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Identification of Compounds that Bind Mitochondrial F1F0 ATPase by Screening a Triazine Library for Correction of Albinism

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Summary

A triazine-based combinatorial library of small molecules was screened in albino murine melanocytes to identify compounds that induce pigmentation. Six compounds (of 1536 screened) produced at least 3-fold increases in pigmentation. Immunohistochemical studies demonstrated that the compounds conferred correct routing of the mistrafficked enzyme tyrosinase, which is critical to normal melanogenesis. Affinity matrices of the immobilized compounds allowed the cellular target to be identified as the mitochondrial F1F0-ATP synthase. Oligomycin and aurovertin B, small molecules known to inhibit the mitochondrial ATP synthase, were shown to compete with the triazine-based compounds for their cellular target in albino melanocytes and confer similar effects on pigmentation and tyrosinase rerouting. This is the first demonstration of the mitochondrial ATP synthase as a potential therapeutic target for restoring pigmentation in albino melanocytes.

Introduction

Oculocutaneous albinism type 2 (OCA2), the most common form of albinism, results from mutations in the *pink-eyed dilution* gene (*p*) [1]. Unlike oculocutaneous albinism type 1, which results from mutations in tyrosinase, the rate-limiting enzyme in melanogenesis, patients with OCA2 express active tyrosinase [2]. Previous studies in *p* null melanocytes (melan-p1 cells), derived from the murine equivalent of OCA2, demonstrate incorrect trafficking of tyrosinase and increased retention in the endoplasmic reticulum [3–5]. This misrouting can be corrected by transfection with an expression vector encoding wild-type *p*, or by incubating melan-p1 cells with the vacuolar ATPase inhibitor bafilomycin A1, the ionophore monensin, or ammonium chloride [6].

Chemical genetics is a relatively new field of research that has the potential to provide powerful tools for identifying novel drug candidates and their cellular target(s) [7–13]. Small molecules replace the mutation-inducing agents or X-ray irradiation employed by classical genetics. Combinatorial techniques [14–17] allow for the rapid screening of large numbers of small molecules that can be identified by the production of novel phenotypes in a cellular or embryonic system. An affinity matrix made of the immobilized active compound is used to identify biological targets.

Traditionally, active molecules selected and modified after biological screening are fitted with a linker, to provide an attachment point to the affinity bead. This modification has the potential to cause a loss of activity, requiring time-consuming and laborious structure-activity relationship (SAR) studies. The incorporation of the linkers before biological screening allows for a straightforward method of isolation of the target protein and avoids potential compromise of the lead compound's activity. The triazine scaffold was chosen due to its ease of manipulation and structural similarity to purine and pyrimidine, since purine and pyrimidine binding sites are common in proteins [7]. Purine- and pyrimidine-based libraries have already been demonstrated to be active in various biological systems [18–22].

Thus, this study utilizes a novel tagged library approach to accelerate the conversion of a hit compound to an efficient affinity matrix. Six active small molecules obtained from library screening in albino melanocytes demonstrate that inhibition of the F1F0 mitochondrial ATPase is a potential therapeutic target for the correction of OCA2.

Results

Identification of Triazine-Based Compounds that Induce Pigmentation in Melan-p1 Cells

Twenty-one compounds were identified as candidate active drugs after an initial survey of a 1536 compound library. Candidates were rescreened in triplicate, resulting in six confirmed active compounds (Figure 1A). Three inactive but structurally related compounds were chosen as controls (Figure 1B). The ability of the active compounds to stimulate pigmentation in melan-p1 cells is shown in Figure 2. Plotting the induction of pigmentation as either "melanin/mg protein × total mg protein per well" or "melanin/mg protein" produced similar results (compare Figures 2Ai and 2Aii). This illustrates that the enhanced pigmentation is due to an absolute increase in the amount of melanin in the cell population, rather than selective toxicity of the compounds on cells expressing reduced levels of melanin. Differences were observed in the nature of the induction of pigmentation by the six compounds. MPc11, MPd11, APc25, and APc32 were more potent at inducing pigmentation (Figures 2A and 2B) and showed greater levels of pigment induction when melan-p1 cell received repeat doses of the drug (Figures 2D and 2E). The compounds PPa01 and PPj01 failed to display a redosing effect (Figures 2C and 2F).

Identification of the Mitochondrial F1F0-ATP Synthase as a Cellular Target

Eluted proteins from affinity matrix-conjugated compounds included a 55 kDa doublet that was specific for

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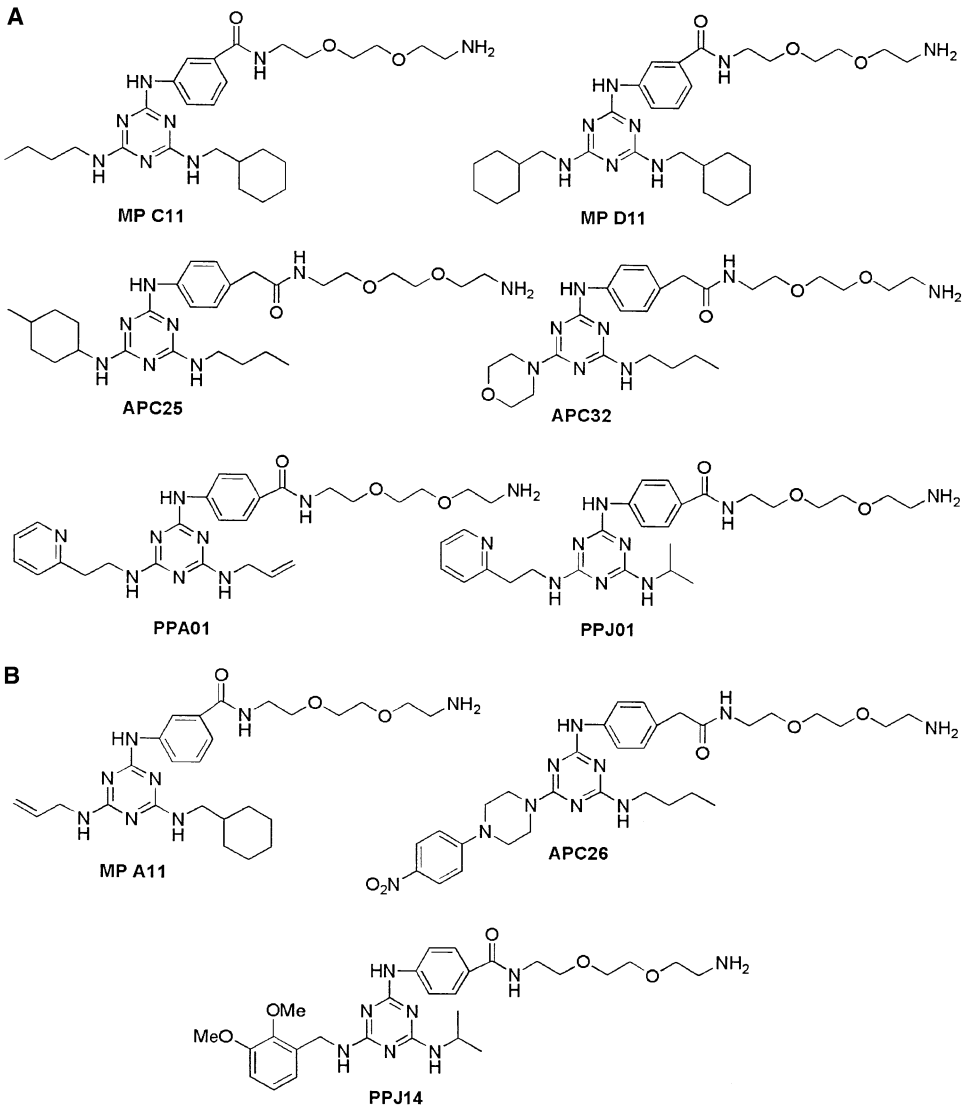

Figure 1. Chemical Structure of the Triazine Compounds that Induce Pigmentation in Melan-p1 Cells

(A) Structure and designation of screened compounds that pigment albino melan-p1 melanocytes.

(B) Structure and designation of the inactive compounds used as controls in this study.

pigmenting drugs (Figures 3A and 3B). In addition, a 30 kDa protein was bound specifically to the affinity matrix-conjugated compounds MPc11, MPd11, APc25, and APc32. However, this band was less prominent among the eluted protein from matrices conjugated with the compounds PPa01 and PPj01 (data not shown). Preincubating the cell lysate with free compound diminished the presence of the 55 kDa doublet in the eluate (Figure 3C). The free compound acts as a competitor of target binding to the matrix-conjugated compound. Ion-Trap mass spectrometry identified the 55 kDa doublet as the α and β subunits of the mitochondrial F1F0-ATP synthase (Figure 3D). Two inhibitors of the mitochondrial ATPase, oligomycin and aurovertin, were also found to compete with the binding of the α and β ATPase subunits to the matrix-conjugated compounds (Figure 3C).

To further demonstrate that the cellular target is the mitochondrial ATPase, proteins bound to the matrix-conjugated compounds were blotted and probed with antibodies against the α and β subunits of the ATPase (Figures 3E and 3F). The β subunit sometimes migrated as a doublet. Oligomycin was a more potent competitor of drug target binding than aurovertin. In keeping with the ATPase as a target for pigmentation induction, both oligomycin and aurovertin induced pigmentation in melan-p1 cells (Figure 3G); however, oligomycin was the more potent stimulator of pigmentation.

Active Compounds Induce Pigmentation in Melan-p1 Cells by Correcting Tyrosinase Trafficking via Targeting of the Mitochondrial ATPase

Previous immunohistochemical analyses of the melanosomal proteins tyrosinase and tyrosine-related protein-1 (TRP-1) in melan-p1 cells demonstrated that both proteins reside in different cellular compartments from that observed in normal melanocytes [3–6]. TRP-1 and tyrosinase immunostaining of normal melanocytes reveals a

punctuate, cytoplasmic distribution of both tyrosinase and TRP-1, and this distribution pattern is consistent with their location in melanosomes. However, in melan-p1 cells, tyrosinase in restricted to the perinuclear region while TRP-1 shows increased misrouting to the cell periphery (Figure 4A) [3–6]. Treating melan-p1 cells with the pigmenting compounds caused an alteration in the trafficking of tyrosinase and TRP-1 and produced increased colocalization of the two proteins that resembled the distribution seen in normal melanocytes (Figures 4C and 4D). Western blot analysis of lysates obtained from melan-p1 cells at 72 hr after drug treatment showed that there was no significant increase in the amount of tyrosinase and TRP-1 protein (data not shown). Thus, the triazine compounds appear to induce pigmentation in melan-p1 cells by correcting tyrosinase and TRP-1 trafficking. This effect could be seen at relatively early time points after the addition of drug and before the onset of detectable increases in melanin synthesis. Treatment of melan-p1 cells with oligomycin produced a similar correction of tyrosinase and TRP-1 trafficking (Figure 4E).

The MitoFluor594 mitochondrial membrane potential-sensing dye demonstrated that the novel pigmentation-inducing triazine compounds influenced mitochondrial membrane potential in a similar manner to the mitochondrial F1F0-ATP synthase inhibitor, oligomycin. This effect was seen as soon as 3 hr after drug treatment (Figure 5). By contrast, bafilomycin, a vacuolar ATPase inhibitor, had a minimal influence on mitochondrial membrane potential, as detected by the MitoFluor594 dye.

Discussion

In this study, by screening a tagged triazine-based, small-molecule library we demonstrated that the mitochondrial F1F0-ATP synthase (mtATPase) is a potential target protein for rescuing melanocytes from the albinism phenotype. This is achieved by restoring tyrosinase transport to melanosomes, the normal site of melanin synthesis and deposition. In addition, we report that oligomycin and aurovertin B, compounds that also target and inhibit the mitochondrial ATPase, both induce melanin synthesis in melan-p1 cells.

Melan-p1 cells have defective early processing of the key melanogenic enzyme, tyrosinase [3, 4]. Previous research has shown that a variety of compounds are capable of restoring melanin synthesis in melan-p1 cells [3, 6]. These compounds include tyrosine, ammonium chloride, bafilomycin A1, concanamycin, monensin, and nigericin. Results from our laboratory suggest that bafilomycin A1 and monensin induce melanin synthesis in melan-p1 cells by facilitating tyrosinase processing from the ER to the Golgi. This is achieved by increasing the pH in either the ER or the ER-Golgi intermediate compartment [6]. Bafilomycin A1 and concanamycin A are vacuolar-type (H+)-ATPase (V-ATPase) inhibitors [23]. V-ATPases transport H+ ions into various endomembrane systems and cellular organelles, such as endosomes and lysosomes. Monensin and nigericin are ionophores that induce melanin synthesis in melan-p1 cells at micromolar concentrations [6, 24]. Monensin and nigericin are known to disrupt Na^+/H+ exchange and K+/H+ exchange in the Golgi, respectively [25]. NH_4Cl is also capable of melanin induction in melan-p1 cells, presumably by directly increasing the pH of various endomembrane systems [3, 26]. We propose that our screened compounds induce melanin synthesis in melan-p1 cells by inhibiting mtATPase-mediated transport of H^+ ions, resulting in the alkalination of the cytosol. This hypothesis is supported by the pigmenting effects of two known inhibitors of the mtATPase, oligomycin and aurovertin B. We are confident that inhibition of the mtATPase, rather than general mitochondrial toxicity, induces melan-p1 cell pigmentation because previous results from our laboratory have shown that neither FCCP (a mitochondrial uncoupler) nor arsenical compounds (which target the mitochondria) induce pigmentation in these melanocytes (data not shown).

It is interesting to compare the induction of pigmentation induced by an inhibitor of the V-ATPase, bafilomycin, and that produced by inhibitors of the mtATPase. Bafilomycin is more effective at inducing pigmentation and, typically, could produce levels of melanin accumulation in melan-p1 cells that are 10-fold above the basal level. The triazine-based compounds and oligomycin can, on average, produce increases in melan-p1 cell pigmentation that are 3- to 4-fold above the basal level.

Both aurovertin B and oligomycin were shown to be capable of inducing pigmentation in melan-p1 cells. Oligomycin could induce pigmentation at significantly lower doses than aurovertin (Figure 3G). This difference was also reflected in the ability of oligomycin and aurovertin to compete with mtATPase binding to affinity matrices of the triazine-based compounds. A greater amount of aurovertin was required to reduce the binding of the triazine-based compounds to the β subunit of the mtATPase (Figure 3F). In addition, it was difficult to achieve aurovertin competition for the binding of the triazine-based compounds to the α subunit of the mtATPase (Figure 3E).

Differences in the ability of the six triazine-based compounds to induce pigmentation in melan-p1 cells can be seen after redosing studies. Typically, melan-p1 cells were incubated with one dose of drug for 72 hr prior to melanin assay. We have found that the compounds MPc11, MPd11, APc25, and APc32 can produce a significantly greater induction of pigmentation when cells are given three doses of drug, spaced 24 hr apart, over a 72 hr period. Using this dosing regime, even suboptimal doses of drug can then induce levels of pigmentation in melan-p1 cells that rival that induced by bafilomycin. This suggests that these compounds are unstable in the tissue culture environment and degrade over a 24 hr period. It is interesting to note that two compounds that induce pigmentation in melan-p1, PPa01 and PPj01, did not show a redosing effect and are, presumably, stable in tissue culture for at least 72 hr.

A number of studies of melan-p1 pigmentation were completed to investigate whether the triazine-based compounds could act synergistically with themselves or other known inducers of pigmentation. However, no synergistic effects were recorded, although some additive effects could be seen when combining oligomycin dosing with the triazine-based compounds MPc11, MPd11, and APc25 (data not shown).

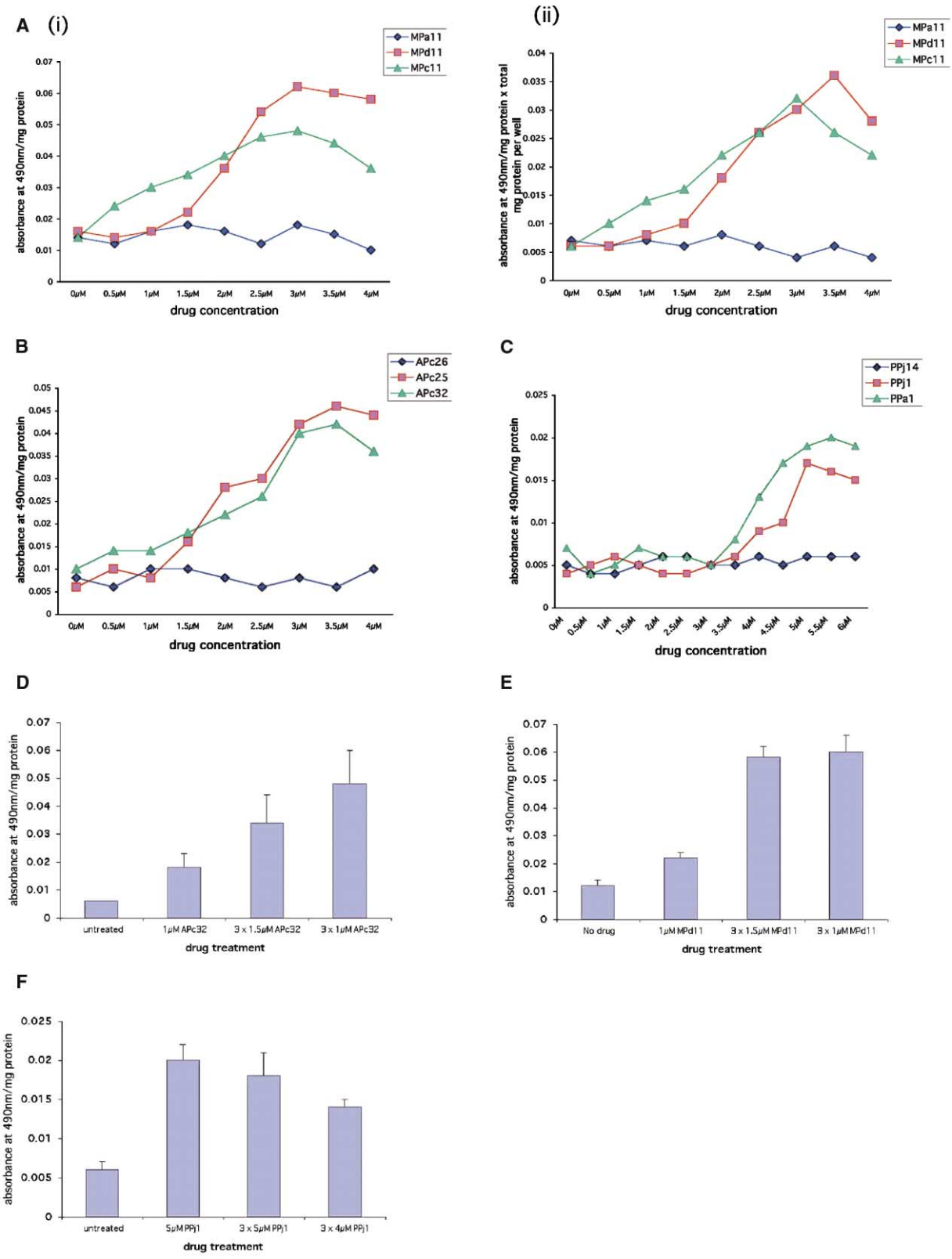

Figure 2. Quantitative Assessment of the Induction of Pigmentation in Melan-p1 Cells by Screened Triazine-Based Compounds

(A) (i) Dose-response study for compounds MPc11 and MPd11. Dose-response data for MPa11, an inactive compound of similar structure, are included for comparison. Cytotoxicity reaches ≥25% at doses of ≥3 μM MPc11 or MPd11, but not MPa11. (ii) Adjusting the dose-response data for total milligrams of protein per well does not diminish the degree of pigmentation induction.

(B) Dose-response study for compounds APc25 and APc32. Dose-response data for APc26, an inactive compound of similar structure, is

Further studies of these novel melan-p1 pigmenting compounds include the detailed characterization of their binding to the mtATPase. The mtATPase consists of two parts, F1 and F0. F1 is the catalytic portion and consists of five subunits. F0 consists of 10 subunits in mitochondria and is arranged as a membrane-embedded, proton-conducting channel [27]. It is known that aurovertin B binds to subunit β of the mtATPase [28]. The exact subunit targeted by oligomycin is not known, although one subunit (the oligomycin sensitivity-conferring protein) is essential for conferring sensitivity to inhibition by oligomycin [27]. Further detailed biochemical studies will be needed to decipher which mtATPase subunit(s) is the precise molecular target for our triazine-based pigmenting compounds. In addition, elongated melanosomes and melanin deposition in endosome-like structures were observed in melan-p1 cells treated with 50 nM bafilomycin A1. Whether similar structures are seen in melan-p1 cells after treatment with our mtATPase binding pigmenting compounds is not yet known.

We have successfully demonstrated the power of a tagged library approach for efficient forward chemical genetics, in this case demonstrating a novel role for the mtATPase in restoring pigmentation in albino melanocytes. Our small-molecule library facilitates the connection of a hit compound to the affinity matrix by incorporating a linker directly to the compounds before their phenotypic screening. The same tagged library approach can be readily applied to other cellular screens.

Significance

We have adopted a chemical genetics approach to identify a novel potential drug target for the most common form of albinism, oculocutaneous albinism type 2. A triazine-based combinatorial library of 1536 small molecules was screened in albino melanocytes. Six compounds were identified that could induce significant levels of pigmentation. The cellular target of each compound was identified as the mitochondrial F1F0-ATP synthase. Two known inhibitors of the ATP synthase were shown to also induce pigmentation in albino melanocytes and to compete with the triazine compounds for binding to the ATP synthase. Oculocutaneous albinism type 2 is characterized by perturbed intracellular trafficking of two melanocyte-specific proteins, tyrosinase and tyrosinase-related protein-1. The triazine-based compounds were shown to correct this defect and their ability to inhibit the mitochondrial F1F0-ATP synthase was demonstrated by a mitochondrial inner membrane potential-sensitive dye. We propose that these novel triazine-based pigmenting compounds rescue albino melanocytes by perturbing intracellular pH, similar to another pigmenting compound, bafilomycin, which inhibits the vacuolar ATP synthase. This study demonstrates the power of chemical genetics for discovering potential new drugs and drug targets for the treatment of diseases.

Experimental Procedures

Development of the Small-Molecule Library

The design and production of the tagged triazine library have been reported previously [29]. Briefly, a solid-phase method was used to construct a tagged triazine library, where three building blocks were prepared separately and assembled orthogonally to yield 1536 highly pure compounds. Each library compound contained one of a variety of triethyleneglycol (TG) linkers at one of the diversity sites of the triazine scaffold.

Cell Culture

Melan-a (*a*/*a*, *P*/*P*) is an immortalized melanocyte line derived from C57BL/6J mice wild-type at the *p* locus. Melan-p1 is an immortalized melanocyte line from C57BL/6J mice lacking *p* gene transcripts due to overlapping deletions [30]. Melan-p1 cells were maintained in RPMI culture medium containing 2 mM L-glutamine, 100,000 IU/L penicillin, 100 mg/L streptomycin, 5% fetal bovine serum and 200 nM tetradecanoyl phorbol acetate at 37°C and 5% CO_2.

Small-Molecule Screening in Melan-p1 Cells

Lyophilized library compounds were resuspended in DMSO to a final concentration of 10 mM. Each compound was added to one well of a 24-well tissue culture plate containing 1×10^5 melan-p1 cells at a final concentration of 5 μM. Cells were incubated with each compound for 72 hr. Changes in pigmentation were detected by melanin assay (see below). Cytotoxicity was assessed initially by visual inspection of the cells and defined by the presence of dead, floating, or misshapen cells with reduced dendricity. A protein assay was then completed if cytotoxicity was apparent. Toxic compounds were rescreened at 1 μM.

Melanin Assay

Cells were rinsed with phosphate-buffered saline (PBS) and lysed with an extraction buffer (50 mM Tris [pH 7.5], 2 mM ethylenediamine tetraacetic acid [EDTA] [pH 7.8], 150 mM NaCl, 1% Triton X-100) with protease inhibitor cocktail at 4°C. Cell extracts were then spun at 12,000 rpm for 10 min at 4°C. The remaining pellet was assayed for melanin by rinsing twice with ethanol-ether (1:1) and dissolving in 200 μl 2 N NaOH in 20% DMSO at 60°C. A 100 μl aliquot of the resulting solution was then measured for absorbance at 490 nm.

Isolation and Identification of Drug Cellular Target(s)

Protein was extracted from cells by incubation with extraction buffer (1 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris [pH 7.4], 1% Triton X-100, 1 mM PMSF, plus one tablet of protease inhibitor cocktail [Roche] per 20 ml buffer) for 5 min on ice. Crude lysate was centrifuged at 13,000 rpm for 10 min. The protein concentration of the supernatant was measured by the Bradford assay (Bio-Rad) and adjusted to a final concentration of 1 μg/μl prior to affinity chromatography.

25–50 μl agarose affinity matrix-conjugated compound was washed with 1 ml bead buffer (10 mM Tris [pH 7.4], 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Triton X-100 plus protease

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included for comparison. Cytotoxicity reaches ≥25% at doses of ≥4 μM APc25 or APc32, but not APc26.

(C) Dose-response study for compounds PPa1 and PPj1. Dose-response data for PPj14, an inactive compound of similar structure, is included for comparison. The induction of pigmentation begins to level off at doses of ≥ 6 μM PPa1 or PPj1.

(D) Repeated dosing of the compound APc32 produces greater increases in pigmentation than a single dose alone. Repeat doses were spaced 24 hr apart over a 72 hr time course. Two different doses were used: three doses of 1 μM triazine compound (labeled on the x axis as "3 × 1 μM") or three doses of 1.5 μM triazine compound (labeled on the x axis as "3 × 1.5 μM").

(E) A similar repeat dosing effect could be seen for the compound MPd11 and was also observed for the compounds MPc11 and APc25 (data not shown).

(F) This repeat dosing effect was not a feature of the compound PPj1 and was also absent from the compound PPa1 (data not shown).

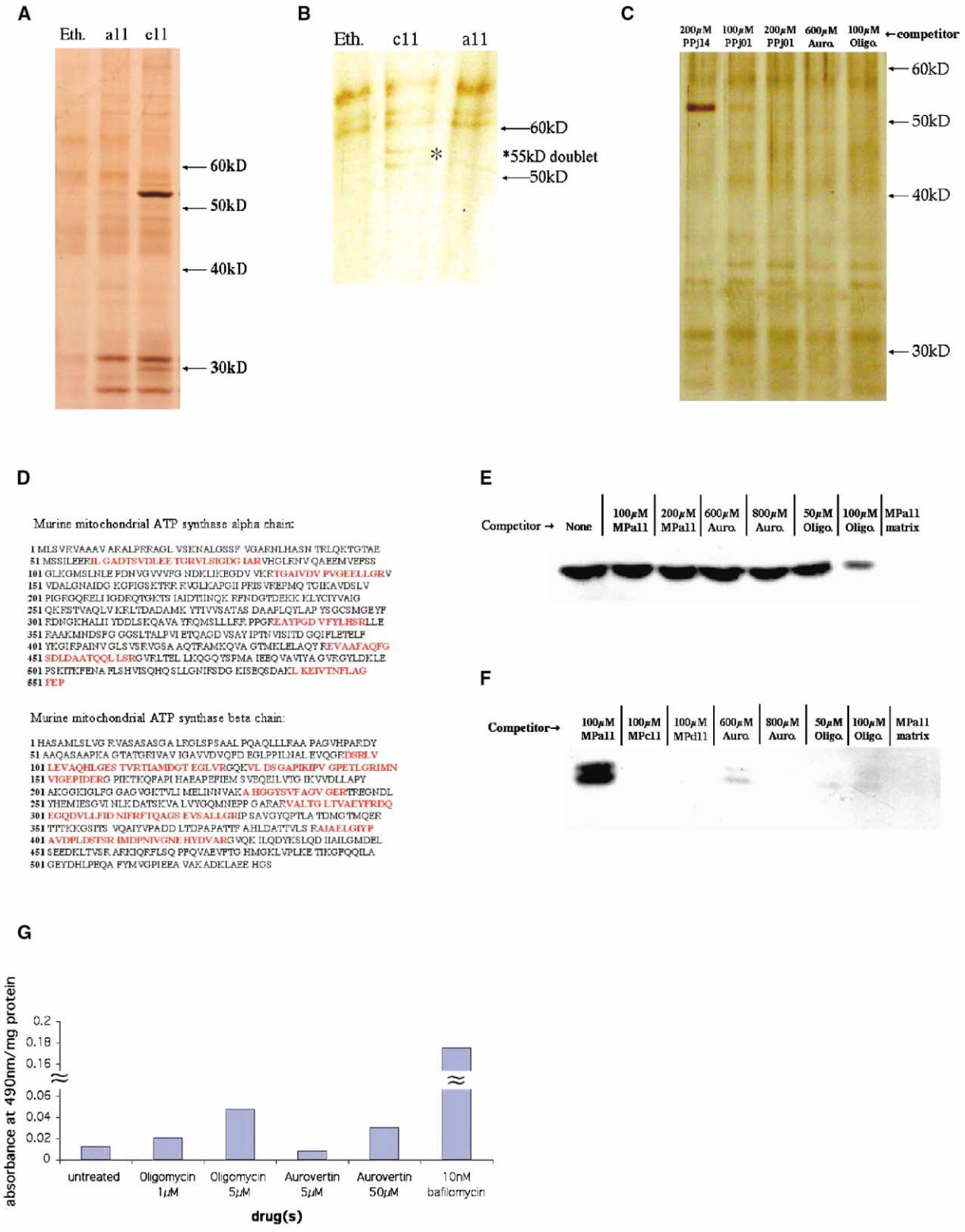

Figure 3. Studies to Identify the Target as the Mitochondrial F1F0-ATP Synthase; Demonstration of Competition for the Drug Target by Known Inhibitors of the Mitochondrial F1F0-ATP Synthase; Demonstration that Known Inhibitors of the Mitochondrial F1F0-ATP Synthase Can Induce Pigmentation in Melan-p1 Cells

(A) Drug target study for the compound MPc11 in melan-p1 cells. Eluted proteins were separated by 7.5% SDS-PAGE. Lanes: Eth., ethanolamine-conjugated affinity matrix; a11, MPa11 (inactive compound)-conjugated affinity matrix; c11, MPc11-conjugated affinity matrix.

(B) The prominent 55 kDa band that is specific for pigmenting compounds can be resolved into a doublet by 6%–12% gradient SDS-PAGE.

inhibitor cocktail [Roche]). Matrices were incubated with 50–200 μg of protein extract, plus an identical volume of bead buffer at 4°C or 30°C. For studies of competition of drug binding to cellular target, the competitor was added to the mixture of protein extract/bead buffer and incubated at 4°C for 30 min prior to incubation with the matrix. The supernatant containing unbound proteins was removed by centrifugation, and the matrices were washed seven times with 1 ml bead buffer. Proteins bound to the matrices were eluted by incubation with 50 μl Laemmli buffer (Bio-Rad) at 94°C for 3 min.

Eluted proteins were separated by 7.5% or 10% SDS-PAGE and visualized by silver staining (Amersham). Prominent protein bands specific to active matrices were excised from each gel and identified by Ion Trap mass spectrometry (NYU Protein Analysis Facility, Skirball Institute of Biomolecular Medicine).

Antibodies and Reagents

Monoclonal antibodies against the α subunit and β subunit of the ATP synthase (F1F0) were purchased from Molecular Probes Inc. The monoclonal antibody against tyrosinase-related protein-1 (Mel5) was purchased from Sigma-Aldrich Co. Pep-7 antibody against tyrosinase was obtained from Dr. Vincent Hearing, National Institutes of Health, Bethesda, MD.

Oligomycin and aurovertin B were purchased from Sigma-Aldrich. Bafilomycin A1 was purchased from Wako Pure Chemical Industries Ltd.

Western Blot Analysis

Proteins were separated by 7.5% or 10% SDS-PAGE and transferred onto membranes (Immobilon-P; Millipore).

Immunofluorescence Microscopy

Cells were grown on coverslips (Fisher Scientific) for 48 hr, washed three times with ice-cold PBS, and fixed with 20°C methanol for 5 min and processed as described previously [31]. The slides were analyzed with either a confocal microscope (LSM510; Carl Zeiss) or a digital fluorescence microscope (Axiophot; Carl Zeiss). All data were analyzed with a 100× oil lens and processed with Adobe Photoshop 7.0 (Adobe Systems).

Measurement of Mitochondrial Membrane Potential

MitoFluor594 (Molecular Probes, Inc.) is a mitochondrial membrane potential-sensing dye. Live cells, grown in 4-well chamber slides (Nalge Nunc Int.), were incubated with 500 nM MitoFluor594 in growth medium for 30 min in a tissue culture incubator at 37°C and 5% CO_2. The slides were analyzed with a digital fluorescence microscope (LSM510; Carl Zeiss). All data were analyzed with a 20× lens and processed with Adobe Photoshop 7.0 (Adobe Systems).

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Lanes: Eth., ethanolamine-conjugated affinity matrix; c11, MPc11-conjugated affinity matrix; a11, MPa11 (inactive compound)-conjugated-affinity matrix.

(C) Competition study for the compound PPj01 in melan-p1 cells. Lanes: 200 μM PPj14, PPj01-conjugated affinity matrix with 200 μM PPj14 (inactive compound) as competitor; 100 μM PPj01, PPj01-conjugated affinity matrix with 100 μM PPj01 as competitor; 200 μM PPj01, PPj01-conjugated affinity matrix with 200 μM PPj01as competitor; 600 μM Auro., PPj01-conjugated affinity matrix with 600 μM aurovertin as competitor; 100 μM Oligo., PPj01-conjugated affinity matrix with 100 μM oligomycin as competitor.

(D) Sequencing results for the 55 kDa protein band specific for immobilized melan-p1 active compounds. The data shown were derived from the protein bound to the immobilized compound MPc11. Peptides detected by Ion Trap mass spectrometry are shown in red (aligned according to the complete sequence derived from the Swissport-Mouse database).

(E and F) Two competition studies for the compound MPd11 in melan-p1 cells. (E) Eluted proteins were blotted and probed with antibodies against the α subunit of the mitochondrial ATPase. Lanes: None, MPd11-conjugated affinity matrix with no competitor; 100 μM MPa11, MPd11-conjugated affinity matrix with 200 μM MPa11 (inactive compound) as competitor; 200 μM MPa11, MPd11-conjugated affinity matrix with 200 μM MPa11 (inactive compound) as competitor; 600 μM Auro., MPd11-conjugated affinity matrix with 600 μM aurovertin as competitor; 800 μM Auro., MPd11-conjugated affinity matrix with 800 μM aurovertin as competitor; 50 μM Oligo.1, MPd11-conjugated affinity matrix with 50 μM oligomycin as competitor; 100 μM Oligo.2, MPd11-conjugated affinity matrix with 100 μM oligomycin as competitor; MPa11 matrix, MPa11 (inactive compound)-conjugated affinity matrix with no competitor. (F) Eluted proteins were blotted and probed with antibodies against the β subunit of the mitochondrial ATPase. Lanes: 100 μM MPa11, MPc11-conjugated affinity matrix with 100 μM MPa11 (inactive compound) as competitor; 100 μM MPc11, MPc11-conjugated affinity matrix with 100 μM MPc11 as competitor; 100 μM MPd11, MPc11-conjugated affinity matrix with 100 μM MPd11 (an active compound with structural similarity to MPc11 that also binds the mitochondrial F1F0-ATP synthase) as competitor; 600 μM Auro., MPc11-conjugated affinity matrix with 600 μM aurovertin as competitor; 800 μM Auro., MPc11-conjugated affinity matrix with 800 μM aurovertin as competitor; 50 μM Oligo., MPc11-conjugated affinity matrix with 50 μM oligomycin as competitor; 100 μM Oligo., MPc11-conjugated affinity matrix with 100 μM oligomycin as competitor; MPa11 matrix, MPa11 (inactive compound)-conjugated affinity matrix with no competitor.

(G) Melanin production in melan-p1 cells is increased after 72 hr of incubation with oligomycin or aurovertin. Melanin production induced by 24 hr of incubation with bafilomycin, an inhibitor of the vacuolar ATPase, is shown for comparison. The y axis has been modified to illustrate the greater pigmenting effect of bafilomycin alongside the pigmentation induced by aurovertin and oligomycin.

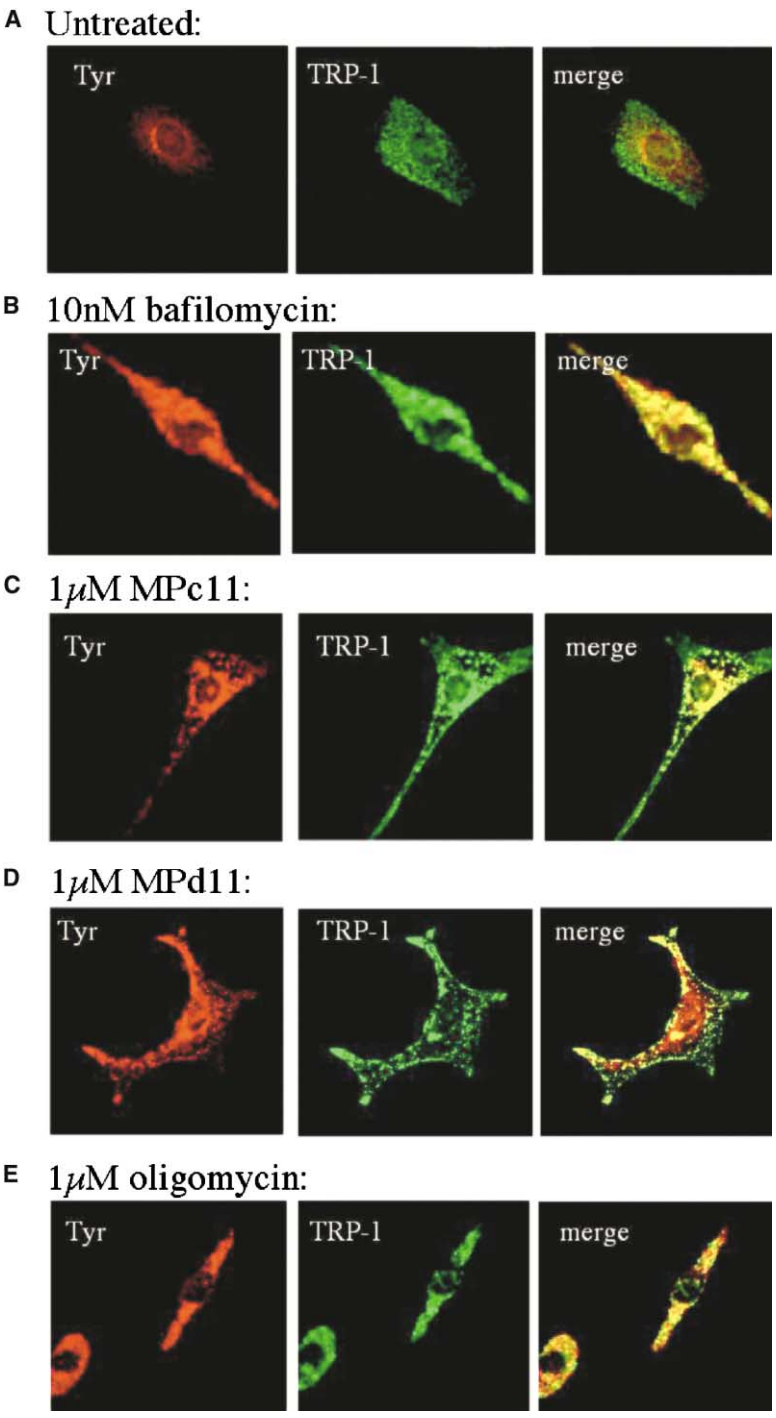

Figure 4. Immunomicroscopic Analysis of the Effect of Triazine-Based Pigmentation Inducers on the Distribution of Tyrosinase and Tyrosinase-Related Protein-1 in Melan-p1 Cells

Immunohistochemical analysis of tyrosinase (detected by the pep-7 antibody) and TRP-1 (detected by the mel-5 antibody) trafficking in melan-p1 cells. All images are taken 12 hr after the addition of the drug to cells. The yellow color indicates areas of overlapping signal from tyrosinase (Tyr) and TRP-1. Compounds were used at doses that typically increase pigmentation in melan-p1 cells (magnification ×600).

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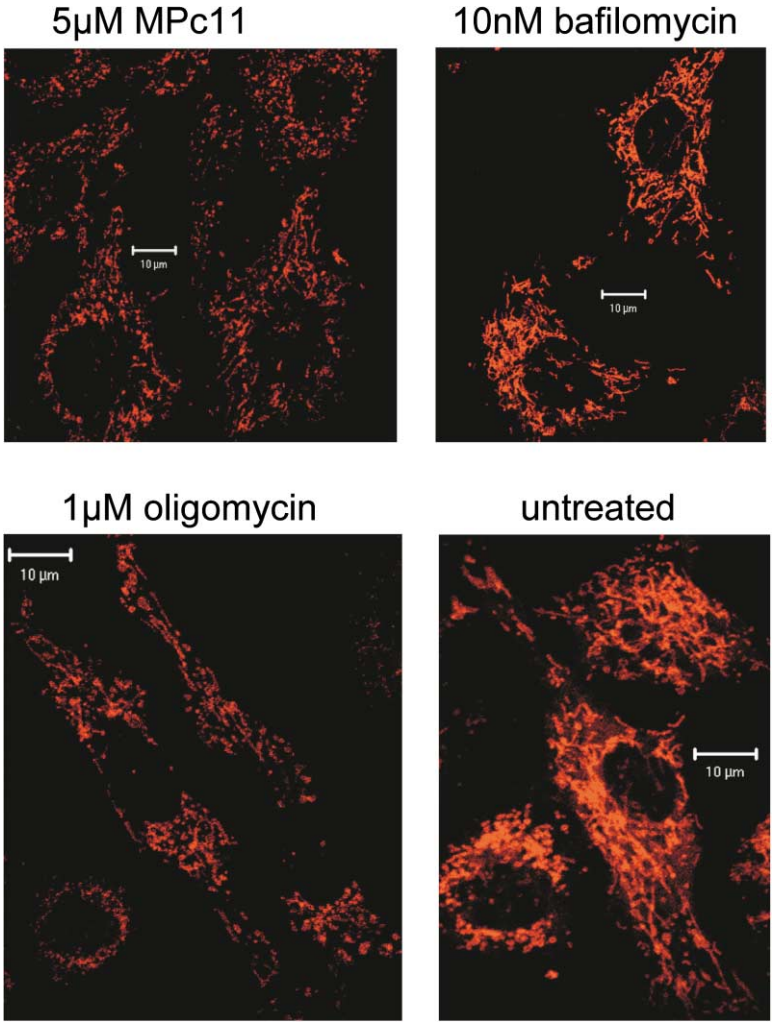

Figure 5. Characterization of the Effect of Triazine-Based Pigmentation Inducers and Oligomycin on Mitochondrial Membrane Potential in Melan-p1 Cells Using MitoFluor594

Mitochondria show altered membrane potential 3 hr after treatment with MPc11, a pigmentation-inducing drug that binds the mitochondrial ATPase. The effect is similar to that produced by the mitochondrial ATPase inhibitor, oligomycin, with diminished intensity of the MitoFluor594 signal and qualitative changes in mitochondrial morphology. By contrast, the vacuolar ATPase inhibitor, bafilomycin, produced a minimal effect on mitochondrial membrane potential. Oligomycin, MPc11, and bafilomycin were used at concentrations that typically induce pigmentation in melan-p1 cells (the scale bar represents 10 μM).

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