Letter

A mitochondrial Cl--selective fluorescent probe for biological applications

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A mitochondrial Cl^--selective fluorescent probe for biological applications

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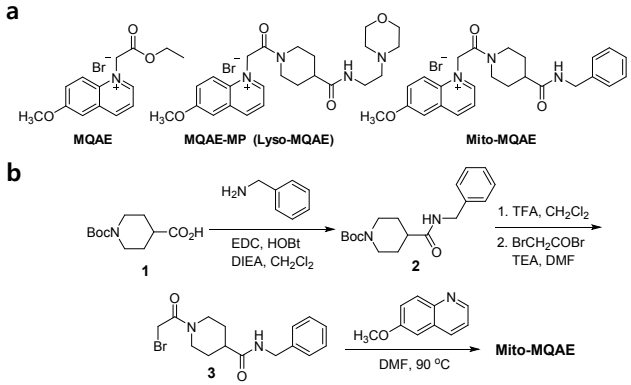
ABSTRACT: Herein we describe the development of the first mitochondrial Cl^--selective fluorescent probe, Mito-MQAE, and its applications in biological systems. Fluorescence of Mito-MQAE is insensitive to pH over the physiological pH range and is quenched by Cl^- with a Stern-Volmer quenching constant of 201 M^{-1} at pH 7.0. The results of cell studies using Mito-MQAE show that substances with the ability to disrupt mitochondrial membranes cause increases in the mitochondrial Cl^- concentration.

The mitochondrion is a double-membrane organelle that plays a major role in ATP production for the cellular energy supply and is also responsible for many other biological events including cell signaling, growth and death.[1] Mitochondrial membranes contain several cation and anion channels.[2,3] Among them, mitochondrial chloride channels are involved in regulation of the mitochondrial volume, maintenance of the mitochondria membrane potential and apoptosis.[4] The chloride transport activity of these channels is known to be dependent on the mitochondrial pH, with their activity increasing under acidic conditions and decreasing at pH > 7.0.[5]

Owing to important roles that chloride ions play in diverse mitochondria-associated biological processes, methods for determination of the levels of Cl^- in biological systems are of great importance. In particular, the ability to determine the effects of substances, which influence the function of mitochondria, on mitochondrial Cl^- concentrations is exceptionally valuable. However, studies aimed at this goal have not been performed because appropriate probes to monitor mitochondrial Cl^- have not been available. To remedy this deficiency, we conducted the investigation that was aimed at the development of the first mitochondrial Cl^--selective fluorescent probe, Mito-MQAE. The results of the study show that Mito-MQAE can be utilized to evaluate the effects of various substances on the levels of Cl^- in mitochondria.

MQAE (*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide, Figure 1a) is a widely used fluorescent probe for intracellular Cl^-.[6-10] Recently, using MQAE as a platform we designed the lysosomal Cl^--selective fluorescent probe MQAE-MP (or Lyso-MQAE, Figure 1a) and employed it to elucidate lysosome-associated biological events.[11] Because of the success of this earlier study, we utilized MQAE as a scaffold for the design of the new mitochondrial Cl^--selective fluorescent probe, Mito-MQAE.

It has been shown that lipophilic quinolinium cation moieties serve as a mitochondria-targeting motif as do triphenylphosphonium cation moieties.[12-15] However, we found that the quinolinium derivative MQAE detects cytosolic Cl^- without displaying selectivity for detection of this ion in mitochondria (*vide infra*).[11] We surmised that the mitochondria-directing property of the quinolinium cation in MQAE might be lost as a consequence of the formation of a zwitterion through rapid hydrolysis of the ester group to form carboxylate inside cells.[16] This possibility led us to propose that replacement of the ester group in MQAE by amide would promote hydrolytic stability. We inserted a rigid isonipecotic acid moiety as the tether between the quinolinium moiety and the lipophilic benzyl group into the probe to avoid the possible effect of the benzyl group on the fluorescence of a quinolinium group.[11] Thus, we expected that a stable amide-



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pH	3.0	4.0	5.0	6.0	7.0	8.0
Mito-MQAE (M^{-1})	201	202	206	204	201	203

Figure 1. (a) Chemical structures of fluorescent Cl^- probes. (b) Synthesis of Mito-MQAE. (c) Fluorescence spectra of Mito-MQAE in the absence of Cl^- at pH 3.0-8.0 (Ex = 350 nm). (d) Fluorescence spectra of Mito-MQAE in the presence of various concentrations of Cl^- at pH 7.0 (Ex = 350 nm). (e) Stern-Volmer constants for quenching Mito-MQAE by Cl^- at various pHs.

containing MQAE derivative would locate preferentially in mitochondria because of the presence of a lipophilic quinolinium cation moiety. To test this postulate, we prepared Mito-MQAE (Figure 1a) and evaluated its ability to monitor Cl^- in mitochondria of cells.

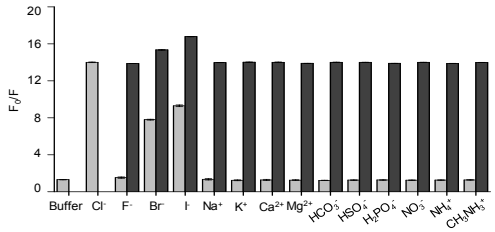

Figure 2. Fluorescence response of Mito-MQAE (100 μM) to indicated ions (50 mM) in buffer (Ex = 350 nm). Gray bars represent F_0/F ratios after addition of indicated ions to a solution of Mito-MQAE (mean ± s.d., n = 3). Dark bars represent F_0/F ratios after addition of Cl^- (50 mM) to a solution of Mito-MQAE in the presence of competing ions (F_0: fluorescence of buffer only; F: fluorescence of buffer containing Cl^-).

Mito-MQAE was synthesized by using the procedure shown in Figure 1b and analyzed by employing NMR and MS techniques (Figure S1-S5). The absorption spectrum of Mito-MQAE displayed two maxima at 320 nm and 350 nm (Figure S6), which are similar to those in the spectra of MQAE and Lyso-MQAE.[11] The results of studies of the fluorescence response of Mito-MQAE to pH and Cl^- showed that the emission intensity of the probe at 460 nm (excitation at 350 nm) gradually decreases as the Cl^- concentration increases and that it remains unchanged over a pH range of 3.0-8.0 (Figure 1c-1d and S7a). In addition, fluorescence of Mito-MQAE was quenched by Cl^- in the range of 0–250 mM with a Stern-Volmer constant (quenching sensitivity) of 201 M^{-1} at pH 7.0 (Figure 1e and S7b), a value that is close to those of MQAE (202 M^{-1}) and Lyso-MQAE (204 M^{-1}).[11] As anticipated based on the results of the previous study using MQAE and Lyso-MQAE,[11] cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+ and $CH_3NH_3^+$), F^- and non-halide anions (bicarbonate, sulfate, phosphate and nitrate) neither influenced the emission of Mito-MQAE nor did they interfere with its fluorescence response to Cl^- (Figure 2 and Figure S8).

Because its fluorescence is pH-insensitive and Cl^--sensitive in aqueous solutions, the use of Mito-MQAE to detect Cl^- in mitochondria of cells was evaluated. For this purpose, HeLa cells were exposed to non-cytotoxic Mito-MQAE (Figure S9), along with MQAE as a control, followed by treatment with MitoTracker red. Analysis of confocal fluorescence microscopy images of treated cells showed the presence of fluorescence signals emanating from Mito-MQAE, which overlap with those of MitoTracker (Pearson coefficient 0.92) (Figure 3). In contrast, fluorescence arising from MQAE was distributed throughout the entire cytosol. The time-dependent detection of mitochondrial Cl^- by Mito-MQAE was also examined by incubating HeLa cells with the probe during a 2 h time period. The intensity of fluorescence arising from Mito-MQAE in cells was found to increase rapidly and to approach a maximum after 30-40 min (Figure S10). Collectively, the findings indicate that Mito-MQAE is capable of selectively and efficiently monitoring mitochondrial Cl^-.

Mito-MQAE was then utilized to assess the effect of Cl^- deficiency on the level of mitochondrial Cl^-. In this study, HeLa cells cultured in Cl^--deficient or normal media were treated independently with Mito-MQAE, MQAE and Lyso-MQAE. Consistent with previous results,[11] cells cultured in Cl^--deficient media displayed larger fluorescence intensities arising from MQAE and Lyso-MQAE than those incubated in normal media (Figure 3c). The reason for this observation is that cytosolic and lysosomal Cl^- concentrations are reduced when cells are cultured in Cl^--deficient media.[11,17] However, culturing cells in Cl^--deficient media did not lead to changes in the mitochondrial Cl^- concentration. The findings suggest that although chloride ions exit from lysosomes to the cytosol and then to extracellular media under Cl^--deficient conditions,[18] they do not efficiently cross mitochondrial membranes.

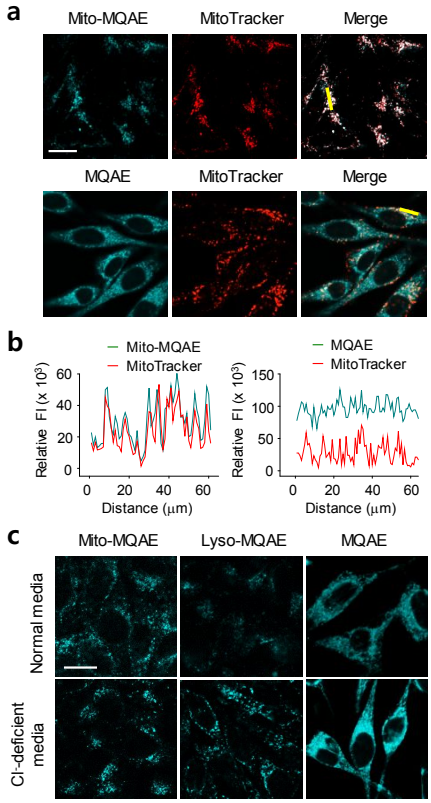

Figure 3. Detection of intracellular Cl^- using fluorescent probes. (a) HeLa cells were treated with (upper) Mito-MQAE or (lower) MQAE and then exposed to MitoTracker deep red. Cell images were obtained using confocal fluorescence microscopy (scale bar: 10 μm). (b) Relative fluorescence intensity (FI) of (left) Mito-MQAE and MitoTracker or (right) MQAE and MitoTracker at positions along the yellow line across cells in (a). Experiments were repeated three times, giving similar results. (c) HeLa cells were cultured in (upper) normal media and (lower) Cl^--deficient media for 6 h and then treated separately with Mito-MQAE, Lyso-MQAE and MQAE. Cell images were obtained by using confocal fluorescence microscopy (scale bar: 10 μm)

Changes in levels of mitochondrial Cl^- induced by substances that directly or indirectly affect the function of mitochondria were next evaluated. For this purpose, HeLa cells were first separately incubated in culture media for 8 h with FCCP (a potent inhibitor of mitochondrial oxidative phosphorylation),[19] apoptozole-triphenylphosphonium (Az-TPP, an inhibitor of mitochondrial Hsp70 (named mortalin)),[20]

Bam7 (an activator of a proapoptotic protein Bax),[21] apoptozole (Az, an inhibitor of lysosomal Hsp70),[20,22] methylamine (a lysosomotropic amine),[23] and bafilomycin A1 (BfA1, an inhibitor of the proton pump V-ATPase) (Figure 4).[24] Then, the cells were exposed to Mito-MQAE along with Lyso-MQAE.

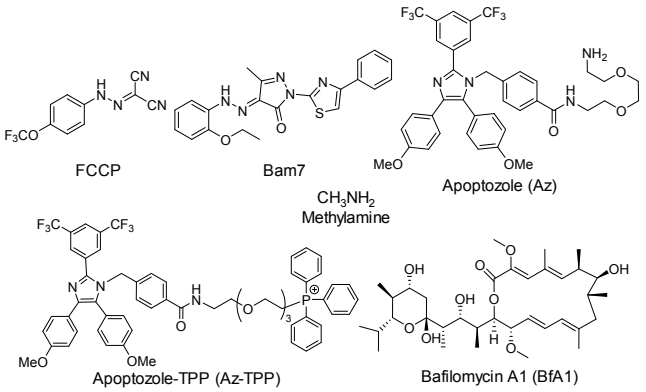

Figure 4. Chemical structures of substances used in this study.

FCCP, Az-TPP and Bam7 are known to disrupt the function of mitochondria by inducing mitochondrial outer membrane permeabilization (MOMP), thereby leading to apoptosis.[25] Analysis of confocal fluorescence microscopy images showed that cells treated with these substances display increased mitochondrial Cl^- concentrations (Figure 5). The findings serve as evidence to support the notion that because FCCP, Az-TPP and Bam7 disrupt mitochondrial membranes, they enhance the movement of Cl^- from the cytosol where its concentration is > 20 mM to mitochondria where the Cl^- concentration is < 20 mM.[26] However, these substances did not affect lysosomal Cl^- concentrations.

Az is known to induce lysosomal membrane permeabilization (LMP) by inhibiting lysosomal Hsp70, which subsequently promotes MOMP for apoptosis induction.[20] Methylamine induces lysosomal osmotic stress, leading to induction of LMP and subsequently MOMP.[23] Cell images analyses revealed that treatment of either Az or methylamine results in a decrease in the lysosomal Cl^- concentration and an increase in levels of mitochondrial Cl^- (Figure 5). The findings suggest that these effects are a consequence of the ability of these substances to induce LMP and subsequently MOMP.

Finally, we explored the mitochondrial Cl^- concentration effect of BfA1, a substance that causes an increase in the lysosomal pH concomitant with a decrease in the lysosomal Cl^- concentration to maintain electroneutrality.[11] The results showed that, as expected, BfA1 decreases the lysosomal Cl^- concentration,[11] but it does not influence the level of mitochondrial Cl^- presumably because it does not induce MOMP (Figure 5).[24] Taken together, the observations demonstrate that substances inducing MOMP directly or indirectly cause increases in mitochondrial Cl^- concentrations.

In conclusion, in the effort described above we developed the first mitochondrial Cl^--selective fluorescent probe Mito-MQAE. The fluorescence arising from this probe is quenched by Cl^- with a Stern-Volmer constant of 201 M^{-1} at pH 7.0 but is not affected by other biologically relevant ions. Because Mito-MQAE responded efficiently to mitochondrial Cl^-, it was utilized to determine the effects of several substances on the levels of mitochondrial Cl^-. The results show that substances with the ability to induce MOMP increase mitochondrial Cl^- concentrations (Table S1), indicating that the mitochondrial Cl^- concentration is lower than that in the cytosol as has been shown in previous studies using isolated mitochondria.[26] The present study demonstrates that Mito-MQAE has a great potential for use as a mitochondrial Cl^--selective fluorescent probe for studies of biological processes associated with mitochondria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthetic procedures of Mito-MQAE, cell study, Table S1, summary of substances; Figure S1-S4, NMR spectra; Figure S5, HPLC profile of Mito-MQAE; Figure S6, absorption spectra of Mito-MQAE; Figure S7, fluorescence emission of Mito-MQAE; Figure S8, visual emission of Mito-MQAE;

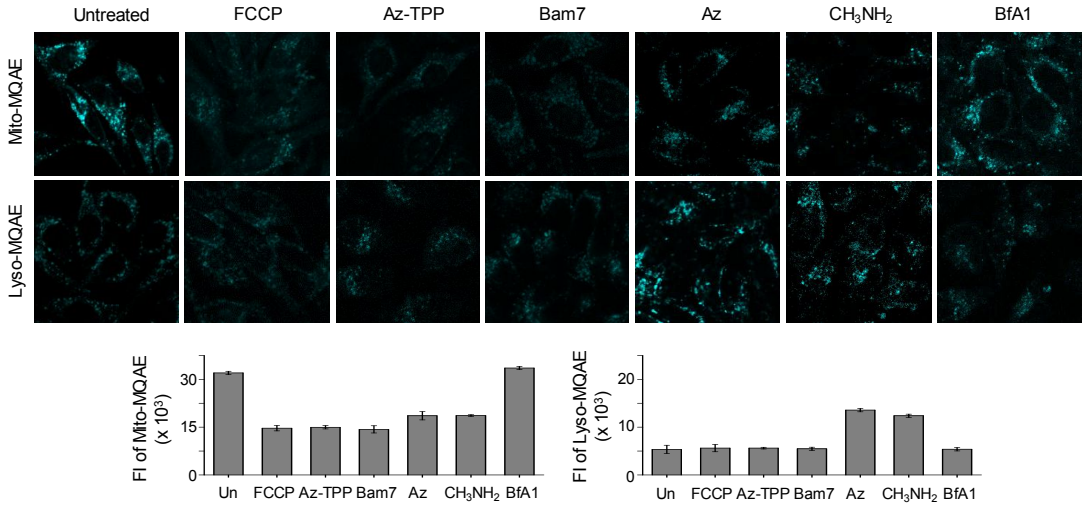

Figure 5. HeLa cells were separately incubated for 8 h with 20 μM FCCP, 1 μM Az-TPP, 20 μM Bam7, 6 μM Az, 30 mM CH_3NH_2 and 5 nM BfA1, followed by treatment with (upper) Mito-MQAE or (lower) Lyso-MQAE. Cell images were obtained using confocal fluorescence microscopy. Graphs show fluorescence intensity of (left) Mito-MQAE and (right) Lyso-MQAE in cells treated with each substance.

Figure S9, cytotoxicity of Mito-MQAE; Figure S10, time-dependent detection of Mito-MQAE (PDF).

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Notes

The authors declare no competing financial interest.

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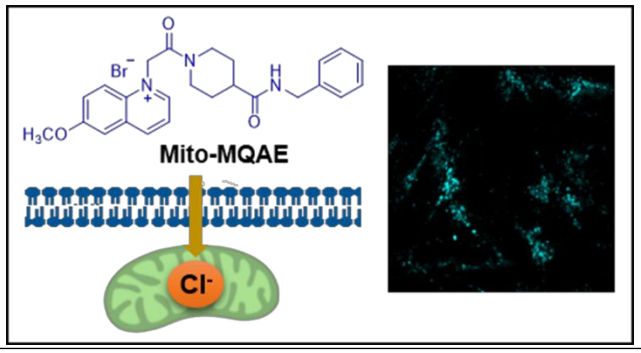

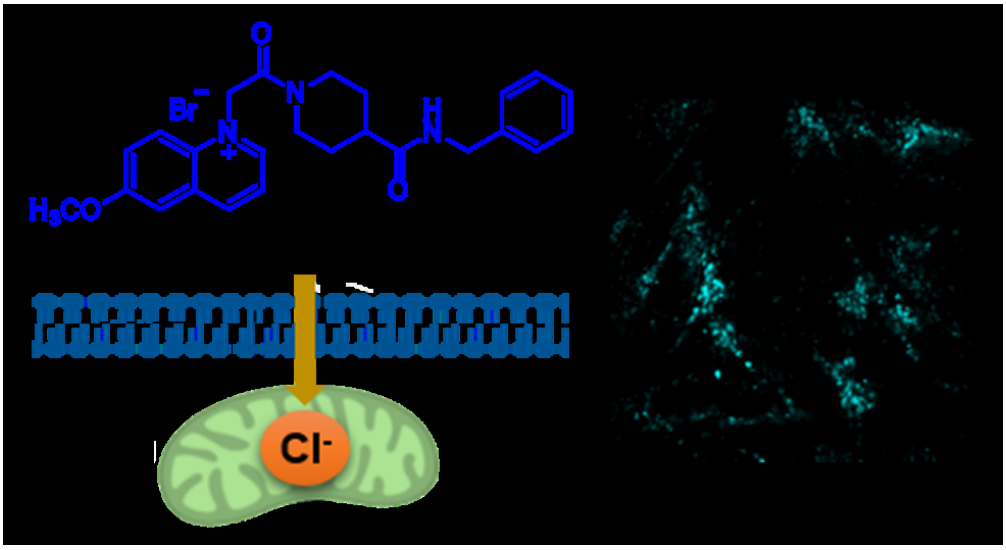

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