Substrate specificity of the TIM22 mitochondrial import pathway revealed with small molecule inhibitor of protein translocation

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The TIM22 protein import pathway mediates the import of membrane proteins into the mitochondrial inner membrane and consists of two intermembrane space chaperone complexes, the Tim9-Tim10 and Tim8-Tim13 complexes. To facilitate mechanistic studies, we developed a chemical-genetic approach to identify small molecules that cause synthetic lethal interactions at a temperature-sensitive mutant for the TIM22 import pathway. Taking advantage of our large collection of temperature-sensitive mutants for the TIM22 import pathway, we conducted a chemical-genetic screen with a temperature-sensitive mutant to identify small molecules that caused synthetic lethality in yeast. Our results indicate that the substrate specificity of the small Tim proteins is important for understanding the molecular basis of deafness-dystonia syndrome.

Mitochondrial assembly has been studied extensively using classical yeast genetics and biochemical assays with purified mitochondria. However, new strategies are needed to elucidate the details of protein translocation and its role in development and human disease. Important questions about the substrate specificity of the small Tim proteins and the mechanism by which the small Tim proteins bind substrate have been unresolved. These studies would be facilitated by drug-like inhibitors that modulate protein import. Here we report the development of a small molecule screening approach to identify inhibitors of the TIM22 import pathway. Taking advantage of our large collection of temperature-sensitive mutants for the TIM22 import pathway, we conducted a chemical-genetic screen with a temperature-sensitive mutant to identify small molecules that caused synthetic lethality at the permissive temperature of 25 °C (16–19). Our results indicate that a new set of tools for mechanistic studies in protein translocation can be developed and may be useful for characterizing protein translocation in mammalian mitochondria, where tools are lacking.

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Results

A Screen to Identify Inhibitors of Mitochondrial Protein Translocation.

We exploited a large collection of temperature-sensitive mutants for the TIM22 import pathway (10, 16–18) and developed a composite synthetic lethal screen to identify small molecule inhibitors that blocked the TIM22 import pathway (19). The tim10-1 mutant was used as the starting strain (16); the strains used in this study are described in Table S1. The rationale in this screen was that small molecules might be identified that target the mutant Tim10 protein or other components of the TIM22 pathway and thereby cause lethality of the tim10-1 mutant at the permissive temperature of 25 °C. This approach uses the well-characterized synthetic growth defects of the tim10-1 mutant to guide the design of cells genetically sensitized for inhibition of the TIM22 pathway.

To generate a suitable strain for screening, genes for the multidrug resistance pumps PDR5 and SNQ2 were disrupted to increase the steady-state intracellular concentration of the drugs in yeast (19). The tim10-1 mutant grew similar to the parental strain (designated TIM10) at 25 °C but failed to grow at the restrictive temperature of 37 °C (Fig. 1A). Growth was inhibited on media that contained glucose (YPD, supporting fermentable growth) or ethanol-glycerol (YPEG, supporting nonfermentable growth) as the sole carbon source. We verified that the abundance of the mutant Tim10 was decreased in the tim10-1 strain; however, the abundance of other mitochondrial proteins was not markedly decreased in mitochondria when the strain was grown at 25 °C (Fig. S1A) (16). In addition, deletion of the multidrug resistance pumps did not compromise growth or the mitochondrial protein profiles of the tim10-1 mutant. In contrast, when we investigated assembly of the soluble 70 kDa Tim9-Tim10 complex in the tim10-1 mutant, the complex was not detected by immunoblot analysis (Fig. S1B). Moreover, in vitro import of the TIM22 pathway substrate, AAC, was inhibited in comparison to mitochondria from the parental strain (Fig. 1B). The tim10-1 mutant thus has excellent growth properties for conducting a synthetic genetic screen with small compounds to target the TIM22 import pathway.

For subsequent testing of the compounds in biochemical assays with isolated mitochondria, a suppressor strain, designated tim10-1 tim9S, was used because growth of the tim10-1 mutant (Fig. 1A) and import of the carrier proteins were restored (Fig. 1B). Suppression in this strain is caused by a Ser → Cys mutation in Tim9; the mutated serine residue is nine amino acids after the second CXXC motif (17). Whereas the specific mechanism of suppression is not understood, the mutant Tim9 protein restored the abundance of Tim10 (Fig. S1A) and the assembly of Tim9-Tim10 complexes, albeit of aberrant size (Fig. S1B).

The screen was conducted with an integrated robotic system with plate scheduling. Briefly, diversity-oriented commercial libraries of drug-like compounds from Chembridge and Asinex were screened against the tim10-1 strain at a concentration of approximately 10 μM. The screen encompassed a total of approximately 40,000 compounds dissolved in DMSO. Yeast in YPD medium was aliquoted into 384-well plates followed by compound addition with robotic pinning into the assay wells. DMSO was the vehicle for the small molecules, and several plate columns that contained only 1% DMSO were included as a control with the pinned compounds. As a negative control for growth, wells pinned with the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which caused lethality, were also included. After 2 d of incubation at 25 °C, cultures in each well were measured for optical density (OD) as a measure of growth. A typical reading for the positive control was OD500 = 0.7. Wells in which the growth was inhibited by >50% were deemed as potential inhibitors and chosen for further analysis. Approximately 600 inhibitors from the primary screen were selected for hit confirmation and secondary screens.

To identify possible specific inhibitors of mitochondrial protein translocation from the pool of hit compounds, two counter screens were executed. In the first round, the initial hit compounds were incubated with the tim10-1 mutant and the isogenic control strain carrying an integrated version of the TIM10 gene at the leu2 locus. Small molecules that inhibited growth of the mutant but not the control strain at 10 μM were advanced to the second counter screen. In a second round, compounds were assayed for selective growth inhibition of the tim10-1 mutant, but not the tim10-1 mutant harboring a plasmid containing the wild-type TIM10 gene. The second counterscreen was a test for chemical-genetic rescue. Compounds that showed inhibition of only the tim10-1 mutant in both counter screens were dubbed “MitoBloCK” compounds based on their potential to inhibit protein translocation in mitochondria. Of 25 potential “lead” inhibitors, MitoBloCK-1 was chosen for additional analysis.

MitoBloCK-1 Inhibits Protein Import of TIM22 Substrates into Mitochondria. MitoBloCK-1 is a tetrahydrodibenzofuran derivative that was identified from the Chembridge library (Fig. 2A). The MIC50 for MitoBloCK-1 that inhibited growth of the tim10-1 mutant was approximately 1 μM (Fig. 2B). MitoBloCK-1 had a similar MIC50 with another temperature-sensitive tim10 mutant, tim10-73. In contrast, the MIC50 for the isogenic control was greater than 200 μM. To understand the cell-based activity of MitoBloCK-1, we also determined the MIC50 with other yeast mutants that also were disrupted for PDR5 and SNQ2 (Table 1). For mutants within the TIM22 pathway, MitoBloCK-1 displayed an MIC50 concentration of 11 μM for the tim9-3 mutant and 10 μM for the tim10-1 tim9S suppressor strain, respectively. In contrast, the MIC50 for MitoBloCK-1 in the tim23 mutant was

![Fig. 1.](image-url) Phenotypic analysis of the strains used for the chemical synthetic-lethality screen for inhibitors of the TIM22 protein import pathway. (A) Growth phenotypes of the control (TIM10), the tim10-1 mutant, and tim10-1 suppressor (tim10-1 tim9S) strains used in the screen. Strains were plated on rich glucose (YPD) or ethanol-glycerol (YPEG) media and incubated at 25 °C or 37 °C. All of these strains were isogenic except for their denoted genetic variation. (B) Radiolabeled AAC was imported into isolated mitochondria in the presence and absence of a membrane potential (Δψ). Aliquots were removed at the indicated time points and samples were treated with carbonate extraction to confirm that AAC was inserted into the IM.
greater than 200 μM. Overexpression of import components, TIM8, TIM9, TIM13, TIM22, and TIM23, in the tim10-1 mutant did not alter the ability of MitoBloCK-1 to inhibit growth. Interestingly, strains lacking the mitochondrial genome (denoted as rho null) were also sensitive to MitoBloCK-1. Thus, MitoBloCK-1 specifically inhibited growth of the tim10-1 mutant (Table 1) and import of the model substrate, AAC, was restored in comparison to the tim10-1 strain (Table 1) and import of substrates Tim9, Tim10, Tim13, and Tim23, in the tim10-1 mutant was not inhibited. However, AAC import was markedly decreased in the tim10-1 tim9S mutant, even in the presence of the suppressing mutation in Tim9; this growth analysis suggests MitoBloCK-1 targets the Tim9-Tim10 complex.

The ability of MitoBloCK-1 to inhibit import of mitochondrial precursors was tested using the in vitro import assay with radiolabeled substrates. For this analysis, mitochondria from the tim10-1 tim9S strain were used because MitoBloCK-1 inhibited growth of this strain (Table 1) and import of the model substrate, AAC, was restored in comparison to the tim10-1 mutant (Fig. 1B). An import time course was performed in the presence of the vehicle DMSO or varying concentrations of MitoBloCK-1 (Fig. 3). In the presence of DMSO, the import of the TIM22 substrate, AAC, was not inhibited. However, AAC import was markedly decreased in the tim10-1 tim9S mitochondria in the presence of 1 μM MitoBloCK-1 or greater (Fig. 3A). In contrast, MitoBloCK-1 did not inhibit import into WT mitochondria (Fig. S2). Thus, the MIC<sub>50</sub> in the import assays agree well with the cell growth assays (Table 1 and Fig. 2B).

### Table 1. Chemical-genetic analysis of MitoBloCK-1 activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tim10-1 rho null</td>
<td>0.75 ± 0.02</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>tim10-1</td>
<td>1.00 ± 0.05</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>tim10-73</td>
<td>2.00 ± 0.06</td>
<td>0.74 ± 0.02</td>
</tr>
<tr>
<td>tim9-3</td>
<td>11.34 ± 1.56</td>
<td>4.18 ± 0.57</td>
</tr>
<tr>
<td>tim23-1</td>
<td>&gt;200</td>
<td>&gt;74</td>
</tr>
<tr>
<td>TIM10 rho null</td>
<td>12.39 ± 0.9</td>
<td>4.56 ± 0.33</td>
</tr>
<tr>
<td>TIM10</td>
<td>&gt;200</td>
<td>&gt;74</td>
</tr>
<tr>
<td>tim10-1 TIM10</td>
<td>&gt;200</td>
<td>&gt;74</td>
</tr>
<tr>
<td>tim10-1 tim9S</td>
<td>9.91 ± 0.24</td>
<td>3.65 ± 0.09</td>
</tr>
<tr>
<td>tim10-1 TIM9 (2μ)</td>
<td>1.48 ± 0.08</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>tim10Ts TIM8 (2μ)</td>
<td>2.42 ± 0.15</td>
<td>0.89 ± 0.05</td>
</tr>
<tr>
<td>tim10-1 TIM13 (2μ)</td>
<td>1.26 ± 0.03</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>tim10-1 TIM22 (2μ)</td>
<td>8.35 ± 0.27</td>
<td>3.07 ± 0.1</td>
</tr>
<tr>
<td>tim10-1 TIM23 (2μ)</td>
<td>1.37 ± 0.04</td>
<td>0.51 ± 0.02</td>
</tr>
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*mean ± s.d. (n = 3).

MitoBloCK-1 also inhibited the import of an additional carrier protein, PIC, and the outer membrane protein Tom40, which requires the small Tim proteins for import (7) (Fig. 3 B, C). However, for dihydrofolate reductase (DHFR) fusion constructs Su9-DHFR and cyt b<sub>5</sub>-DHFR as well as Hsp60 that use the TIM23 pathway, MitoBloCK-1 did not impair import (Fig. 3D, S3 A, B). In addition, the import of substrates Tim9, Tim10, and Mia40 that use the Mia40/Erv1 import pathway (20) was not inhibited in the presence of MitoBloCK-1 (Fig. S3 C–E). Finally, MitoBloCK-1 did not inhibit the import of AAC into tim12-1 mutant mitochondria (16), indicating that import inhibition is specific for the tim10-1 mutant (Fig. S3F). Therefore, MitoBloCK-1 seems to specifically block the import of the carrier proteins and Tom40, which rely on the TIM22 pathway for translocation.

MitoBloCK-1 Does Not Nonspecifically Damage Mitochondria. A potential mechanism by which MitoBloCK-1 may inhibit protein translocation indirectly is by the disruption of oxidative phosphorylation or dissipation of the membrane potential. We therefore used a battery of tests to determine if MitoBloCK-1 nonspecifically altered mitochondrial integrity or function. As a first test, the ability of MitoBloCK-1 to interfere with respiration was measured (Fig. S4A–C) (21). Mitochondria were incubated in a chamber with an oxygen electrode and respiration was initiated by the addition of NADH. The rate of oxygen consumption was representative of mitochondria that were well coupled. The subsequent addition of vehicle DMSO (Fig. S4A) or 25 μM MitoBloCK-1 (~25-fold above the biochemical MIC<sub>50</sub>) did not significantly alter the rate of respiration (Fig. S4A–C) (p = 0.72). As a control, mitochondria were treated with the proton ionophore CCCP, and respiration increased drastically, indicative of uncoupled mitochondria (Fig. S4A–C).

The membrane potential (Δψ) of mitochondria was measured with the fluorescent dye rhodamine 123, which is taken up by mitochondria and then released when the Δψ is dissipated (22 and 23). The relative change of fluorescence between dye uptake and release is a relative measure of the Δψ; the dye that loads into coupled mitochondria (causing quenching and a decrease in fluorescence) is released when treated with an uncoupling agent such as CCCP (causing an increase in fluorescence). The fluorescence did not change with addition of either DMSO (Fig. S4D) or 25 μM MitoBloCK-1 (Fig. S4E) in contrast to the sharp increase in fluorescence upon CCCP addition. Taken together, the oxygen electrode and dye uptake assays support that MitoBloCK-1 is not a mitochondrial uncoupler.

Another potential mechanism that may alter protein translocation is that the small molecules may nonspecifically permeabilize mitochondrial membranes, and proteins may be released from the mitochondrion, particularly those in the IMS. We therefore incubated mitochondria with MitoBloCK-1 for 30 min followed by centrifugation at 8,000 × g (Fig. S4 F, G). Released proteins were recovered in the supernatant fraction and analyzed by immunoblot assays for key proteins and Coomassie staining for the collective release of proteins. As a positive control, MitoBloCK-2, another compound from the screen that permeabilized mitochondrial membranes, was included. Immunoblots revealed that the release of marker proteins Tom40 (OM), cytochrome c and Tim10 (IMS), AAC (IM), and Hsp60 (matrix) was similar when mitochondria were treated with MitoBloCK-1 or DMSO (Fig. S4F). In contrast, MitoBloCK-2 treatment resulted in release of the marker proteins from mitochondria, and Coomassie blue staining confirmed the extensive release of mitochondrial proteins (Fig. S4G). Finally, MitoBloCK-1 did not alter steady-state stability of the Tim9-Tim10 complex because the complex migrated as a 70 kDa complex in the presence of the small molecule (Fig. S4H). From the aforementioned analysis, MitoBloCK-1 does not alter mitochondrial function or membranes.
nonspecifically and seems to be a specific inhibitor of protein import for the TIM22 pathway.

**MitoBloCK-1 Impairs Substrate Binding by the Tim9-Tim10 Complex.** MitoBloCK-1 can be used for mechanistic studies in protein translocation. From our previous analysis of the tim10-1 and tim12-1 mutants, we showed that Tim10 was required to mediate translocation of AAC across the outer membrane and Tim12 was required at a later step to mediate insertion of the AAC into the IM (16); this analysis was determined by monitoring protease sensitivity of the AAC precursor. We adapted this methodology to determine where MitoBloCK-1 impaired AAC translocation. In wild-type mitochondria, a small fraction of the AAC was trapped in the IMS when protease was added to mitochondria in the absence of a membrane potential (Fig. 4A, lane 4). However, in tim10-1 mutant mitochondria, AAC failed to enter the IMS. Therefore, AAC that accumulated at the outer membrane was degraded upon protease addition (Fig. 4A, lane 6, 8), confirming that Tim10 is required for a very early step in protein translocation (24 and 25). We added MitoBloCK-1 in this assay. In the presence of MitoBloCK-1, AAC was sensitive to protease in the presence of a membrane potential (Fig. 4A, lane 12), similar to that of the tim10-1 mutant (Fig. 4A, lane 6). This result implies that MitoBloCK-1 blocks protein translocation at a step similar to the block observed with the tim10-1 mutant, namely translocation across the outer membrane.

The early obstruction in protein translocation by MitoBloCK-1 suggested that binding between the Tim9-Tim10 complex and substrate might be abrogated. We have previously used a cross-linking and immunoprecipitation approach in tim10-1 tim955 mitochondria to show that Tim9 binds to substrate during translocation (18). MitoBloCK-1 was therefore added to import assays that were subjected to cross-linking and immunoprecipitation (Fig. 4B). In the absence of MitoBloCK-1, antibodies against Tim9 immunoprecipitated a cross-linked product between Tim9 and AAC (Fig. 4B, lane 9). However, the presence of MitoBloCK-1 altered the cross-linking pattern such that the cross-link to Tim9 decreased in abundance (Fig. 4B, compare lane 4,6); instead another cross-linked band, indicative of an interaction with another protein, became more prevalent (Fig. 4B, lane 6 denoted by *). Following immunoprecipitation, the cross-linked Tim9-AAC product was decreased in the presence of MitoBloCK-1 (Fig. 4B, compare lane 9, 12). Additional immunoprecipitation assays with antibodies against Tom22 and Tom40 failed to immunoprecipitate cross-linked AAC, regardless of whether MitoBloCK-1 was present. This lack of cross-linking may indicate that the homobifunctional crosslinker BMH, which is reactive to free sulfhydryls, did not have adequate sites for reactivity. As an additional control, AAC with uncoupled mitochondria (incubated with CCCP) lacked abundant cross-links (Fig. 4B, lane 5). Therefore, this analysis supports that MitoBloCK-1 impedes protein translocation at an early stage by obstructing the substrate binding site of the Tim9-Tim10 complex.

**MitoBloCK-1 Can Be Used to Determine Substrates of the Tim9-Tim10 Complex.** A central question about the TIM22 pathway has been the specificity of the small Tim complexes. Yeast contain both the Timm-Tim13 complex and the Tim9-Tim10 complex and a variety of studies have suggested that they might have different substrate specificities (10, 11, 13). Most precursors including the carriers,
Tim22, and Tim17 require the Tim9-Tim10 complex, whereas Tim23 and the aspartate-glutamate carriers require the Tim8-Tim13 complex. In addition, the small Tim proteins facilitate the import of outer membrane proteins (5 and 7). We therefore examined whether MitoBloCK-1 could be used to determine substrate specificity of the Tim9-Tim10 complex with precursors Tim22, Tim23, and Tafazzin (Fig. 5). The import of Tim22 but not Tim23 was impaired in the presence of MitoBloCK-1, indicating that Tim23 seems to require the Tim8-Tim13 complex for translocation across the outer membrane (Fig. 5A, B). Tafazzin import was impaired in mitochondria lacking functional Tim10 (6). When Tafazzin was imported in the presence of MitoBloCK-1, import was inhibited, confirming a role for the Tim9-Tim10 complex in the biogenesis of Tafazzin (Fig. 5C). Studies with MitoBloCK-1 thus support a role for the Tim9-Tim10 complex in the import of Tafazzin and Tim22, but not Tim23.

Taking advantage of commercially available compounds similar to MitoBloCK-1, we purchased additional compounds for an abbreviated structure-activity relationship (SAR) study (Fig. 6A). Similar compounds to MitoBloCK-1 were available in which the side chain was substituted or the tricyclic ring was changed from a dihydrobenzofuran to a carbazole. Analogs A and D were similar to MitoBloCK-1 except that the thiourea of the side chain was modified. Analogs B and C contained changes in the ring (carbazole) as well as the side chain. These compounds were tested in the import assay and Analog D was the only compound to inhibit import of AAC but required an increased concentration of 50 μM (Fig. 6A). A limited SAR analysis showed that properties of the ring structure and side chain are important for MitoBloCK-1 activity.

The long-term goal with these MitoBloCK compounds is to develop small molecules that inhibit protein translocation in mammalian systems for mechanistic studies and for developing tools to alter mitochondrial function with the objective of developing disease models. As a first step, we tested whether MitoBloCK-1 might affect general mitochondrial function in mammalian cells and measured cell viability in mammalian cells using a 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay (Fig. S5A). Given that mitochondrial protein import is essential for cell survival, a reduction in translocation would be expected to reduce cell viability. When cells were treated with 25 μM and 50 μM MitoBloCK-1, viability significantly decreased in a dose-responsive manner. We then tested whether MitoBloCK-1 inhibited import into isolated mouse liver mitochondria (Fig. 6B). In the presence of 25 mM MitoBlock-1, the import of AAC was inhibited. In contrast, the import of Su9-DHFR and Hsp60 was not altered in the presence of MitoBloCK-1 (Fig. S5 B, C). Thus, the addition of MitoBloCK-1 to mammalian mitochondria disrupts the import of AAC, albeit at a higher concentration than with yeast mitochondria.

Discussion

MitoBloCK-1 is a unique small molecule inhibitor that blocks the import of substrates that use the TIM22 import pathway. We started this screen with a genetic approach by developing a composite synthetic lethal screen to identify small molecules that inhibited growth of the tim10-1 mutant at the permissive temperature of 25 °C. Although MitoBloCK-1 may have many potential targets within a yeast cell, we devised a battery of tests using growth analyses followed by biochemical assays to determine the specific site of inhibition by MitoBloCK-1. Because the small molecules may nonspecifically alter mitochondrial function, we determined its effect on membrane potential, respiration, and mitochondrial integrity; MitoBloCK-1 does not generally damage mitochondria. Moreover, import assays showed that import of TIM22 substrates was specifically inhibited and cross-linking and immunoprecipitation assays showed that the Tim9-Tim10 complex did not bind to substrate effectively. The combination of these assays indicated that MitoBloCK-1 inhibits an early step in protein translocation, when the Tim9-Tim10 complex binds to substrate during translocation across the outer membrane (Fig. 6C) (3, 16, 25).

The characterization of MitoBloCK-1 supports that the chemical-genetic approach is important for developing probes to study assembly of mitochondrial membranes. Mechanistic studies for the assembly of outer and IM proteins still need refinement (1). Our analysis shows that Tim9-Tim10 is important for the import of Tafazzin, Tom40, the carrier proteins, and Tim22, but not Tim23, which support that the small Tim complexes have different substrate specificity (3, 4, 10, 13). Therefore, develop-
ment of these probes will yield a new set of tools for studying mitochondrial membrane biogenesis.

A potential drawback of MitoBIOCK-1 is that import is inhibited in the tim10-1 tim95 mitochondria but not wild-type mitochondria. The small SAR studies suggest that particular properties of MitoBIOCK-1, such as the length of the side chain and the dihydrobenzofuran ring, may be important for its function. Therefore MitoBIOCK-1 may serve as a starting point for developing more potent analogs that inhibit protein import in wild-type yeast mitochondria. In addition, the overall structure of the human small Tim proteins is highly conserved with the yeast homologs (2), and we clearly show that import into isolated mammalian mitochondria is inhibited. Following the initial import assays in mammalian mitochondria with an extended SAR approach may lead to the refinement of small molecules that inhibit function of the different mammalian small Tim proteins.

Mitochondria now have been implicated in a wide array of degenerative diseases including Parkinson’s and Alzheimer’s (27–30). For example, a defect in import has been linked to Alzheimer’s when the amyloid precursor protein arrests in the Tom40 translocon (30). These latest developments indicate that alteration of protein translocation pathways may be important for (1) mechanistic studies in these diseases and (2) to create model systems to recapitulate the disease. Thus, having new and specific tools available such as the MitoBIOCK compounds may be important for broad research in understanding how mitochondrial dysfunction contributes to disease. The development of small molecule inhibitors also serves as a technological advance over general mitochondrial inhibitors (uncouplers and inhibitors of respiration) that uncouple mitochondria or irreversibly inhibit respiration.

Materials and Methods
High-Throughput Screening. A primary screen was performed using freshly streaked tim10-1 diluted in YPD to an OD600 of approximately 0.0002 and kept on ice throughout the screening run. A Titertek multidrop was used to dispense 40 μL of cell suspension to all wells of each clear 384-well plate (Greiner Bio One). After yeast suspension warmed to room temperature, a Biomek FX (Beckman Coulter) was used to pin transfer 0.5 μL of compound from 1 mM stock or DMSO to respective wells. Approximate screening concentration was 12.5 μM. All operations were performed by an automated plate scheduler to ensure consistency across the screening run. After completion of compound transfer, all plates were incubated in a humidified incubator until the OD600 reached approximately 0.8 in the control wells; the control consisted of the tim10-1 mutant with the vehicle 1% DMSO. Each plate was shaken in a Beckman orbital shaker to resuspend settled cells, and the OD600 in each well was read by a Wallac Victor plate reader (Perkin Elmer). The top 600 growth inhibitory compounds were determined and assessed into two plates. Using a similar screening methodology, hit compounds were reconfirmed with the tim10-1 strain and growth inhibition was compared to the WT strain (TIM10) as well as the “rescued” strain (tim10-1 TIM10 that contained a copy of the wild-type TIM10 genes on a centromeric plasmid) strains. Compounds reordered from Asinex and Chembridge were assayed for MIC4 using a similar automated technique in 384-well plates as previously described. Serial dilutions of purchased compounds were performed with robotic automation in 100% DMSO. Subsequently, compounds were pinned into assay plate wells containing 50 μL of the respective yeast strain in YPD medium (starting OD600 = 0.0002). Growth duration and conditions were similar to the original screen.

Biochemical Assays with Mitochondria and Additional Methods. Detailed methods are listed in the SI Text.

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Supporting Information

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SI Text

Plasmids and Strains. In general, a standard set of genetic and molecular techniques were used to generate the strains in this study (1 and 2). Screening strains were generated based on previously characterized temperature-sensitive mutants (see Table S1). The snq1 and pdr5 deletions were introduced in each strain by strain mating with MDY326 or PCR-mediated deletion (3 and 4). Overexpression strains were generated by transforming 2μ yeast shuttle vectors carrying the gene of interest with the native promoter into the am10-1 strain using a standard LiCl protocol (5). Transformed yeast was maintained on selective media appropriate for the plasmid’s auxotrophic marker. Strains lacking mitochondrial DNA (rho null) were generated by two rounds of selection of the parent on YPD plates supplemented with ethidium bromide (40 μg/mL) followed by two rounds of single colony selection on YPD plates.

Media and Reagents. Media used in this study was purchased from EMD Biosciences and US Biological. Chemical reagents were from Chembridge, Asinex, and Sigma unless otherwise noted. YPD medium is 1% Bacto-peptone, 2% Bacto-yeast extract, and 1% glucose with appropriate amino acid dropout mixture. YPD and YEPD plates used in growth analysis included 2% agar. For 1-(4,5-Dimethylthiazol-2-yl)-3-(3-Hydroxyphenyl)triazol (MTT) assays, cultured HeLa were grown in DMEM high glucose medium (Invitrogen) with glutamine, sodium pyruvate, 10% FBS, and penicillin-streptomycin (complete medium).

Analysis and Statistics. Unless otherwise stated, all results reported are representative of three experimental replicates. Quantitative analysis was performed in GraphPad Prism 5 software (GraphPad Software, Inc.) unless otherwise stated. Statistical tests for significant deviation between samples were performed with unpaired, two-tailed t-tests. The alpha threshold for significance was <0.05 for all tests. In graphs, error bars represent standard deviation from a given mean. Data transformation of rhodamine 123 fluorescence data was performed by setting the maximum 2g = 530 nanometer (nm) value from a particular trace to 100%. All fluorometry data was scaled to the 0–100% range using GraphPad Prism’s “normalize” function.

Purification of Mitochondria. Mitochondria were purified from yeast cells grown in YPEG or selective SEG medium as described in previous studies (6). Yeast cultures were kept at a constant 25 °C with vigorous shaking during growth. After concentration was determined by BCA assay, mitochondrial preparations and were either in YPEG (1% yeast dextrose added to 2% after sterilization. Yeast cultures for mitochondrial preparation and were either in YPEG (1% yeast extract, 2% peptone, 3% glycerol, 3% ethanol) or selective ethanol-glycerol SEG medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 3% glycerol, 3% ethanol) with appropriate antibiotic and with appropriate amino acid dropout mixture. YPD and YEPD plates used in growth analysis included 2% agar. For 1-(4,5-Dimethylthiazol-2-yl)-3-(3-Hydroxyphenyl)triazol (MTT) assays, cultured HeLa were grown in DMEM high glucose medium (Invitrogen) with glutamine, sodium pyruvate, 10% FBS, and penicillin-streptomycin (complete medium).

Membrane Potential and Oxygen Consumption. Oxygen consumption of tim10-1 tim9S mitochondria was measured using methods of microtubular tubes. Next, supernatant material was spun for 10 min at 800 × g (this process was repeated a second time). The supernatants from these steps were subjected to a high-speed (12,000 × g for 20 min) spin to pellet heavy membrane fractions. Pellets were washed in isolation buffer and spun again (12,000 × g for 20 min). After the final centrifugation step, supernatants were discarded and heavy membrane fraction was resuspended in mammalian import buffer (250 mM sucrose, 5 mM magnesium acetate, 80 mM potassium acetate, 10 mM sodium succinate, 1 mM dithiothreitol, 0.1 mM ADP, 20 mM Hepes-KOH, pH 7.4) as described in (7) and kept on ice. All subsequent imports were performed within 1 h of isolation of mammalian mitochondria. Mitochondrial concentrations were determined by BCA assay.

Blue-Native Gel Electrophoresis. Steady-state levels of the small Tim complexes were analyzed from mitochondria isolates from TIM10, tim10-1, and tim10-1 tim9S strains following established methods (8). Approximately 200 μg of mitochondria from each strain was solubilized at 5 mg/mL in 0.16% n-dodecylmaltoside (Anatrace) for 30 min on ice. Following removal of insoluble material (30 min centrifugation at 14,000 rpm), solubilized protein supernatants were analyzed by blue-native gel electrophoresis on a 6–16% linear polyacrylamide gradient (9–11).

Import of Radiolabeled Proteins into Mitochondria and Cross-linking. Prior to import into purified mitochondria, 35S-methionine and cysteine labeled proteins were generated with TNT Quick Coupled Transcription/Translation kits (Promega) and plasmids carrying the gene of interest. Transcription of the genes was driven by either a T7 or SP6 promoter. Import reactions were conducted according to established methods. After frozen mitochondria aliquots were thawed and added to the import buffer at a final concentration of 100 μg/mL, drug or DMSO vehicle was added as indicated. A final vehicle concentration of 1% was used in all experiments. Following 15 min incubation at 25 °C, import reactions were initiated by the addition of 5–10 μL of translation mix. Aliquots were removed at intervals during the reaction course and import was terminated with either cold buffer, 25 μg/mL trypsin, or a combination of both. If trypsin was added to digest unimported precursor protein, soybean trypsin inhibitor was subsequently added in excess after 15 min incubation on ice. After a final recovery by centrifugation (8,000 × g, 5 min), mitochondria were disrupted in Laemmli sample buffer. Import of membrane proteins (AAC, Pic, Tom40, Tom22, and Tom23) included a carbonate extraction step to remove proteins that had not inserted into the membrane (12). Samples from import reaction time points were resolved by SDS-PAGE and gels were dried prior to exposing to film.

Cross-linking and immunoprecipitation experiments were derived from procedures previously utilized (8) with the inclusion of MitoBloCK-1 or DMSO. Following import, a portion of the reaction was subjected to cross-linking with 0.5 mM bis-maleimido-dodecane (BMH) for 30 min on ice. After quenching cross-linking reactions with 1 mM β-mercaptoethanol, a fraction of each sample was subjected to immunoprecipitation with polyclonal antibodies against either Tim9, Tom22, or Tom40. For each immune precipitation, 20 μL of antisera was bound to 50 μL of protein A-Sepharose slurry according to established protocols (8).
previously described (13). Briefly, purified \textit{tim10-1 tim9S} mitochondria aliquots (25 mg/mL) were thawed on ice and tested within 2 h. A Clark-type oxygen electrode in a stirred, thermostatically controlled 1.5-mL chamber at 25 °C (Oxytherm; Hansatech) facilitated measurement. State II respiration was induced on a suspension of 100 mg/mL mitochondrial in 0.25 M sucrose, 20 mM KCl, 20 mM Tris-Cl, 0.5 mM EDTA, 4 mM KH$_2$PO$_4$, and 3 mM MgCl$_2$, pH 7.2 after adding 2 mM NADH. Consumption rate was monitored for approximately 2 min. Drug or DMSO was then added to a final vehicle concentration of 1% and respiration was measured for another approximately 1.5 min. Uncoupled respiration was achieved by adding 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to the chamber.

Membrane potential measurement assays were conducted with a SPEX spectrophotometer system (HORIBA) with a magnetically stirred cuvette held at 25 °C. The quenching of rhodamine 123 fluorescence ($\lambda_{em}$ = 530 nm and $\lambda_{ex}$ = 485 nm) was used as previously described (14) to detect changes in mitochondrial membrane potential. Purified \textit{tim10-1 tim9S} mitochondrial aliquots were thaw and resuspended in respiration buffer (0.65 M mannitol, 0.3 mM EGTA, 3 mM tris-phosphate, 10 mM tris-malate, pH 6.75). Trials were started by adding 100 nM of rhodamine 123 to respiration buffer. After a period of signal stabilization, mitochondria were added to a concentration of 100 μg/mL. Either drug or DMSO was then added to a final vehicle concentration of 1% following the establishment of baseline quenched fluorescence. Finally, mitochondria were uncoupled with 3 μM CCCP.

\textbf{Cell Viability Assays.} Measurements of cell viability/toxicity were made with a MTT based toxicity assay kit (Sigma-Aldrich). HeLa cells were grown in 24-well tissue culture dishes to 80% confluency. Following this cells were either left untreated or treated with 1% DMSO or drug in complete medium for 12 h. Following drug treatment, cells were rinsed with phosphate buffered saline and incubated with complete media with MTT solution supplement for additional 4 h as described in manufacturer’s protocols. This media was removed and 500 μL of MTT solubilization solution was added to dissolve the formazan crystals. The formazan absorbance was measured at $\lambda$ = 570 nm on a Wallac Victor plate reader (Perkin Elmer) along with a turbidity measurement at $\lambda$ = 630 nm. After turbidity subtraction, the percent viability of each cell sample was calculated as: $\frac{\text{absorbance of vehicle treated cells} - \text{absorbance of drug treated cells}}{\text{absorbance of vehicle treated cells}} \times 100$.

\textbf{Miscellaneous.} Steady-state levels of mitochondrial proteins from lysed aliquots of isolated mitochondria were resolved using SDS-PAGE. Western blotting was performed using standard protocols with polyclonal antibodies raised towards highly purified antigens. Proteins were transferred to nitrocellulose membranes and immune complexes were visualized with HRP labeled Protein A in a chemiluminescence assay (Pierce). Chemiluminescent and autoradiographic imaging was performed on film unless otherwise noted. Unless otherwise stated, all results reported are representative of three experimental replicates.

Fig. S1. Phenotypic analysis of the strains used for the chemical synthetic-lethality screen for inhibitors of the TIM22 protein import pathway. (A) Steady-state levels of mitochondrial proteins determined by immunoblot analysis. Equivalent amounts of purified mitochondria were prepared from each strain and mitochondrial proteins were subsequently immunoblotted with polyclonal antibodies. The antibody against AAC also cross-reacted with porin (denoted by *) (B) Mitochondria were solubilized in buffer with 1.6 mg/mL n-dodecylmaltoside and separated on a 6–16% blue-native gel. Proteins were transferred to a PVDF membrane and blotted with antibodies against Tim9 and Tim10.

Fig. S2. MitoBloCK-1 does not inhibit AAC import into wild-type mitochondria. Import of AAC was performed as described in Fig. 3A into wild-type mitochondria. The rate of import was similar in the presence of the vehicle DMSO or MitoBloCK-1.
Fig. S3. MitoBloCK-1 inhibits the import of substrates that use the TIM22 import pathway but not the TIM23 and Mia40/Erv1 import pathways. Import assays were performed as described in Fig. 3. Precursors include (A) Su9-dihydrofolate reductase (DHFR), (B) cytochrome b$_2$-DHFR, (C) Tim10, (D) Tim9, (E) Mia40. A–B are proteins that use the TIM23 import pathway and C–D are intermembrane space proteins that use the Mia40 import pathway. (F) Import assays were performed with AAC into tim12-1 mutant mitochondria. p, precursor; i, intermediate; m, mature.
Fig. S4. MitoBloCK-1 does not impair general mitochondrial function. Respiration measurements were performed with an oxygen electrode using yeast mitochondria (M) from the tim10 tim9S suppressor strain in the presence of (A) 1% DMSO (vehicle control for drug) and (B) MitoBloCK-1. Respiration was initiated with NADH addition. 25 μM MitoBloCK-1 or 1% DMSO was added once steady-state respiration had been established. As a control, CCCP was added to uncouple the electron transport chain. (C) Respiration for series with DMSO or MitoBloCK-1 addition was quantitated (n = 3). Bars represent mean rates with standard deviations as error bars. (D) Membrane potential (Δψ) measurements of purified mitochondria were performed with the fluorescent dye rhodamine 123 using a fluorimeter. Coupled mitochondria (M) sequestered and quenched the dye fluorescence; 1% DMSO was added to determine its effect on the Δψ. Collapse of the Δψ initiated by CCCP was included as a control. (E) As in D, but 25 μM MitoBloCK-1 was added to determine its effect on the Δψ. (F) 50 μM MitoBloCK-1 (MB-1) was added to purified 100 μg/mL tim10-1 tim9S mitochondria for 30 min at 25 ºC in import buffer and released proteins (S) were separated from mitochondria (P) by centrifugation at 8,000 x g for 5 min. Immunoblot analysis was performed to determine fractionation for Hsp60, Tom40, AAC, cyt c, and Tim10. As a control, treatment with the vehicle (1% DMSO) and MitoBloCK-2 (MB-2, disrupts mitochondrial membranes) was included. (G) As in F, but integrity was investigated with Coomassie staining. (H) As in Fig. S1B, MitoBloCK-1 (25 and 50 μM) was incubated with mitochondria and the steady-state stability of the Tim9-Tim10 complex was monitored by blue-native gels and immunoblotting with antibodies against Tim10.
Fig. S5. MitoBlock-1 inhibits import into mammalian mitochondria and growth of HeLa cells. (A) The effect of MitoBlock-1 (MB-1) on HeLa cells was demonstrated with an MTT cell viability assay. Cultured cells were treated for 24-h with DMSO or 25 and 50 μM MitoBlock-1. Bars display mean cell viability where 100% was defined as signal from untreated samples. Error bars are standard deviations (n = 3 trials). P value for t-tests between DMSO and MitoBlock-1 is illustrated with bracket lines. (B, C) As a control for Fig. 7B, the import of Hsp60 and Su9-DHFR that are targeted to the matrix was also tested in isolated mouse liver mitochondria in the presence and absence of a membrane potential. Note for Su9-DHFR import, the mitochondria were not treated with protease after import to remove nonimported Su9-DHFR because the DHFR is resistant to protease degradation. The processed form (mature, m) indicates the amount of precursor that has been imported. p, precursor; m, mature.
### Table S1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>tim10-1 rho null</strong></td>
<td>his3, leu2, ura3, trp1, ade8,Δpdr5Δ0::HGR, snq2Δ0::KANMX, rho null</td>
<td>Strain used for the primary screen. A tetrad containing the tim10-1 allele and drug pump deletions were selected.</td>
<td>(1); this study</td>
</tr>
<tr>
<td><strong>tim10-1</strong></td>
<td>his3, leu2, ura3, trp1, ade8,Δpdr5Δ0::HGR, snq2Δ0::KANMX, pdr5Δ0:URA, snq2Δ0::KANMX</td>
<td>Strain was incubated on ethidium bromide to remove mitochondrial DNA. Original tim10-1 strain was mated to MDY326 and sporulated.</td>
<td>(1); this study</td>
</tr>
<tr>
<td><strong>tim10-73</strong></td>
<td>ade8, his3, leu2, ura3, Δtrp1::LEU2, Δtim10::HIS3, pdr5Δ0::URA, snq2Δ0::KANMX, [pTim10-73::TRP1 CEN]</td>
<td>Original tim10-73 strain was mated to a version of MDY326-trp1. After sporulation, a tetrad containing tim10-73 allele and drug pump deletions were selected.</td>
<td>(1); this study</td>
</tr>
<tr>
<td><strong>tim9-3</strong></td>
<td>ade8, his3, leu2, ura3, Δtim9::TRP1, Δpdr5::HIS3, Δsnq2::URA3, [ptim9-3::LEU2 CEN]</td>
<td>Original tim9-3 strain was deleted for PDR5 and SNQ2 using PCR mediated deletion.</td>
<td>(2); this study</td>
</tr>
<tr>
<td><strong>tim23-2</strong></td>
<td>ade8, his3, leu2, ura3, trp1, ura3, Δpdr5::HIS3, Δsnq2::LEU2</td>
<td>Original tim23-2 strain was deleted for PDR5 and SNQ2 using PCR mediated deletion.</td>
<td>(3)</td>
</tr>
<tr>
<td><strong>TIM10 rho null</strong></td>
<td>his3, leu2, ura3, TIM10:URA3, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, rho null</td>
<td>Strain was incubated on ethidium bromide to remove mitochondrial DNA. Strain used as primary screen control. The tim10-1 strain used for screening was restored to wild-type at the TIM10 locus by integration of the TIM10 allele to replace the mutant allele.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>TIM10</strong></td>
<td>his3, leu2, ura3, TIM10:URA3, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX</td>
<td>Strain was incubated on ethidium bromide to remove mitochondrial DNA. Strain used as primary screen control. The tim10-1 strain used for screening was restored to wild-type at the TIM10 locus by integration of the TIM10 allele to replace the mutant allele.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim10-1 TIM10</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [pTim10-1:URA3 CEN]</td>
<td>Centromeric plasmid carrying TIM10 under the control of its native promoter was transformed into the tim10-1 screening strain. This was used as a second control strain in screening.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim10-1 tim95</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [ptim9-5::URA3 CEN]</td>
<td>Centromeric plasmid carrying a suppressing allele tim95 under the control of the TIM9 promoter was transformed into the tim10-1 screening strain.</td>
<td>(4, 5)</td>
</tr>
<tr>
<td><strong>tim10-1 TIM9 (2µ)</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [pTim9-1::URA3 2µ]</td>
<td>A 2µ plasmid carrying TIM9 under the control of its native promoter was transformed into the tim10-1 screening strain.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim10-1 TIM8 (2µ)</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [pTim8-1::URA3 2µ]</td>
<td>A 2µ plasmid carrying TIM8 under the control of its native promoter was transformed into the tim10-1 screening strain.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim10-1 TIM13 (2µ)</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [pTim13-1::URA3 2µ]</td>
<td>A 2µ plasmid carrying TIM13 under the control of its native promoter was transformed into the tim10-1 screening strain.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim10-1 TIM22 (2µ)</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [pTim22-1::URA3 2µ]</td>
<td>A 2µ plasmid carrying TIM22 under the control of its native promoter was transformed into the tim10-1 screening strain. Plasmid contained a high copy 2µ origin of replication.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim10-1 TIM23 (2µ)</strong></td>
<td>his3, leu2, ura3, trp1, ade8, Δtim10::LEU2, Δtim10::HIS3, pdr5Δ0::HGR, snq2Δ0::KANMX, [pTim23-1::URA3 2µ]</td>
<td>A 2µ plasmid carrying TIM23 under the control of its native promoter was transformed into the tim10-1 screening strain.</td>
<td>This study</td>
</tr>
<tr>
<td><strong>tim12-1</strong></td>
<td>his3, leu2, ura3, Δtrp1, ade8, Δtim12::LEU2, Δtim12::HIS3</td>
<td>Strain used as a control for import studies. The strain is deleted for TIM12 and contains the tim12-1 mutant allele integrated at the LEU2 locus.</td>
<td>(1)</td>
</tr>
<tr>
<td><strong>MDY326</strong></td>
<td>his3, leu2, ura3, pdr5Δ0::URA, snq2Δ0::KANMX</td>
<td>Strain with multidrug pumps deleted.</td>
<td>(6)</td>
</tr>
<tr>
<td><strong>MDY326-trp1</strong></td>
<td>his3, leu2, ura3, Δtrp1::LEU2 pdr5Δ0::URA, snq2Δ0::KANMX</td>
<td>The trp1 allele was deleted with LEU2 in MDY326.</td>
<td>This study</td>
</tr>
</tbody>
</table>