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Control of Muscle Differentiation by a Mitochondria-Targeted Fluorophore

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Abstract: During muscle differentiation, mitochondria undergo dramatic changes in their morphology and distribution to prepare for the higher rate of energy consumption. By applying a mitochondria-targeted rosamine library in C2C12 myogenesis, we discovered one compound that controls muscle differentiation. When treated to undifferentiated myoblasts, our selected compound, **B25**, inhibited myotube formation, and when treated to fully differentiated myotubes, it induced fission of multinucleated myotubes into mononucleated fragments. Compared to myoseverin, which is known for inducing myotube fission by destabilizing microtubules, **B25** affects neither microtubule stability nor cell cycle. Further investigation identified that **B25** induces myotube fission through the activation of NF-κB, which is one of the important signaling pathways linked to skeletal muscle differentiation. So far, the use of small-molecule fluorophores is limited in the discovery of labeling agents or sensors. In addition to their potential as a sensor, here we show the application of fluorescent small molecules in the discovery of a bioactive probe that induces a specific cellular response.

Introduction

Mitochondrial functions are closely related with various cellular processes including apoptosis, aging, and differentiation.[1] Accordingly, mitochondrial dysfunctions are responsible for a wide range of human diseases such as diabetes, cancers, and neurodegenerative diseases. A small-molecule library that targets mitochondria and manipulates mitochondrial function will be a valuable tool to investigate mitochondrial functions and diseases. For that purpose, we have developed a diversity-oriented rosamine library, which contains derivatives of rhodamine.[2] Rhodamine dyes have long been used as mitochondrial probes. Their aromatic and cationic properties direct them to mitochondria due to mitochondrial membrane potential.[3] By removing the 2′-carboxylic acid, which plays a key role in the structural rigidity of rhodamine, our rosamine library compounds are endowed with structural flexibility and the monovalent cationic property required for mitochondrial localization (Figure 1). When applied to live cells, a majority of compounds in the library showed selective localization to mitochondria, as we envisioned (Table S1, Supporting Information). In comparison, neutral BODIPY[4] or NBD[5] library compounds are mainly localized in intracellular vesicles,[5] and another cationic styryl library showed about 59% mitochondrial staining.[6] Rosamine library compounds were more efficient in mitochondrial localization, with 80% co-localization with MitoTracker (Pearson's coefficient $R > 0.7$).

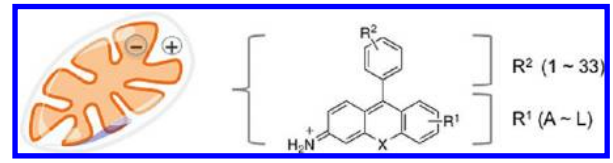

Figure 1. Rosamine library targeting mitochondria.

Skeletal muscle differentiation is an ideal system to investigate mitochondrial function. Mitochondria undergo dramatic changes during myogenesis to prepare for the higher rate of energy consumption needed for muscle contraction.[7] A trademark of skeletal muscle cell differentiation is the fusion of

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mononucleated myoblasts into multinucleated myotubes. Recently we have shown the possible use of the rosamine library in distinguishing the distinct states of muscle differentiation.[8] In addition to its potential as a sensor, here we show the use of the rosamine library for the discovery of bioactive probes that modulate muscle differentiation.

Experimental Procedure

Myogenic Cell Culture and Differentiation. The C2C12 myoblast cell line was obtained from American Type Culture Collection (Rockville, MD). Undifferentiated myoblasts were grown in Dulbecco's modified eagle medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (FBS). After 2 days, myoblasts were stimulated to differentiate by replacing the medium with DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 2% heat-inactivated horse serum. Differentiation was allowed to continue for 5 days to obtain differentiated myotubes, with media replacement every 2 days.

PC12 Culture and Neuronal Differentiation. The PC12 cell line was obtained from American Type Culture Collection. PC12 cells were grown in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 15% FBS. For neuronal differentiation, PC12 cells were plated on 96-well plates in growth medium. After overnight incubation, growth medium was replaced with differentiation medium [DMEM supplied with penicillin (100 U/mL), streptomycin (100 μg/mL), 5% FBS, 10% heat inactivated horse serum, and 100 ng/mL of nerve growth factor]. On the third day, half of the medium was discarded and replaced with fresh medium.

Screening of Rosamine Library in Myogenic Differentiation. Myoblasts grown in 96-well plates were incubated with library compounds at 500 nM for 5 days in differentiation medium. On the third day, half of the medium was discarded and replaced with fresh medium. On the fifth day, fluorescence images were taken in three optical channels using a Leica 2000 fluorescence microscope (λ_{ex} = 540/25 nm and λ_{em} = 605/55 nm; λ_{ex} = 480/40 nm and λ_{em} = 527/30 nm; λ_{ex} = 420−490 nm, $\lambda_{em} \geq$ 515 nm).

Compound Treatment and Fluorescence Microscopy. For live-cell imaging, myoblasts and myotubes grown in a black optical 96-well plate were incubated with 500 nM of **A25** or **B25**, or 20 μM of myoseverin. Fluorescence images of active compounds were taken by using a Leica 2000 fluorescence microscope (λ_{ex} = 540/25 nm and λ_{em} = 605/55 nm). MitoTracker Deep Red 633 or MitoTracker Red CMXRos (Invitrogen) was added to cells according to the manufacturer's instructions.

Immunostaining. For myosin heavy-chain staining, C2C12 myotubes differentiated in a 96-well plate were incubated with each compound for 48 h in growth medium. Cells were fixed with 3.8% formaldehyde, labeled with anti-myosin antibody (Sigma), and then detected by Cy5-conjugated secondary antibody. For cytoskeleton staining, C2C12 myoblasts grown in a 96-well plate were incubated with each compound for 18 h and fixed with 3.8% formaldehyde. For microtubule staining, anti-microtubule antibody (Gene Tex. Inc.) was used, and then the cells were detected by Cy5-conjugated secondary antibody. For actin staining, palloidin-fluorescein isothiocyanate (Invitrogen) was used.

NF-κB Luciferase Assay. C2C12 myoblasts were transfected with lentiviral NF-κB reporter (SABiosciences) and selected with puromycin (2 μg/mL) for two passages. pNF-kB-Luc myoblasts were plated in 12-well plates and differentiated for 5 days. Cells were then incubated with each compound for 24 h in growth medium: **B25** (500 nM), curcumin (20 μM), myoseverin (20 μM), and tumor necrosis factor (TNF; 2 ng/mL). Luciferase activity was normalized by total protein concentration.

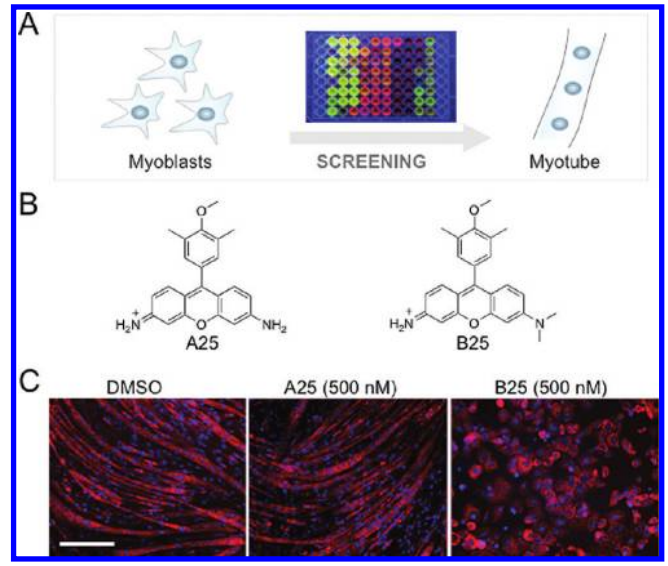

Figure 2. **B25** inhibits skeletal muscle differentiation. (A) Screening of rosamine library. Myoblasts were incubated with 500 nM concentrations of library compounds for 5 days under differentiation conditions. (B) Chemical structures of a hit compound, **B25**, and a control, **A25**. (C) Fluorescence images of myoblasts differentiated with **A25** or **B25** for 5 days (red, λ_{ex} = 540/25 nm, λ_{em} = 605/55 nm). DMSO-treated cells were stained with MitoTracker Red immediately before imaging. Nuclei are stained with Hoechst (blue). Scale bar = 100 μm.

Characterization of B25. B25 was synthesized as described in previous literature.[2] **B25**: ^{1}H NMR (MeOH-d_4) δ 7.34 (dd, J = 9.64, 9.06 Hz, 2H), 7.03 (m, 2H), 7.00 (d, J = 2.05 Hz, 1H), 6.88 (d, J = 2.05 Hz, 1H), 6.77 (m, 2H), 3.78 (s, 3H), 2.32 (s, 6H), 1.21 (s, 6H); ^{13}C NMR (MeOH-d_4) δ 133.80, 133.06, 131.29, 125.02, 117.84, 116.22, 115.34, 114.96, 98.53, 97.48, 80.69, 60.36, 51.25, 40.86, 30.73, 20.88, 16.26 ppm; MS (ESI) m/z 373 (M + 1).

Results and Discussion

Phenotypic Screening in C2C12 Myotube Differentiation. Initially the rosamine library was screened for molecules that inhibit muscle cell differentiation. Murine C2C12 myoblasts were incubated with rosamine library compounds for 5 days under differentiation conditions (Figure 2A). One member of the library, **B25**, blocked myotube formation, keeping cells undifferentiated (Figure 2B). Fluorescence microscopic images showed that **B25**-treated myoblasts remained undifferentiated mononucleated cells while cells treated with a structurally similar, but inactive compound, **A25**, formed long and cylindrical multinucleated myotubes as in the dimethylsulfoxide (DMSO) control (Figure 2C). High-resolution fluorescence microscopic images showed that **B25** remained localized in mitochondria even after 5 days of incubation (Figure S2, Supporting Information).

Myotube Fission. When applied to fully differentiated myotubes, **B25** induced fission of myofibers (Figure 3B). While **A25**-treated myotubes kept their long and straightened structure, **B25**-treated myotubes fragmented into mononucleated cells (Figure 3B). Myotube culture is actually a mixture of myotubes and undifferentiated myoblasts. To ensure that the mononucleated cells originated from myotubes, not from myoblasts, compound-treated myotube cultures were fixed and stained with myosin antibody. Myosin is a muscle differentiation marker and selectively stains differentiated myotubes, as seen in **A25**-treated

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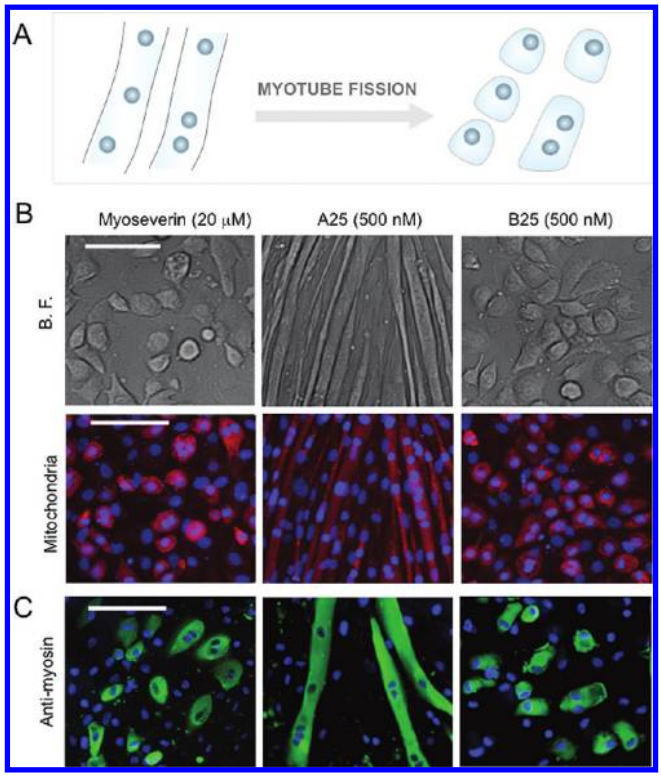

Figure 3. B25 induces myotube fission. (A) To induce myotube fission, fully differentiated myotube cultures were incubated with compounds for 48 h. (B) Phase contrast images and fluorescence images of the selected compound (red). Myoseverin-treated culture was stained with MitoTracker Red before microscopic observation. (C) Compound-treated myotube cultures were fixed and stained with myosin antibody. Scale bar = 100 μm.

myotube culture (Figure 3C). In contrast, myosin-positive mononucleates were observed in **B25**-treated myotube cultures. The number of myosin-positive mononucleates, expressed as a percentage over total number of myosin positive nuclei, was used as an index of myotube fragmentation (**B25**: 39.8 ± 2.0%, 750 myosin-positive mononucleates; $n = 2$). As a positive control, we treated myoseverin,[9] which is known to induce myotube fission by disrupting microtubules (myoseverin: 31.6 ± 1.8%, 590 myosin-positive mononucleates; $n = 2$). For comparison, the percentage of myosin-positive mononucleates from DMSO-treated control is around 1% of the total myosin positive nuclei (DMSO: 1.1 ± 0.4%, 46 myosin-positive mononucleates; $n = 2$). This result clearly indicates that **B25** induces the fragmentation of multinucleated myotubes to mononucleates efficiently even at sub-micromolar concentration (Figure S2). In both cases, compound-induced mononucleated cells were still expressing high levels of a differentiation marker, which implied that the fragmented cells did not reverse back to myoblasts as reported.[10] In fact, when we observed the fragmented cells with a time-lapse microscope, they did not enter the cell cycle even after 70 h (Figure S3, Supporting Information).

Comparison with Myoseverin. Myoseverin has been known to induce myotube fission by destabilizing microtubules.[10] To investigate the mechanism of **B25** action compared with

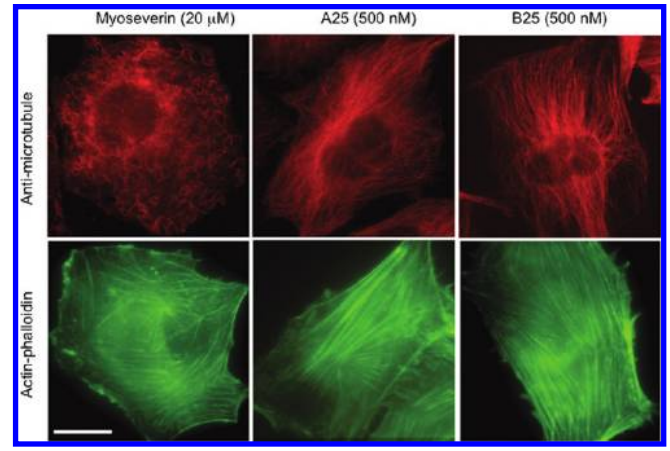

Figure 4. Immunostaining of microtubules. Myoblasts were incubated with each compound for 18 h and fixed for microtubule or actin staining. While a myoseverin-treated cell shows randomly oriented microtubule filaments, a **B25**-treated cell shows parallel bundles of microtubules aligned with the long axis of a cell. In both cases, actin microfilaments were not affected. Scale bar = 20 μm.

myoseverin, C2C12 myoblasts were incubated with each compound and stained with anti-microtubule antibody. Myoseverin-treated myoblasts showed randomly oriented microtubule filaments as reported, but **B25** did not affect microtubule network (Figure 4). **B25**-treated cells showed parallel bundles of microtubules aligned with the long axis of a cell, as shown in **A25**-treated control cells. Additionally, **B25** did not inhibit polymerization of purified tubulin *in vitro*, up to 10 μM treatment (data not shown). Microtubule dissociation is directly linked to cell cycle arrest. Accordingly, when applied to cell cycle analysis, myoseverin-treated C2C12 myoblasts were strongly arrested in G2/M transition. In addition, when PC12 cells were treated under differentiation conditions, myoseverin inhibited neuronal differentiation (Figure 5). As such, myoseverin-induced myotube fission might result in the dissociation of microtubules in general, regardless of cell types or cell states. In contrast, **B25** affects neither cell proliferation nor neuronal differentiation. These results strongly suggest that **B25** would have a distinct mechanism of myotube fission, different from that of microtubule-disrupting drugs such as myoseverin. Further investigation identified that **B25** activates the NF-κB pathway.

Activation of NF-κB Pathway. NF-κB is one of the important signaling pathways linked to skeletal muscle differentiation.[11] A number of studies have shown that NF-κB activation by TNF blocks muscle differentiation in primary human myoblasts or murine C2C12 myoblasts.[12] To investigate the involvement of NF-κB activation in **B25**-mediated myotube fission, NF-κB reporter assay was conducted. C2C12 myoblasts were trans-

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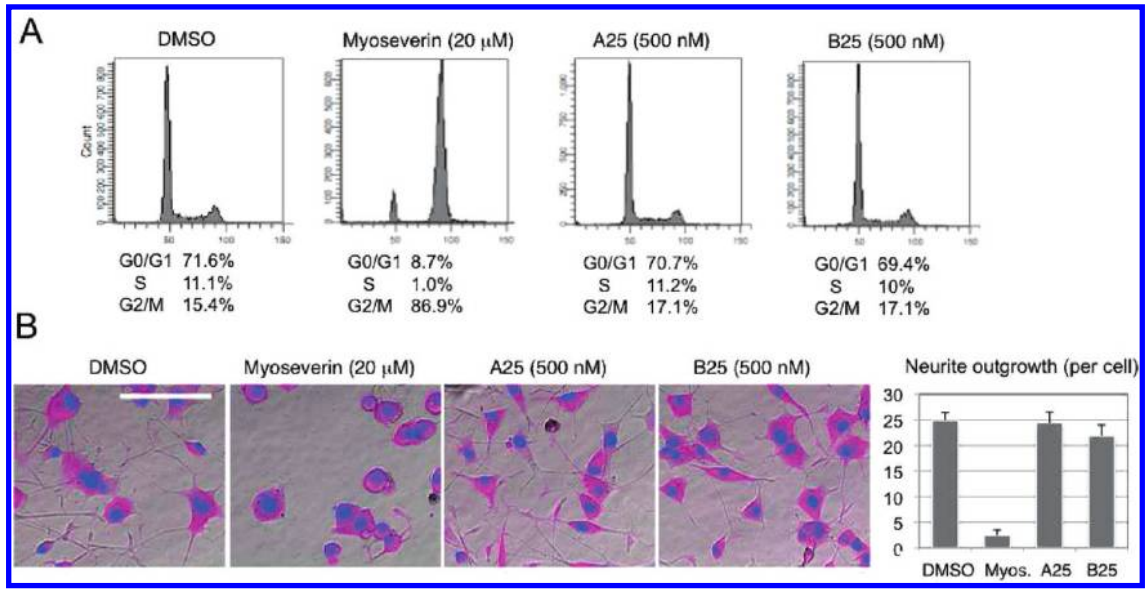

Figure 5. Cell cycle analysis and neuronal differentiation. (A) Cell cycle analysis. C2C12 myoblasts were incubated with each compound for 24 h. While myoseverin caused strong cell cycle arrest at G2/M transition, **B25** did not affect cell cycle. (B) Neuronal differentiation of PC12 cells. PC12 cells were grown in 96-well plates, with differentiation for 6 days in the presence of each compound. Neuronal cell bodies were stained with MitoTracker Deep Red 633, and neurite outgrowth was analyzed by using MetaXpress software (Molecular Devices). Size bar = 100 μm.

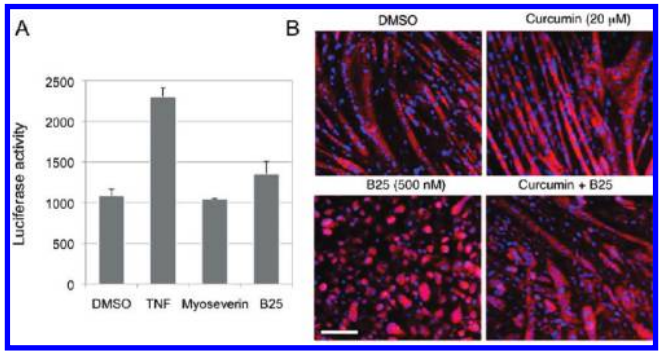

Figure 6. Effect of B25 on the activation of NF-κB. (A) C2C12 myoblasts were stably transfected with NF-κB reporter. Fully differentiated myotubes were incubated with each compound for 24 h: **B25** (500 nM), myoseverin (20 μM), and TNF-α (2 ng/mL). Luciferase activity, expressed as mean $\pm$ standard error of mean from triplicate experiments, was normalized by total protein concentration. **B25**-treated cells were obviously increased in NF-κB activity ($P < 0.01$). (B) Myotubes were incubated with each compound for 18 h in growth medium. For comparison with **B25**-treated cells, DMSO- or curcumin-treated cells were stained with MitoTracker prior to fluorescence imaging. Size bar = 100 μm.

fected with Lentivirual pNF-κB Luc and selected by puromysin. For luciferase assay, differentiated pNF-κB-Luc myotubes were incubated with each compound for 24 h. TNF-α was treated as a positive control. The result clearly showed that NF-κB is activated by **B25** ($P < 0.01$) (Figure 6A and Figure S4, Supporting Information). Moreover, **B25**-induced myotube fission was suppressed by co-treatment with curcumin, a potent inhibitor of up-regulated NF-κB (Figure 6B).[13] These results strongly suggest that NF-κB activation is a requisite for **B25**-mediated myotube fission. However, treatment of TNF-α itself did not induce myotube fission in spite of showing elevated NF-κB activity. It suggests that NF-κB activation is necessary but not sufficient to induce myotube fission by itself. As such, there is substantial evidence that the NF-κB pathway is involved in the control of cellular differentiation.[11] However, its detailed mechanism remains largely unknown. Our selective probe, **B25**, will be a valuable tool to investigate the connection between NF-κB signaling and muscle differentiation.

Concluding Remarks

Fluorescent small molecules have long been used for live cell imaging, but mainly as a labeling reagent for another binding motif such as antibody.[15] As most fluorescent molecules are rigid and hydrophobic, it has been generally believed that the fluorophores may bind to hydrophobic proteins in the cells without any specificity.[16] This conventional wisdom, however, has not been systematically tested due to the lack of diverse dye sources. Here, we show the first fluorescent small-molecule library targeting mitochondria and demonstrate that fluorescent small molecules could induce specific cellular responses.

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Supporting Information Available: Two tables and four supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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