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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 molecule agonists that caused lethality to a *tim10-1* yeast mutant at the permissive temperature. One molecule, MitoBloCK-1, attenuated the import of the carrier proteins including the ADP/ATP and phosphate carriers, but not proteins that used the TIM23 or the Mia40/Erv1 translocation pathways. MitoBloCK-1 impeded binding of the Tim9-Tim10 complex to the substrate during an early stage of translocation, when the substrate was crossing the outer membrane. As a probe to determine the substrate specificity of the small Tim proteins, MitoBloCK-1 impaired the import of Tim22 and Tafazzin, but not Tim23, indicating that the Tim9-Tim10 complex mediates the import of a subset of inner membrane proteins. MitoBloCK-1 also inhibited growth of mammalian cells and import of the ADP/ATP carrier, but not TIM23 substrates, confirming that MitoBloCK-1 can be used to understand mammalian mitochondrial import and dysfunction linked to inherited human disease. Our approach of screening chemical libraries for compounds causing synthetic genetic lethality to identify inhibitors of mitochondrial protein translocation in yeast validates the generation of new probes to facilitate mechanistic studies in yeast and mammalian mitochondria.

chemical biology | chemical genetics

The mitochondrion has an outer (OM) and inner (IM) membrane that separates the matrix from the intermembrane space (IMS). The mitochondrion has developed an elaborate translocation system to orchestrate the import and subsequent sorting of proteins to the correct compartment (1). Proteins destined for the mitochondrion, termed precursors until they reach their correct location, utilize Translocase of the Outer Membrane (TOM) and Translocase of the Inner Membrane (TIM) complexes, TIM23 and TIM22, to cross the OM and IM, respectively. Proteins with a typical N-terminal targeting sequence use the TIM23 translocation system, whereas proteins destined for the IM use the TIM22 translocation system.

Components of the TIM22 translocation system include the small Tim proteins, Tim8, Tim9, Tim10, Tim12, and Tim13, and the membrane components Tim18, Tim22, and Tim54. The small Tim proteins assemble in 70-kDa hexameric complexes (referred to as small Tim complexes) in the IMS in which three Tim9 polypeptides partner with three Tim10 polypeptides, and three Tim8 polypeptides partner with three Tim13 polypeptides. Structural studies reveal that the overall structure is similar to that of the Skp and prefoldin chaperones (2), although the sequences are not conserved. The small Tim proteins function as chaperones to maintain the hydrophobic membrane proteins in an import competent state (3 and 4). The 300-kDa insertion complex in the IM consists of a fraction of Tim9 and Tim10 with Tim12, Tim22, Tim18, and Tim54. The small Tim proteins escort substrates to the insertion complex, which mediates protein insertion into the membrane.

Substrates of the TIM22 complex include the carrier proteins such as the ADP/ATP carrier (AAC) and the phosphate carrier (PiC) and IM proteins Tim17, Tim22, and Tim23. In addition, the small Tim proteins facilitate the insertion of outer membrane proteins Tom40 and porin and the cardiolipin remodeling enzyme Tafazzin (5–7). The substrates cross the TOM complex as a loop in an unfolded state and then the small Tim proteins bind to the substrate at an early stage of translocation (4, 8, 9).

The Tim8-Tim13 and Tim9-Tim10 complexes display different substrate binding preferences. The Tim9-Tim10 complex can be efficiently cross-linked to carrier proteins and the import components Tim17, Tim23, and Tim22 (10–12). The Tim8-Tim13 complex can be cross-linked to Tim23 and the aspartate–glutamate carriers (10–13). Mutations in the human homolog of Tim8, DDP1, cause the X-linked disease deafness-dystonia syndrome (14 and 15), and the disease may be caused by a decrease in specific IM proteins (13). Therefore, understanding 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 not been resolved. 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 *tim10-1* mutant to identify small molecules that caused a 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. 1*A*). 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. S1*A*) (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. S1*B*). Moreover, in vitro import of the TIM22 pathway substrate, AAC, was inhibited in comparison to mitochondria from the parental strain (Fig. 1*B*). The *tim10-1* mutant thus has excellent growth properties for conducting a synthetic genetic screen with small compounds to target the TIM22 import pathway.

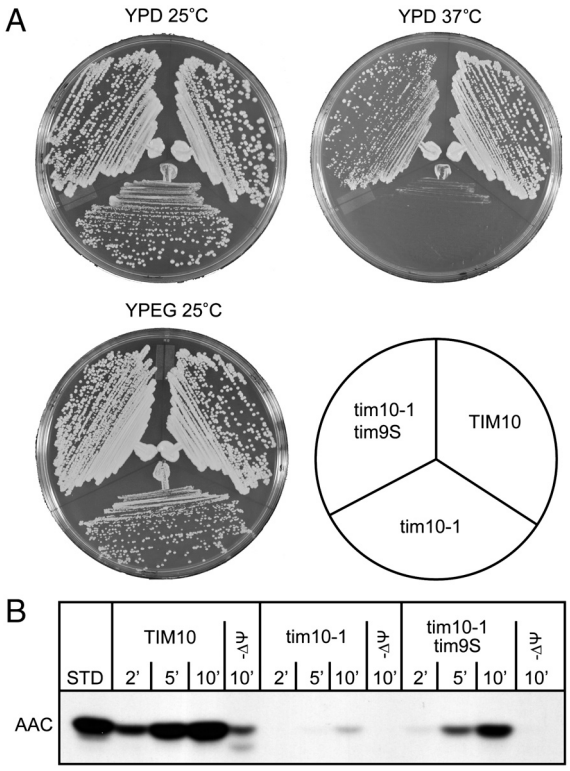

Fig. 1. Phenotypic analysis of the strains used for the chemical synthetic-lethality screen for inhibitors of the TIM22 protein import pathway. (*A*) Growth phenotypes of the control (*TIM10*), the *tim10-1* mutant, and *tim10-1* suppressor (*tim10-1 tim9S*) strains used in the screen. Strains were plated 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 ($\Delta\psi$). Aliquots were removed at the indicated time points and samples were treated with carbonate extraction to confirm that AAC was inserted into the IM.

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. 1*A*) and import of the carrier proteins were restored (Fig. 1*B*). Suppression in this strain is caused by a Ser → Cys mutation in Tim9; the mutated serine residue is nine amino acids after the second CX_3C motif (17). Whereas the specific mechanism of suppression is not understood, the mutant Tim9 protein restored the abundance of Tim10 (Fig. S1*A*) and the assembly of Tim9-Tim10 complexes, albeit of aberrant sizes (Fig. S1*B*).

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 $OD_{600} = 0.7$. 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. 2*A*). The MIC_{50} for MitoBloCK-1 that inhibited growth of the *tim10-1* mutant was approximately 1 μM (Fig. 2*B*). MitoBloCK-1 had a similar MIC_{50} with another temperature-sensitive *tim10* mutant, *tim10-73*. In contrast, the MIC_{50} for the isogenic control was greater than 200 μM. To understand the cell-based activity of MitoBloCK-1, we also determined the MIC_{50} with other yeast mutants that also were disrupted for *prd5* and *snq2* (Table 1). For mutants within the TIM22 pathway, MitoBloCK-1 displayed an MIC_{50} concentration of 11 μM for the *tim9-3* mutant and 10 μM for the *tim10-1 tim9S* suppressor strain, respectively. In contrast, the MIC_{50} for MitoBloCK-1 in the *tim23* mutant was

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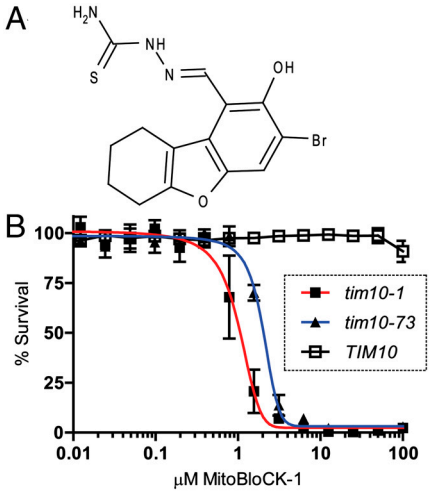

Fig. 2. MitoBloCK-1 exhibits a chemical synthetic lethality with the *tim10-1* mutant. (*A*) The structure of MitoBloCK-1, a tetrahydrodibenzofuran compound. (*B*) MIC_{50} analysis of two *tim10* mutants (*tim10-1* and *tim10-73*) and the parental (*TIM10*) strain with MitoBloCK-1. Average%survival ± SD of $n = 3$ trials. The R^2 value for *tim10-1* and *tim10-73* curve fits were 0.98 and 0.99, respectively.

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 *tim9* and *tim10* mutants, 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. 1*B*). 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. 3*A*). In contrast, MitoBloCK-1 did not inhibit import into WT mitochondria (Fig. S2). Thus, the MIC_{50} in the import assays agree well with the cell growth assays (Table 1 and Fig. 2*B*).

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

Strain	MIC_{50}* (μM)	MIC_{50}* (mg/mL)
tim10-1 rho null	0.75 ± 0.02	0.28 ± 0.01
tim10-1	1.00 ± 0.05	0.37 ± 0.03
tim10-73	2.00 ± 0.06	0.74 ± 0.02
tim9-3	11.34 ± 1.56	4.18 ± 0.57
tim23-1	>200	>74
TIM10 rho null	12.39 ± 0.9	4.56 ± 0.33
TIM10	>200	>74
tim10-1 TIM10	>200	>74
tim10-1 tim9S	9.91 ± 0.24	3.65 ± 0.09
tim10-1 TIM9 (2μ)	1.48 ± 0.08	0.54 ± 0.03
tim10ts TIM8 (2μ)	2.42 ± 0.15	0.89 ± 0.05
tim10-1 TIM13 (2μ)	1.26 ± 0.03	0.46 ± 0.01
tim10-1 TIM22 (2μ)	8.35 ± 0.27	3.07 ± 0.1
tim10-1 TIM23 (2μ)	1.37 ± 0.04	0.51 ± 0.02

*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_2-DHFR as well as Hsp60 that use the TIM23 pathway, MitoBloCK-1 did not impair import (Fig. 3*D*, 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. S3*F*). 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. S4*A*–*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. S4*A*) or 25 μM MitoBloCK-1 (~25-fold above the biochemical MIC_{50}) did not significantly alter the rate of respiration (Fig. S4*A*–*C*) ($p = 0.72$). As a control, mitochondria were treated with the proton ionophore CCCP; and respiration increased drastically, indicative of uncoupled mitochondria (Fig. S4*A*–*C*).

The membrane potential ($\Delta\psi$) of mitochondria was measured with the fluorescent dye rhodamine 123, which is taken up by mitochondria and then released when the $\Delta\psi$ is dissipated (22 and 23). The relative change of fluorescence between dye uptake and release is a relative measure of the $\Delta\psi$; 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. S4*D*) or 25 μM MitoBloCK-1 (Fig. S4*E*) 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 x *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. S4*F*). 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. S4*G*). 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. S4*H*). From the aforementioned analysis, MitoBloCK-1 does not alter mitochondrial function or membranes

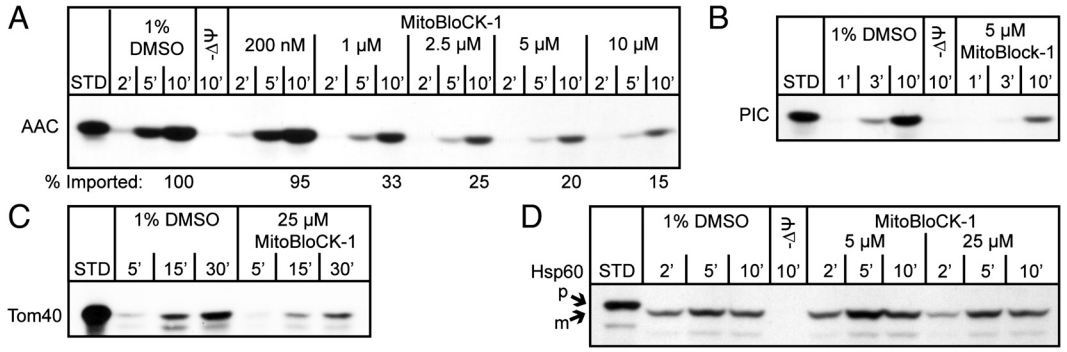

Fig. 3. MitoBloCK-1 inhibits the import of substrates that use the TIM22 import pathway. Import assays were performed with radiolabeled precursors into mitochondria from the *tim10*-1 *tim9S* suppressor strain, which has restored import of AAC. Time course assays were completed with various concentrations of MitoBloCK-1 or the vehicle control (1% DMSO). Nonimported precursor was removed by protease treatment. Precursors include (*A*) AAC, (*B*) the phosphate carrier (PIC), (*C*) Tom40, and (*D*) Hsp60, (*A–C*) represent precursors that use the TIM22 import pathway whereas *D* is a substrate of the TIM23 import pathway. p, precursor; m, mature.

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. 4*A*, *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. 4*A*, *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. 4*A*, *lane 12*), similar to that of the *tim10-1* mutant (Fig. 4*A*, *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 tim9S* 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. 4*B*). In the absence of MitoBloCK-1, antibodies against Tim9 immunopreciptated a cross-linked product between Tim9 and AAC (Fig. 4*B*, *lane 9*). However, the presence of MitoBloCK-1 altered the cross-linking pattern such that the cross-link to Tim9 decreased in abundance (Fig. 4*B*, *compare lane 4,6*); instead another cross-linked band, indicative of an interaction with another protein, became more prevalent (Fig. 4*B*, *lane 6 denoted by* *). Following immunoprecipitation, the cross-linked Tim9-AAC product was decreased in the presence of MitoBloCK-1 (Fig. 4*B*, *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. 4*B*, *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 Tim8-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,

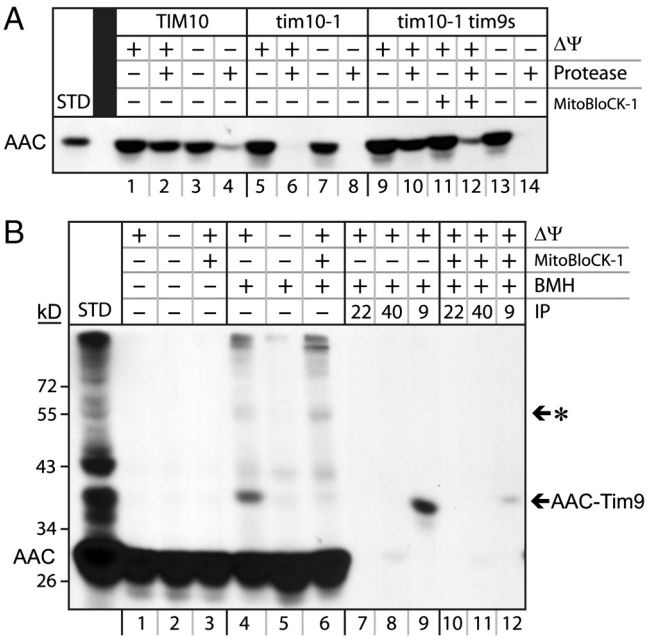

Fig. 4. MitoBloCK-1 impairs substrate binding by the Tim9-Tim10 complex. (*A*) AAC was imported into mitochondria isolated from *TIM10*, *tim10-1*, and suppressor *tim10-1 tim9S* strains in the presence and absence of a $\Delta\psi$. Where indicated, MitoBloCK-1 was included in the *tim10-1 tim9S* mitochondria. After importing AAC 15 min, reactions were stopped with either cold buffer or trypsin (protease). (*B*) AAC was imported into *tim10-1 tim9S* mitochondria in the presence of 25 μM MitoBloCK-1 or uncoupled mitochondria (*lanes 1–3*), A fraction of the import reaction was treated with the irreversible cysteine cross-linker bismaleimidohexane (BMH) (*lanes 4–6*). BMH-treated samples were divided and aliquots were subjected to immunoprecipitation (IP) with either Tim22 (22), Tom40 (40), or Tim9 (9) polyclonal antibodies bound to protein A-Sepharose beads (*lanes 7–12*). In addition to the previously characterized Tim9-AAC cross-link, a second cross-link of approximately 55 kD (denoted by *) was prevalent in the MitoBloCK-1 and BMH treated sample (*lane 6*).

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. 5 *A*, *B*). Tafazzin is a cardiolipin remodeling enzyme that, when mutated, causes the inherited disease Barth Syndrome (26). 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. 5*C*). 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. 6*A*). 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. 6*A*). 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. S5*A*). 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. 6*B*). 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.

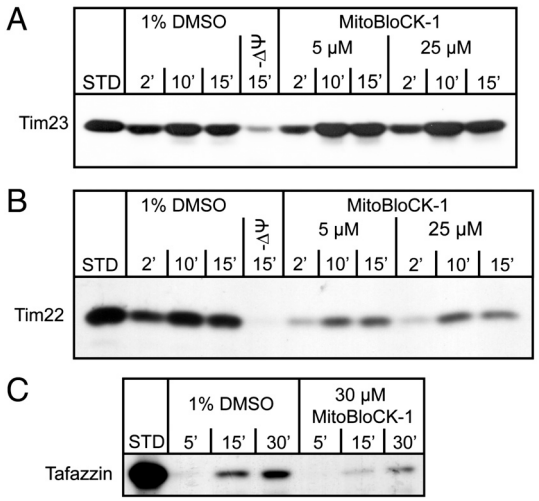

Fig. 5. MitoBloCK-1 facilitates substrate specificity analysis. Tim22 (*A*), Tim23 (*B*), and Tafazzin (*C*) were imported into *tim10-1 tim9S* mitochondria in the presence of MitoBloCK-1 or the vehicle (1% DMSO) followed by carbonate extraction to confirm insertion into the membrane.

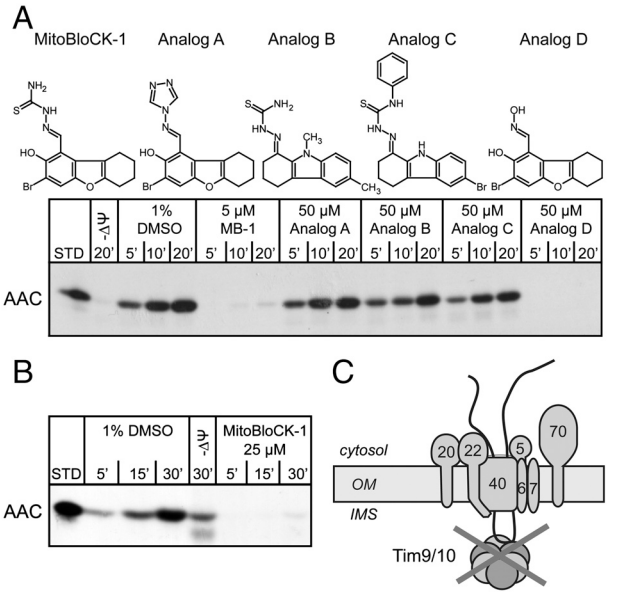

Fig. 6. MitoBloCK-1 activity is influenced by specific chemical characteristics and inhibits AAC imported into mammalian mitochondria. (*A*) Analogs of MitobloCK-1 were purchased from Chembridge and assayed in import assays with radiolabled AAC as previously described. (*B*) AAC was imported into isolated mouse liver mitochondria in the presence of 25 μM MitoBloCK-1 as in Fig. 3*A*. (*C*) Model of MitoBloCK-1 activity from experimental evidence. See text for more details.

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. 6*C*) (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 MitoBloCK-1 is that import is inhibited in the *tim10-1 tim9S* mitochondria but not wild-type mitochondria. The small SAR studies suggest that particular properties of MitoBloCK-1, such as the length of the side chain and the dihydrobenzofuran ring, may be important for its function. Therefore MitoBloCK-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 MitoBloCK 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 OD_{600} 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 completed compound transfer, all plates were incubated at 25 °C in a humidified incubator until the OD_{600} 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 OD_{600} in each well was read by a Wallac Victor plate reader (Perkin Elmer). The top 600 growth inhibitory compounds were determined and assembled 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 MIC_{50} 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 $OD_{600} = 0.0002$). 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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1. Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138:628–644.
2. Webb CT, Gorman MA, Lazarou M, Ryan MT, Gulbis JM (2006) Crystal structure of the mitochondrial chaperone TIM9.10 reveals a six-bladed alpha-propeller. *Mol Cell* 21:123–133.
3. Curran SP, Leuenberger D, Oppliger W, Koehler CM (2002) The Tim9p-Tim10p complex binds to the transmembrane domains of the ADP-ATP carrier. *EMBO J* 21:942–953.
4. Curran SP, Leuenberger D, Schmidt E, Koehler CM (2002) The role of the Tim8p-Tim13p complex in a conserved import pathway for mitochondrial polytopic inner membrane proteins. *J Cell Biol* 158:1017–1027.
5. Hoppins SC, Nargang FE (2004) The Tim8-Tim13 complex of Neurospora crassa functions in the assembly of proteins into both mitochondrial membranes. *J Biol Chem* 279:12396–12405.
6. Brandner K, et al. (2005) Taz1, an outer mitochondrial membrane protein, affects stability and assembly of inner membrane protein complexes: implications for Barth Syndrome. *Mol Biol Cell* 16:5202–5214.
7. Wiedemann N, et al. (2004) Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway. *J Biol Chem* 279:18188–18194.
8. Leuenberger D, Curran SP, Wong D, Koehler CM (2003) The Role of Tim9p in the assembly of the TIM22 import complexes. *Traffic* 4:144–152.
9. Beverly KN, Sawaya MR, Schmid E, Koehler CM (2008) The Tim8-Tim13 complex has multiple substrate binding sites and binds cooperatively to Tim23. *J Mol Biol* 382:1144–1156.
10. Leuenberger D, Bally NA, Schatz G, Koehler CM (1999) Different import pathways through the mitochondrial intermembrane space for inner membrane proteins. *EMBO J* 17:4816–4822.
11. Davis AJ, Sepuri NB, Holder J, Johnson AE, Jensen RE (2000) Two intermembrane space TIM complexes interact with different domains of Tim23p during its import into mitochondria. *J Cell Biol* 150:1271–1282.
12. Davis AJ, Alder NN, Jensen RE, Johnson AE (2007) The Tim9p/10p and Tim8p/13p complexes bind to specific sites on Tim23p during mitochondrial protein import. *Mol Biol Cell* 18:475–486.
13. Roesch K, Hynds PJ, Varga R, Tranebjaerg L, Koehler CM (2004) The calcium-binding aspartate/glutamate carriers, citrin and aralar1, are new substrates for the DDP1/TIM-M8a-TIMM13 complex. *Hum Mol Genet* 13:2101–2111.
14. Jin H, et al. (1996) A novel X-linked gene, DDP, shows mutations in families with deafness (DFN-1), dystonia, mental deficiency and blindness. *Nat Genet* 14:177–180.
15. Koehler CM, et al. (1999) Human deafness dystonia syndrome is a mitochondrial disease. *Proc Natl Acad Sci USA* 96:2141–2146.
16. Koehler CM, et al. (1998) Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. *Science* 279:369–373.
17. Koehler CM, et al. (1998) Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins. *EMBO J* 17:6477–6486.
18. Murphy MP, Leuenberger D, Curran SP, Oppliger W, Koehler CM (2001) The essential function of the small Tim proteins in the TIM22 import pathway does not depend on formation of the soluble 70-kilodalton complex. *Mol Cell Biol* 21:6132–6138.
19. Duncan MC, Ho DG, Huang J, Jung ME, Payne GS (2007) Composite synthetic lethal identification of membrane traffic inhibitors. *Proc Natl Acad Sci USA* 104:6235–6240.
20. Koehler CM, Beverly KN, Leverich EP (2006) Redox pathways of the mitochondrion. *Antioxid Redox Sign* 8:813–822.
21. Claypool SM, Oktay Y, Boontheung P, Loo JA, Koehler CM (2008) Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J Cell Biol* 182:937–950.
22. Goyon V, et al. (2008) Yeast cells depleted in Atp14p fail to assemble Atp6p within the ATP synthase and exhibit altered mitochondrial cristae morphology. *J Biol Chem* 283:9749–9758.
23. Emaus RK, Grunwald R, Lemasters JJ (1986) Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim Biophys Acta* 850:436–448.
24. Koehler CM, Merchant S, Schatz G (1999) How membrane proteins travel across the mitochondrial intermembrane space. *Trends Biochem Sci* 24:428–432.
25. Ryan MT, Müller H, Pfanner N (1999) Functional staging of ADP/ATP carrier translocation across the outer mitochondrial membrane. *J Biol Chem* 274:20619–20627.
26. Claypool SM, McCaffery JM, Koehler CM (2006) Mitochondrial mislocalization and altered assembly of a cluster of Barth syndrome mutant tafazzins. *J Cell Biol* 174:379–390.
27. Silvestri L, et al. (2005) Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum Mol Genet* 14:3477–3492.
28. Mills RD, et al. (2008) Biochemical aspects of the neuroprotective mechanism of PTEN-induced kinase-1 (PINK1). *J Neurochem* 105:18–33.
29. Hansson Petersen CA, et al. (2008) The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. *Proc Natl Acad Sci USA* 105:13145–13150.
30. Devi L, Prabhu BM, Galati DF, Avadhani NG, Anandatheerthavarada HK (2006) Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. *J Neurosci* 26:9057–9068.