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MitoBlue: A Nontoxic and Photostable Blue-Emitting Dye That Selectively Labels Functional Mitochondria

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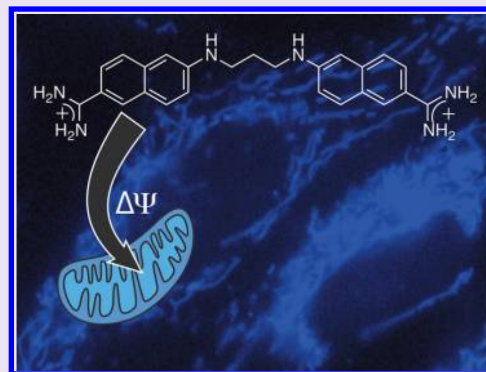
Supporting Information

ABSTRACT: We report the discovery of a fluorogenic dye, N^1,N^3-di(2-aminidonaphthalen-6-yl) propane-1,3-diamine, MitoBlue, which selectively stains functional mitochondria while displaying low toxicity, bright blue emission, and high resistance to photobleaching. Additionally, we show that a biotin-labeled MitoBlue derivative can be used as a handle for the delivery of streptavidin-tagged species to the mitochondria.



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Fluorescence imaging techniques, allowing the visualization of molecular events with extraordinary sensitivity and selectivity, have become a fundamental tool in the study of living cells.[1−3] The success of these techniques derives in great part from the development of cell-permeable probes that associate with particular cellular structures.[4,5] Among them, mitochondria are key organelles that integrate many essential functions in eukaryotic cells.[6,7] As expected for such a metabolic focal point, mitochondria have been linked with a number of diseases and have become a major therapeutic target.[8,9] Consequently, developing new fluorescent probes that can shed light on mitochondrial biology is a major goal in both basic and applied research. Currently used mitochondrial stains are only available in a few emission wavelengths in the green and red channels and suffer from important shortcomings, such as relatively high toxicity and poor (photo)chemical stability.[10] We report a new mitochondrial dye, MitoBlue, which, in addition to being readily accessible, overcomes many of these drawbacks, selectively staining functional mitochondria with both low toxicity and high photostability. Furthermore, MitoBlue displays bright blue emission at 490 nm upon excitation at 329 nm, thus providing a complementary emission channel to commercial Mitotracker dyes.[11,12] Moreover, to our knowledge only two blue-emissinve mitochondrial dyes have been described: one relying on the use of well-established triphenylphophonium targeting groups,[13,14] and the other, a coumarine derivative, which displays relatively poor mitochondrial specificity.[14]

MitoBlue was initially designed as a fluorogenic DNA binding agent. We observed that DNA-binding *aza*-bisbenzamidines display enhanced fluorescence emission upon insertion into the minor groove of A/T-rich DNA sequences,[15−19] but despite its good solvatochromic properties, the aminobenzamidine fluorophore displays relatively short excitation and emission wavelengths that are not ideal for cellular imaging applications. Therefore, we decided to explore *aza*-propamidine analogues with extended aromatic units, such as the bisnaphthalene derivative 6-({3-[(6-carba mimidoylnaphthalen-2-yl)amino]propyl}amino)naphthalene-2-carboximidamide (**1**), as potential fluorogenic DNA probes with improved spectroscopic properties.[20]

The target bisamidine **1** was readily synthesized following the procedure outlined in Figure 1. Methyl 6-bromo-2-naphthoate (**2**) was hydrolyzed, and the resulting acid transformed into the corresponding 6-bromo-2-naphthamide (**3**). Dehydration of **3**,[21] followed by Hartwig−Buchwald amination[22] and conversion of the nitriles to amidines,[19,23] yielded the desired product (**1**), which was purified by reverse-phase chromatography and isolated as a TFA salt with an overall yield for the whole synthetic sequence of ∼14%. The control monoamidines **5** and **6** were synthesized following similar procedures from the intermediate **4** (Figure 1).

Compound **6** was used to characterize the photophysical properties of the 6-amino-2-naphthimidamide fluorophore. As expected for a push−pull conjugated system,[24] this fluorophore is environment-sensitive, displaying a relatively weak emission in aqueous media ($\Phi[H_2O]$ = 0.49), but increasing its quantum yield in more hydrophobic solvents (Φ[iPrOH] = 0.78;

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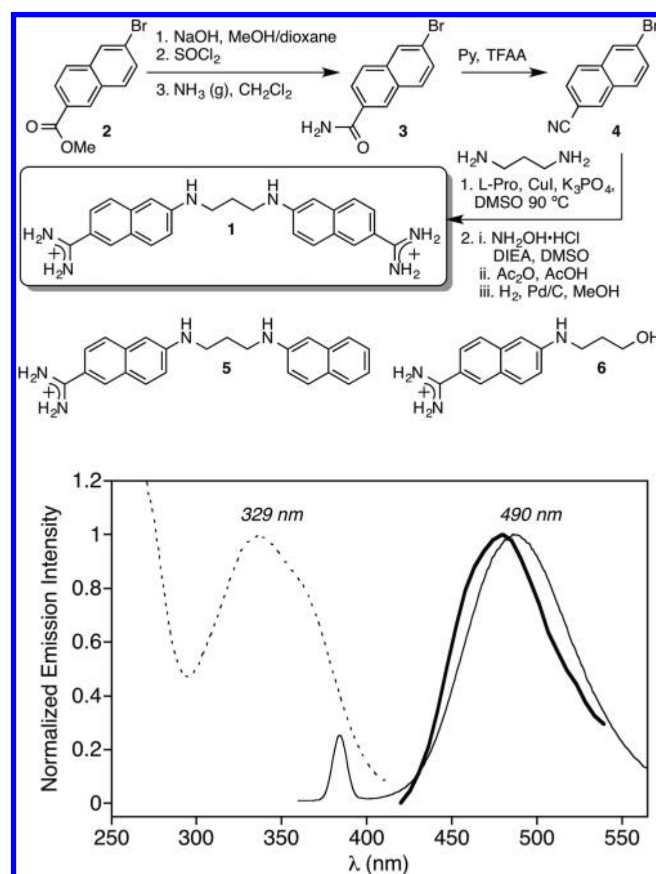

Figure 1. Top: Synthesis of amino-naphthimidamides. Bottom: Normalized excitation (dashed line) and emission spectra (thin solid line) of 6·TFA in 20 mM TrisHCl, pH 7.5, and 100 mM NaCl, and lambda scan (thick solid line) of chicken embryo fibroblasts (CEF) incubated with 5 μM bisamidine **1** for 20 min.

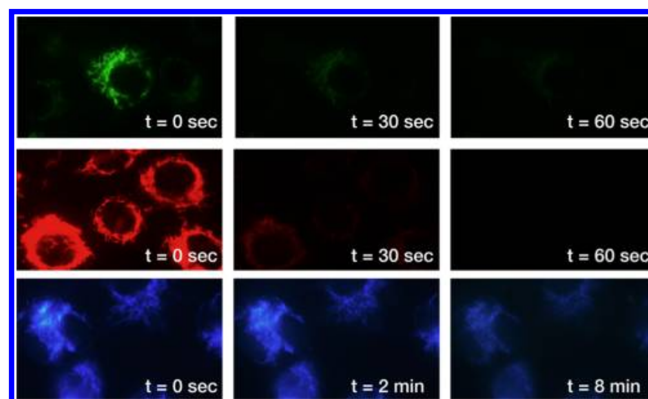

Figure 3. Vero cells incubated with rhodamine 123 (top row), Mitotracker Red (middle row), and MitoBlue (bottom row). Images were taken after the indicated irradiation times. All micrographs were taken using the same settings for comparison.

Φ[EtOH] = 0.67) and particularly in nonprotic media (Φ[dioxane] = 0.94; Φ[THF] = 0.92). Application of the Lippert–Mataga model allowed us to calculate the difference between the dipole moments in the ground and the excited states as a measure of the sensitivity to the polarity.[25−28] The resulting value of $\Delta\mu$ = 7.8 D is of the same order as those measured for most environment-sensitive fluorophores, such as NBD ($\Delta\mu$ = 3.6)[29] or 6-DMN ($\Delta\mu$ = 5.5),[30] and accounts for the significant sensitivity to the solvent polarity. Furthermore, as expected for this type of cationic bisamidines,[15,31] compound **1** behaved *in vitro* as a DNA minor groove binder, exhibiting good affinity for extended A/T-rich sites (Supporting Information).

Following the photophysical and *in vitro* characterization of the fluorophore, we next studied the behavior of **1** in cellular settings. Therefore, cells from a primary culture of chicken embryo fibroblasts (CEF) were incubated with 5 μM of bisamidine **1** for 20 min. The cells were then washed and directly observed under the microscope without fixation. Surprisingly, despite its significant *in vitro* DNA binding affinity, the intracellular emission was not concentrated in the cell nuclei, but showed instead a cytosolic filamentous pattern, consistent with mitochondrial localization of the dye (Figure 2, top row, left panel). Indeed, counter-stained cells with the mitochondrial marker Mitotracker Red (Figure 2, top row, middle) confirm that both dyes display superimposable distributions (Figure 2, top right panel), thus demonstrating

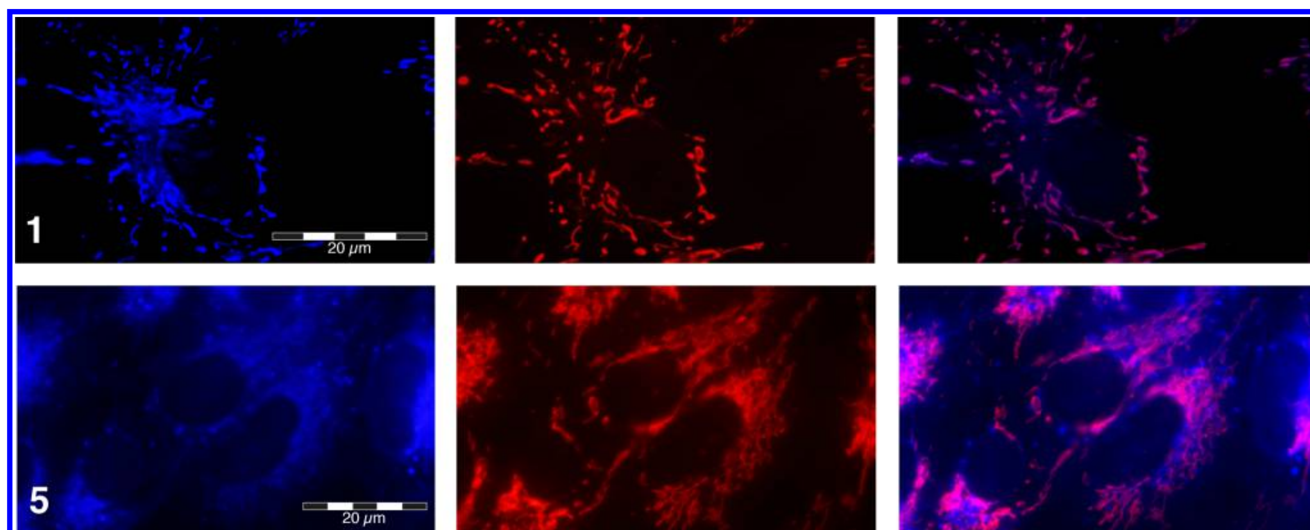

Figure 2. Double staining of living CEF cells with Mitotracker Red. Top row images correspond to MitoBlue (**1**), and bottom row to mononaphtylamidine **5**. The Mitotracker images and the corresponding overlays are in the center and right columns, respectively.

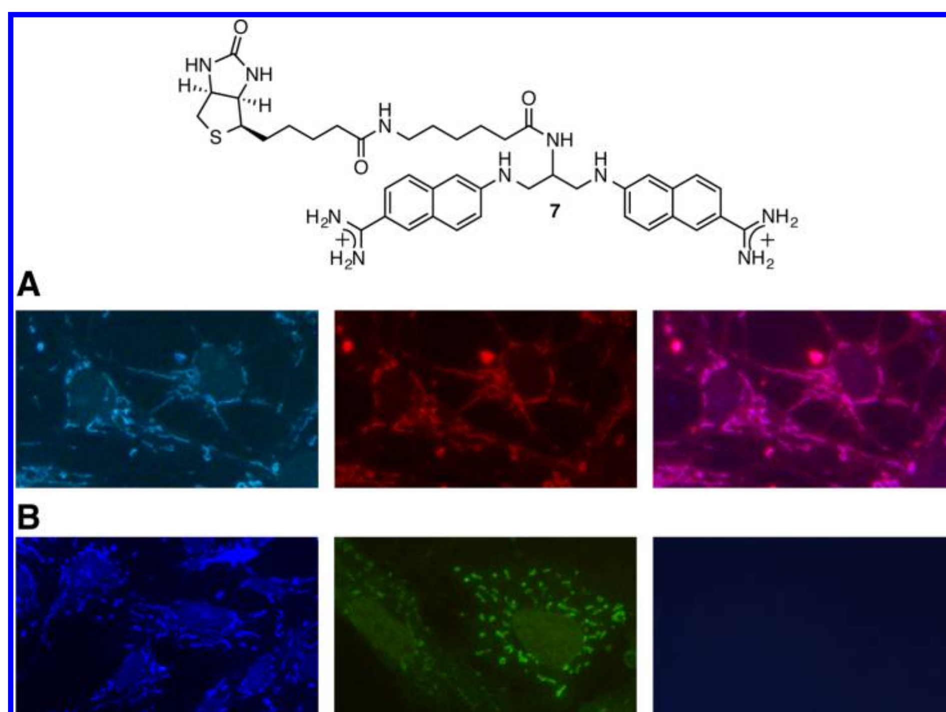

Figure 4. Biotinylated MitoBlue (bt-MitoBlue) is synthesized from intermediate **4** in five operational steps (see Supporting Information). Mitochondrial capture with bt-MitoBlue. (A) CEF cells treated with MitoBlue and bt-MitoBlue using stvAF594 as secondary label. Left image, blue channel; center, red channel; right, superposition of both images. (B) CEF cells treated with bt-MitoBlue. Left image, stvAF405 as secondary label (blue channel); center, Qdot565 as secondary label (green channel); right, no secondary marker (blue channel).

that **1**, henceforward MitoBlue, represents both a new mitochondrial-targeting structural motif and a selective mitochondrial dye. Quantitative analysis of the colocalization of both dyes consistently resulted in Pearson's Correlation Coefficients in the order of 0.9.[32,33] To confirm that MitoBlue can be used as a general marker for mitochondria, we carried out the same double-staining experiment with Vero, BHK, DF1, and HeLa cell lines, and in all cases, the staining is coincident with that of Mitotracker Red (Supporting Information).[34] In contrast with MitoBlue, the control monoamidine **5** shows less selectivity in its mitochondrial staining and significant background emission in the cytoplasm (Figure 2, bottom row); the more hydrophilic monoamidine **6** does not show any intracellular staining, demonstrating that the dicationic nature of MitoBlue is relevant for its selectivity. Cytotoxicity was evaluated with trypan blue,[35] and no significant differences are observed between the cell culture stained with MitoBlue and control cells (see Supporting Information). Incidentally, unlike many other mitochondrial stains, MitoBlue is well retained after fixation with 4% paraformaldehyde (PFA).[36]

The cellular distribution of MitoBlue is consistent with quantitative structure−activity relationship models that point to mitochondrial targeting for compounds with $pK_a > 10$, charge $(Z) > 0$, largest conjugate fragment (LCF) < 17, conjugated bond number (CBN) < 40, and $2 > \text{Log } P_{\text{cation}} > 0$.[37−39] MitoBlue, with $pK_a \approx 11.3$, $Z = 2$, LCF = 15, CBN = 30, and $\text{Log } P_{\text{cation}} \approx -2.9$,[40] meets all these criteria except the Log P value, which is included in those models as a measure of efficient membrane translocation. However, despite their high polarity, amidinium derivatives exhibit excellent transport properties. Additionally, the distribution of MitoBlue is comparable to that of other delocalized lipophilic cations (DLCs),[41,42] such as triphenylphosphonium derivatives,[43] rhodamine 123,[44] or even certain bisbenzamidines.[45]

As expected for an electrostatic trapping mechanism, no staining is observed with MitoBlue if the mitochondria are previously depolarized with CCCP (carbonyl cyanide 3-chlorophenyl hydrazone).[46,47] In that case, only dead cells seem to efficiently uptake the dye, which is then mainly located in nuclei. Furthermore, the addition of CCCP after the cells have been labeled with MitoBlue leads to dye leakage and complete loss of fluorescence after 30 min (Supporting Information).[48] Interestingly, a lambda scan performed with a confocal microscopy on MitoBlue-labeled CEF cells is consistent with the MitoBlue dye partially inserted in a hydrophobic environment, as evidenced by the slight displacement of the maximum emission to shorter wavelengths (see Figure 1, bottom). Taken together, these data suggest that MitoBlue is electrostatically driven to the mitochondria by the large electrochemical potential generated by the electron transport chain,[49] and most probably accumulates in the mitochondrial membrane, where it displays enhanced emission due to the surrounding hydrophobic environment.

We next compared the photostability of MitoBlue against standard mitochondrial dyes. Vero cells were incubated with 5 μM MitoBlue, 2 μM rhodamine 123, or 0.5 μM Mitotracker Red; each sample was then continuously irradiated with the fluorescence microscope through the 100× objective, and images were collected after specific times. The green rhodamine 123 displays very poor photostability, being almost completely photolyzed after 30 s of irradiation (Figure 3, top row). Mitotraker Red shows more resistance to fading, but photobleaching is complete before 60 s under the light source (Figure 3, center row). In contrast with these, MitoBlue shows almost no degradation in its emission after 2 min under the same conditions and is still clearly visible even after 8 min of steady irradiation (Figure 3, bottom row).

Another advantage of MitoBlue derives from its straightforward and versatile synthesis, which can be easily tailored for introducing new functionalities into its structure. In this context, we decided to synthesize the biotinylated MitoBlue derivative (7, bt-MitoBlue, Figure 4) as a potential affinity tag for capturing streptavidin conjugates in the mitochondria.[50]

In order to assess the viability of this strategy, we incubated CEF cells for 2 h with 20 μM bt-MitoBlue, allowed the cells to stand for 5 h in DMEM medium for its effective intracellular redistribution, and then treated them with 10 μM MitoBlue for 20 min as a control for mitochondrial localization.[51] After washing with PBS buffer, fixation with 4% PFA, and permeabilization with 0.5% Triton X100 for 3 min, cells were blocked with 2% BSA and incubated with the streptavidin-Alexa Fluor 594 conjugate (stvAF594) for 1.5 h.[52] As expected, observation of the blue channel, corresponding to the MitoBlue emission, shows the typical mitochondrial network pattern (Figure 4A, left); more importantly, the distribution of the stvAF594 conjugate is also consistent with mitochondrial targeting (red channel, Figure 4A, center) and fully coincident with that of MitoBlue (merge, Figure 4A, right). This result can be reproduced with other streptavidin conjugates, such as with Alexa Fluor 405 (stvAF405) or the quantum dot conjugate Qdot565, both of which also localize in the mitochondria in the presence of bt-MitoBlue (Figure 4B, left and center, respectively). Importantly, control experiments demonstrated that stvAF405 does not concentrate in the mitochondria in the absence of bt-MitoBlue, but instead displays poor labeling and weak emission, barely visible at the same exposure settings in the microscope (Supporting Information). Also, as expected, no emission is observed with bt-MitoBlue if the secondary labeling with the fluorescent streptavidin conjugates is omitted (Figure 4B, right). Taken together these results support the use of bt-MitoBlue as an effective and versatile mitochondrial affinity tag.

In summary, MitoBlue is a nontoxic, blue-emitting dye that stains functional mitochondria with high selectivity, retaining its localization and emissive properties after cell fixation with PFA. MitoBlue displays high resistance to photobleaching, making it particularly appropriate for experiments requiring long or multiple exposures. This set of advantages makes MitoBlue quite unique among most mitochondrial dyes. In addition to that, MitoBlue represents a new mitochondrial targeting system that can be readily derivatized for mitochondrial delivery of chemical cargoes and, in the form of a biotin conjugate, can be used as an organelle affinity tag, selectively driving streptavidin conjugates into the mitochondria in fixated cell cultures.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and characterization, fluorescence spectroscopy, DNA binding studies, and in vitro cellular experiments, including costaining experiments, toxicity assays, and controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.I.S. and J.M.-C. carried out the experiments; J.M.-C. also designed and analyzed the biological experiments and advised on cell biology; J.M.C., J.L.M., and M.E.V. supervised the work and prepared the manuscript.

Notes

The authors declare the following competing financial interest(s): A patent application describing the synthesis and applications of MitoBlue as mitochondrial stain has been filed.

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