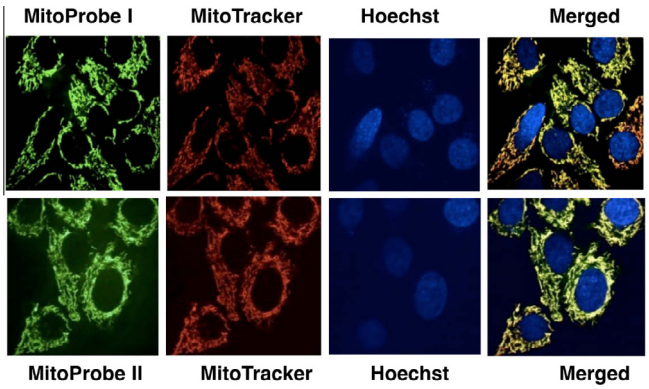

Figure 2. Confocal laser-scanning fluorescent images of HeLa cells incubated with MitoProbe **I** (30 nM, green, 45 min) or MitoProbe **II** (50nM, green, 45 min), and stained with MitoTracker (80nM, 45 min, red), and Hoechst 33242 (0.1 μL/mL, 30 min, blue) and overlay images (yellow). Images were obtained with an confocal laser scanning fluorescent microscope using a 40× objective lens in non-FBS, non-phenol red media.

to avoid reduction of the nitroxide radical by sodium L-ascorbate to the non-paramagnetic hydroxylamine derivative during the click reaction, copper(I) iodide was employed as the copper(I) source instead of the Cu(II)SO_4/sodium L-ascorbate system. An optimal reaction was achieved with the inclusion of 10 mol % Cu(I) iodide. All the ^{1}H NMR signals of the nitroxide-labeled compounds were broadened and the peaks that were associated with the Tempo residue did not appear in the ^{1}H NMR spectra. The signal intensities of the peaks obtained for the triazole moiety were also affected. Because of the paramagnetic broadening effect of the free radical, the target MitoProbes **I**/**II** were further treated with sodium L-ascorbate to give the corresponding hydroxylamines (non-radical), which was further characterized by ^{1}H NMR. All the intermediates were characterized by NMR, and the target compounds were further confirmed by MALDI-TOF MS and high-resolution mass spectrometry. In addition, MitoProbes were analyzed by EPR spectroscopy to further confirm the presence of the intact nitroxide label (Fig. S1). MitoProbes **I**/**II** are stable and were stored at room temperature in sealed bottles and protected from light until use.

*Intracellular localization of MitoProbe **I**/**II***: Compared to non-neoplastic cells, oxidative stress is significantly enhanced in tumor cells. Multiple human tumor cell lines have been reported to produce large amounts of ROS.[1,6] ROS generation by tumor cells may enhance other properties of malignancy, including tissue invasion.[1,6] To analyze the exact intracellular localization of MitoProbe **I**/**II**, counter staining with a mitochondria-specific fluorescent marker MitoTracker (Life Technologies) was performed in living HeLa (human cervical cancer) cells. HeLa cells were treated with MitoProbe **I**/**II** for 45 min, followed by washing with DPBS buffer. HeLa cells were then stained with MitoTracker FM, and subjected to confocal fluorescence microscopy. A high proportion of overlay between MitoProbe **I**/**II** and MitoTracker was clearly visible, indicating a good co-localization (Fig. 2). The presence of MitoProbe **I**/**II** appeared punctately localized within mitochondria, likely due to the presence of the membrane-permeable and cationic rhodamine moiety, which reflects the enhanced negative membrane potential across the inner mitochondrial membrane.

Detection of mitochondrial oxidative stress in human retinal pigment epithelial (ARPE) cells under conditions of stress: The retina is particularly susceptible to oxidative stress because of its high consumption of oxygen, its high proportion of polyunsaturated fatty acids, and its exposure to visible light. Although the vision loss of AMD (age-related macular degeneration) results from photoreceptor damage in the central retina, early pathogenesis involves degeneration of retinal pigment epithelial cells.[19] We used ARPE-19 cells (human retinal pigment epithelial cells) to assess the capacity of MitoProbes to detect mitochondrial oxidative stress in a biological milieu. First, we investigated the effect of phorbol 12-myristate 13-acetate (PMA) stimulation in ARPE-19 cells, and its mitigation with MitoProbe **I**/**II**. Phorbol esters may be involved with redox-sensitive promoter regions. Upon stimulation with PMA, the cytosolic phox proteins translocate and associate with membrane components and resulting in activation of NADPH oxidase.[19] Initially, superoxide (O_2^-) is produced by activated NADPH oxidase. Subsequently, O_2^- is converted into other reactive oxygen species (ROS), such as H_2O_2, ·OH, $^1O_2$ and hypochlorous acid (HOCl). Treatment with 0.19 μM PMA resulted in a 7∼8 fold elevation of ROS levels (Fig. S1), comparable to the ROS increase that occurs in transient ischemia-related disease such as central retinal artery occlusion, angle-closure glaucoma, and carotid artery disease.[19] Furthermore, bright-filed transmission measurements following MitoProbe **I**/**II** incubation with PMA-treated cells verified that the cells were viable throughout the experiments. These results demonstrate that MitoProbe **I**/**II** can passively enter live cells and monitor free radicals generated in the mitochondria (Fig. 2).

Mitochondria undergo distinct morphological changes under oxidative stress: Changes in mitochondrial morphology serve to adapt the cells to an oxidative environment, and are critical for the survival of retinal and retinal pigment epithelial (RPE) cells because of their high oxygen demand and up-regulated metabolism.[19] The consequences of short-term ROS elevation on cell viability and mitochondrial number has not been extensively investigated. To begin to address this gap in our knowledge, we subsequently investigated the effect of oxidative stress on mitochondrial morphology in human retina pigment epithelial (ARPE) cells. Mitochondrial morphology was visualized with MitoProbes **I**/**II** and monitored by confocal fluorescent microscope. We observed that mitochondria undergo distinct morphological changes under conditions of oxidative stress. Following PMA treatment, the morphology of mitochondria in ARPE-19 cells was visualized by MitoProbe application, and could be classified into three categories: tubular (close to normal), intermediate (tubular with swollen regions) and fragmented (small and globular) (Fig. 3).

In the absence of PMA treatment, ARPE-19 cells exhibited tubular mitochondria and showed weak rhodamine fluorescence upon MitoProbe application. Following treatment with low concentrations of PMA (0.5 μM), a proportion of the cells demonstrated enhanced mitochondrial fluorescence, with MitoProbe **I**/**II** being confined to a dispersed network of tubular structures surrounding the nucleus (Figs. 4 and S2). The mitochondrial location of