Adaptation of a Genetic Screen Reveals an Inhibitor for Mitochondrial Protein Import Component Tim44*$$

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Diverse protein import pathways into mitochondria use translocons on the outer membrane (TOM) and inner membrane (TIM). We adapted a genetic screen, based on ura3 mis-targeting from mitochondria to the cytosol, to identify small molecules that attenuated protein import. Small molecule mitochondrial import blockers of the Carla Koehler laboratory (MB)-10 inhibited import of substrates that require the TIM23 translocon. Mutational analysis coupled with molecular docking and molecular dynamics modeling revealed that MB-10 binds to a specific pocket in the C-terminal domain of Tim44 of the protein-associated motor (PAM) complex. This region was proposed to anchor Tim44 to the membrane, but biochemical studies with MB-10 show that this region is required for binding to the translocating precursor and binding to mtHsp70 in low ATP conditions. This study also supports a direct role for the PAM complex in the import of substrates that are laterally sorted to the inner membrane, as well as the mitochondrial matrix. Thus, MB-10 is the first small molecule modulator to attenuate PAM complex activity, likely through binding to the C-terminal region of Tim44.

Protein import into mitochondria is a highly regulated process that is coordinated by translocons on the outer membrane and the inner membrane (1). The translocon of the outer membrane (TOM)3 is the major gateway for proteins to enter mitochondria. Tom70, Tom20, and Tom5 function as receptors to guide the precursor to the translocation pore that is formed by Tom40. The translocase of the inner membrane (TIM23) mediates import for proteins that typically have an N-terminal targeting sequence and reside in the matrix, whereas the TIM22 translocon directs the import of the carrier proteins. Together the translocation and assembly complexes coordinate assembly of the mitochondrion.

The TIM23 core complex consists of membrane-embedded subunits Tim50, Tim23, and Tim17 (see Fig. 7D for schematic) (2). The channel is formed by Tim17 and the C-terminal region of Tim23 (3), whereas the N-terminal regions of Tim23 and Tim50 have a receptor function to guide the precursor from the TOM complex to the TIM23 complex (4). The accessory subunit Tim21 assembles with the TIM23 core complex to serve as a conduit to promote the transfer of substrates with a hydrophobic stop transfer signal into the inner membrane in a membrane-potential dependent manner (5). Complete translocation across the inner membrane and into the matrix requires the membrane potential and the ATP-driven protein-associated motor (PAM) (2). Components of the PAM complex include Tim44, mitochondrial Hsp70, the nucleotide exchange factor mGpE (Mge1), and J-proteins Paml6 and Paml8. mtHsp70 is the central component of the PAM complex because it pulls the precursor into the matrix. Co-chaperone Paml8 stimulates the ATPase activity of mtHsp70, whereas co-chaperone Paml6 regulates Paml8 (6). Tim44 is crucial for MtHsp70 and J-protein binding and association of the PAM complex with the membrane (2). Mge1 is the nucleotide exchange factor that is required for completion of the mtHsp70 reaction cycle. Thus, the TIM23 translocon is a dynamic and highly regulated machine.

Mutations in components of the TIM23 protein import machinery result in a variety of diseases. Mutant DNAJC19, the homolog of Paml8, causes the dilated cardiomyopathy with ataxia syndrome (7). The human homolog of yeast Pam16, Magmas, is overexpressed in adenocarcinoma (8). TIMM44 is up-regulated in diabetes (9), whereas a point mutation (P308Q) in the C-terminal domain of TIMM44 is linked to an autosomal dominant familial oncocytic thyroid carcino-

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§ The abbreviations used are: TOM, translocase of outer membrane; BD, Brownian dynamics; DHFR, dihydrofolate reductase; DSS, disuccinimidyl suberate; MD, molecular dynamics; MitoBloCK or MB, mitochondrial protein import blocker from the Carla Koehler laboratory; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; PAM, protein associated motor; SAR, structure activity relationship; TIM, translocase of inner mitochondrial membrane; TIMM, translocase of inner mitochondrial membrane; TOMM, translocase of outer mitochondrial membrane; AAC, ADP/ATP carrier; cyt, cytochrome; hpf, h post-fertilization; PDB, Protein Data Bank.

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Chemical Modulator of Mitochondrial Protein Import

Results

An in Vivo Screen to Identify Inhibitors of Mitochondrial Protein Translocation—To identify modulators of the TOM/TIM23 translocation system, we adopted the genetic screen that resulted in the identification of import components Tim44, Tim23, and Tim17, based on mislocalization of the Su9-Ura3 fusion protein from mitochondria to the cytosol (14). One hit compound, designated MB-10, was characterized in detail. MB-10 inhibited the import of substrates that use the TIM23 translocon but not those imported via other pathways. MB-10 likely targets Tim44 of the PAM complex and reduced TIM23 translocon but not those imported via other pathways. In mammalian cells, MB-10 treatment supports an important role for TIMM44 in the import of SOD2. Finally, TIMM44 was required for cardiac development in zebrafish. Therefore, MB-10 is a useful tool to investigate the role of TIMM44 in disease studies, as well as the mechanistic role of the PAM complex in protein translocation.

Conclusions

Given the importance of mitochondria in a wide range of cellular functions and diseases, the identification of small molecules that modulate mitochondrial protein import is of great importance. The compound MB-10, identified in our screen, provided insights into the role of Tim44 in mitochondrial protein import and may serve as a valuable tool for further studies in this field. Future investigation of the mechanisms underlying the action of MB-10 and other identified compounds will be crucial for advancing our understanding of mitochondrial protein import and its regulation.
FIGURE 1. MB-10 is a potential attenuator of protein import into mitochondria. A, a plasmid expressing Su9-Ura3-myc was integrated at the LEU2 locus in WT and tim23-2 strains, and the strains were grown at 30 °C. A whole cell lysate from WT and the tim23-2 mutant, either without (−) or with [Su9-Ura3], was separated by SDS-PAGE followed by immunoblotting with an anti-Myc antibody. p, precursor; m, mature. B, growth analysis of the WT and tim23-2 mutant with integrated [Su9-URA3] in synthetic dextrose medium in the absence of uracil. Growth was measured based on the density at A600 (OD600). C, in the presence of uracil. D, 1% DMSO, 100 μM MB-10, or 50 μM MB-2 was added to purified 100 μg/ml WT yeast mitochondria for 30 min at 25 °C in import buffer, and released proteins (S) were separated from mitochondria (P) by centrifugation. The proteins were visualized by Coomassie staining. E, as in B, 1% DMSO or the indicated concentration of MB-10 was added to purified mitochondria from HEK293T cells followed by Coomassie staining. F, as in E, the release of proteins was detected by immunoblotting; proteins included PreP (matrix), YME1L (inner membrane), TOMM40 (outer membrane), and MIA40 (intermembrane space). G, respiration measurements were performed with a Clark-type oxygen electrode using 100 μg/ml WT yeast mitochondria in the presence of 1% DMSO or MB-10. Respiration was initiated with NADH addition. 100 μM MB-10 (with DMSO at a 1% final concentration) or 1% DMSO was added once steady-state respiration was established. As a control, CCCP was added to uncouple the mitochondria. The respiration rate for each treatment has been included. H, the structure of MitoBloCK-10 (MB-10).
[(5-nitro-2-thienyl)methylene] benzohydrazide, a hydrazide-hydrazone derivative (Fig. 1H), and it was selected for detailed characterization.

**MB-10 Targets the TIM23 Translocon**—We repeated the growth assay from the screen with varying concentrations of MB-10 to assess the growth properties of the WT[Su9-URA3] strain (Fig. 2A). At 2 μM MB-10, the growth of the WT[Su9-URA3] strain increased robustly (Fig. 2A). Because a complete block in protein translocation is predicted to be lethal, the minimum inhibitory concentration required to inhibit the growth of 50% of the yeast (MIC50) for MB-10 was determined for the WT, tim23-2, and tim10-1 strains. These strains were tested because they lacked the multidrug resistance pumps Pdr5 and Snq2, and the strains were used previously in a screen to identify modulators for the TIM22 pathway (12). We predicted that a yeast strain with a mutation in the translocon that MB-10 targets would display increased sensitivity to MB-10, based on a previous screening strategy to identify TIM22 modulators (12). The MIC50 of MB-10 was 1 μM for the tim23-2 strain, in contrast to MIC50 values of 7.1 and 4.7 μM for the WT and tim10-1 strains, respectively (Fig. 2B). The synthetic lethal analysis suggests that MB-10 likely targets the TIM23 translocon and not the TIM22 translocation system.

Because MB-10 likely impairs the translocation of a subset of precursors that use a particular translocon, the ability of MB-10 to inhibit protein import was tested using the in vitro import assay with radiolabeled precursors. Mitochondria were preincubated with MB-10, and then substrates Su9-Ura3 and Su9-DHFR (TIM23 substrates), AAC (TIM22 substrate), Tom40 (SAM substrate), and Erv1 (Erv1/Mia40 substrate) were imported. A titration assay with MB-10 indicated that 100 μM inhibited Su9-Ura3 import by ~80% (Fig. 2C). Similarly, 100...
μM MB-10 reduced import of Su9-DHFR by 75% (Fig. 2D). In contrast, MB-10 did not interfere with the import of AAC, Tom40, and Erv1, substrates that do not require the TIM23 translocon (Fig. 2, E–G). Therefore, MB-10 specifically inhibits the import of TIM23 substrates.

The mitochondrial targeting sequence of Su9 from N. crassa has an interesting feature of two processing sites at amino acids 35 and 66 that are cleaved by the matrix processing peptidase (16). The precursor and intermediate forms of Su9-DHFR, and Su9-Ura3 to a lesser extent, were resistant to added protease in the presence of 50–100 μM MB-10 (Fig. 2, C and D). These unprocessed forms may have arrested during translocation as an intermembrane space intermediate, similar to that generated during import under low ATP conditions (17). Alternatively, the precursor form with a tightly folded DHFR domain may be held tightly against the TOM complex and therefore resistant to cleavage by the added protease (18).

We followed the import assay of Su9-Ura3 with osmotic shock to disrupt the mitochondrial outer membrane (generating mitoplasts) and subsequently added protease (19). The precursor and intermediate forms accumulated in the presence of MB-10, but not the vehicle control DMSO (Fig. 3, A and B). The intermediates were mostly degraded, indicating that the precursor and intermediate forms arrest as translocation intermediates in the intermembrane space (Fig. 3B). As a control, samples treated with Triton X-100 were generally degraded, except for a small amount of a lower molecular weight species that may represent a tightly folded domain of Ura3 (indicated by asterisks in Fig. 3, A and B). Thus, MB-10 addition caused the translocating precursor to arrest in transit, potentially in the TIM23 translocon.

**MB-10 Impairs the Import of Precursors Sorted to the Matrix and the Intermembrane Space—**Next, we checked the effect of MB-10 on sorting of precursors to the intermembrane space and matrix, using cytochrome (cyt) b$_2$ variants and cyt c$_1$. The precursor, cyt b$_2$ (167)A63P-DHFR, is targeted to the mitochondrial matrix, because of the mutation at amino acid 63 (a proline is substituted for alanine) in the stop transfer sequence (20) and processed in two stages. Immediately after translocation across the inner membrane, the targeting sequence is cleaved by matrix processing peptidase and the processing intermediate (designated i) is formed. In a subsequent step, eight additional amino acids are removed, yielding the intermediate i$^*$ form (21). In the presence of 10 and 30 μM MB-10, formation of the i$^*$ intermediate seemed to be reduced, resulting in accumulation of the i form (Fig. 3C). In the presence of 100 μM MB-10, import of cyt b$_2$ (167)A63P-DHFR was inhibited (Fig. 3C). The inhibition of i$^*$ formation has been observed with mutants of the components of PAM complex, such as Tim44 and Mge1 (22–24), suggesting that MB-10 may target the PAM complex. In contrast, for precursors cyt b$_2$ (167)-DHFR and cyt c$_1$, which are laterally sorted to intermembrane space by the TIM23$^{SORT}$ complex in an ATP-independent manner (5, 19, 25), generation of the import intermediate was not inhibited by treatment with low concentrations of MB-10 (Fig. 3, D and E).

Only in the presence of 100 μM MB-10 was the import of cyt b$_2$ (167)-DHFR and cyt c$_1$ decreased (Fig. 3, D and E). Note that formation of the mature form of cyt c$_1$ is typically not detected because processing by the inner membrane protease is slow (5). The PAM complex also associates with the laterally inserted precursors even when ATP is not required (26), indicating that the PAM complex plays a role in the import of precursors into the matrix and inner membrane. Taken together, we interpret these results to show that MB-10, in low concentration, inhibits the essential function of the PAM complex to import matrix proteins and that MB-10, in high concentration, further inhibits the PAM complex in facilitating the import of precursors laterally sorted to intermembrane space.

**Specific Properties of MB-10 Inhibit Protein Import—**We purchased an additional five compounds with similarity to MB-10 (Fig. 3F) for structural-activity relationship (SAR) studies. The import of cyt b$_2$ (167)A63P-DHFR was tested in the presence of the analogs at 100 μM. Analogs 1, 2, and 5 decreased the import of cyt b$_2$ (167)A63P-DHFR similarly to MB-10. We renamed these compounds MB-10.2, MB-10.3, and MB-10.4, respectively (Fig. 3G). In contrast, analogs 3 and 4 did not abrogate protein import (Fig. 3G). Analog 3 has a methyl group attached to the carbon atom of the hydrazine moiety, and analog 4 has an alkylation alcohol group that replaces the fluorobenzene moiety and the thiophene exchanged for a furan group. In contrast, MB-10.2, MB-10.3, and MB-10.4 contain side groups similar to those of MB-10. Based on structural comparison, we assumed that the six-membered ring and thiophene linked by hydrazide-hydrazone bond would be critical to MB-10 activity. Methylation of the hydrazine interferes with MB-10 activity. To support this notion, we revisited the screening results. The Chembridge chemical library includes MB-10–like compounds with substitution of the thiophene or with methylation of the hydrazine group; these compounds did not confer the growth of WT[Su9-URA3] strain in the screening assay (supplemental Fig. S1), suggesting that these chemical alterations in MB-10–like compounds abolished their ability to inhibit protein import. Taken together, the substituted rings on MB-10 are important for inhibitory activity toward the PAM complex.

**MB-10 Targets Tim44 of the PAM Complex—**The protein target may display reduced sensitivity to an exogenous protease because the small molecule binds tightly to the protein and stabilizes the protein structure, thereby reducing the ability of the protease to cleave protein (27). We used this assay to determine whether Tim44 was a potential target, because our import studies and the published literature support that Tim44 is a probable target. A battery of proteases at varying concentrations were tested to identify conditions in which the protein was degraded in a stepwise manner that could be captured in a time course assay by immunoblotting. Pronase gave the best results (27). Isolated yeast mitochondria were lysed with buffer containing 0.2% Triton X-100. Then the lysates were incubated in the presence of 1% DMSO, 100 μM MB-10, or analog 4, followed by Pronase treatment and immunoblot analysis. In the presence of DMSO or analog 4, Tim44 was readily degraded to a 30-kDa truncation product after 10 min (marked by an asterisk), whereas MB-10 stabilized Tim44, slowing the rate of protease digestion (Fig. 4A, top panel). In contrast, Tim23 and matrix protein, α-ketoglutarate dehydrogenase, were digested by Pronase at the same rate, even in the presence of MB-10 (Fig. 4A, middle and bottom panels). MB-10 did not alter the
protease degradation of another component of PAM complex, mitochondrial Hsp70 (Fig. 4B; Note that the antibody also identified a lower molecular weight species that was marked by an asterisk). Thus, these data support that MB-10 likely targets Tim44.

Given that the crystal structure of the C-terminal domain of Tim44 has been solved (28), we initiated a collaboration with the Sha laboratory to determine whether MB-10 could be incorporated into crystallization trials. However, MB-10 was not soluble under the experimental conditions, and this line of investigation could not be developed. Instead, we used a genetic approach, based on random PCR mutagenesis, to isolate tim44 mutants that were resistant to MB-10 (29), presumably because the mutations altered interactions with MB-10. Mutants were selected that were resistant to 15\(\mu M\) MB-10, which is higher than the MIC50 of 7.1 \(\mu M\) in WT. A concentration greater than 16 \(\mu M\) MB-10 failed to generate resistant yeast.

3 B. Sha, unpublished results.

FIGURE 3. MB-10 inhibition of import depends on specific chemical characteristics. A and B, Su9-Ura3 was imported as in Fig. 2C. After import in the presence of 1% DMSO (A), or 100 \(\mu M\) MB-10 (B), samples were incubated in hypotonic buffer to swell the outer membrane (generating mitoplasts) in the presence and absence of proteinase K (PK) followed by inactivation with PMSF. Mitoplasts were recovered by centrifugation (P) and separated from supernatant (S), which contains the soluble intermembrane space contents. As a control, samples were treated with Triton X-100 (TX). Asterisks mark lower molecular weight products that were resistant to protease in the presence of Triton X-100. C–E, import assays of precursors cyt b2(167)A63P-DHFR (mis-sorting mutant to matrix) (C), cyt b2(167)-DHFR (intermembrane space) (D), and cyt c1 (intermembrane space) (E) were performed. p, precursor; m, mature; i, intermediate; i*, intermediate* that is generated from secondary processing in the matrix (21). F, structures of MB-10 and analogs for SAR studies. The square and circle indicate the benzene ring and the thiophene ring, respectively. G, import of cyt b2(167)A63P-DHFR was performed as in C for 10 min in the presence of 1% DMSO, 100 \(\mu M\) MB-10, or 100 \(\mu M\) each of the analogs. The analogs that markedly inhibited import have been designated MB-10.2, MB-10.3, and MB-10.4, p, precursor; i, intermediate. Import reactions were quantified by ImageJ software; 100% was set as the amount of precursor imported in the presence of DMSO at the end point in the time course.
immunoblotting with antibodies against Tim44, Tim23, and addition of 0.2% SDS and incubation at 100 °C. Samples were analyzed by Pronase at 25 °C. At the indicated time points, proteolysis was stopped by the addition of 0.2% SDS and incubation at 100 °C. Samples were analyzed by immunoblotting with antibodies against Tim44, Tim23, and α-ketoglutarate dehydrogenase (KDH). The asterisk indicates cleaved Tim44 products. A representative gel is shown; quantification of bands was performed with ImageJ software, and the percentage was calculated relative to the treatment with no protease. B, mitochondria were lysed in buffer containing 0.2% Triton X-100. The lysates were incubated with 1% DMSO, 100 μM MB-10, or 100 μM analog 4 for 15 min followed by treatment with 0.3 μg/ml Pronase at 25 °C. Samples were analyzed by immunoblotting with antibodies against Tim44, Tim23, and α-ketoglutarate dehydrogenase (KDH). The asterisk indicates cleaved Tim44 products. A representative gel is shown; quantification of bands was performed with ImageJ software, and the percentage was calculated relative to the treatment with no protease. B, mitochondria were lysed in buffer containing 0.2% Triton X-100. The lysates were incubated with 1% DMSO, 100 μM MB-10, or 100 μM analog 4 for 15 min followed by treatment with 1.2 μg/ml Pronase at 25 °C. Samples were analyzed by immunoblotting with anti-mtHsp70 antibody. An asterisk marks a nonspecific band of lower molecular weight that was detected by the antibody.

strains. The mutant plasmids were isolated and sequenced. The mutant tim44 plasmids were then transformed into a strain in which the genomic copy of TIM44 was deleted and viability was maintained with TIM44 expressed from a centromeric URA3 plasmid. Using a plasmid shuffling strategy (30), the URA3 plasmid with TIM44 was removed by selection on 5-fluororotic acid, and the ability of the strain with the mutagenized tim44 plasmid to grow was tested on selective medium that included uracil; this strategy was used to confirm that resistance to MB-10 was based on the mutagenized plasmid. Although mild conditions were used for mutagenesis, plasmids typically contained two or three mutations in TIM44. A region that was enriched in point mutations among several plasmids from the strains that were resistant to MB-10 centered on the helix designated α4 in the C-terminal domain that functions in membrane tethering (Fig. 5A). Specific mutations in this region were F284L, T290S, I297V, and V298A (Fig. 5B). This area is highly conserved across species, and residues 284 and 297 are invariant (31). The tim44 mutants with specific mutations, T290S and I297V, were reconstructed with the single mutation, respectively, and resistance to MB-10 was confirmed. The MIC50 of tim44 mutants T290S and I297V shifted from ~8 μM to ~12 and ~16 μM, respectively (Fig. 5C). Protease sensitivity studies indicated that MB-10 had a decreased affinity for Tim44 I297V, because the Tim44 I297V mutant was degraded in the presence of a lower MB-10 concentration (60 μM) than the WT protein (greater than 80 μM; Fig. 5D). The genetic approach in yeast indicates that MB-10 inhibits Tim44 function through a specific interaction with the C-terminal domain of Tim44.

To determine the binding pocket for MB-10 in the C-terminal domain of Tim44, we used computational approaches including Brownian dynamics (BD) simulations and docking and molecular dynamics (MD) simulations (Fig. 6, A and B) (32–35). The GeomBD program was used to search for all potential MB-10 binding sites in Tim44, and a total six possible binding sites were identified (Fig. 6C). Because most binding sites suggested by BD diffusion may be nonspecific binding surfaces for a compound, we additionally used docking simulations to estimate binding affinities and to reveal the most possible binding pocket for MB-10. Fig. 6D illustrates the computed binding affinities for MB-10 and Tim44. Only one pocket...
revealed that residues Ile-297, Phe-284, and Thr-290 have direct contact with MB-10, and Val-298 supports the β-sheets on the back of the major binding pocket to provide a stable three-dimensional structure for MB-10 binding (Fig. 6B). MD simulations and energy calculations (supplemental Table S3) for MB-10 and Tim44 with mutations at I297V, F284L, T290S, or V298A showed that binding of MB-10 and the Tim44 mutants was weakened, compared with MB-10 binding to the wild-type Tim44. The weaker interactions between MB-10 and mutant Tim44 explain the experimentally observed MB-10 resistance to the mutants. The agreement between the modeling work and experiments further confirmed the novel binding pocket identified in this study.

Based on the docking analysis, we inserted conservative point mutations in conserved residues of Tim44 that were predicted to interfere with MB-10 binding. Specific mutations included H292A and H292Y of helix α4, W316A and W316Y of helix α5, and W417A and W417Y of the loop that connects the β3 and β4 strands. The plasmids were transformed into the shuffling strain in which viability was maintained with TIM44 on a centromeric URA3 plasmid. When the cells were initially transformed, viable colonies expressing Tim44 H292A, W316A, W316Y, and W417A were not recovered despite numerous attempts (supplemental Fig. S2), signifying that these mutations likely yielded a dominant negative phenotype. In contrast, the Tim44 H292Y and W417Y strains grew similar to the WT strain (supplemental Fig. S2 and data not shown) and were amenable to plasmid shuffling to remove the plasmid for WT TIM44. The MIC\textsubscript{50} of Tim44 H292Y and W417Y strains was ~8 μM, similar to the WT strain (Fig. 5C). Thus, the H292Y and W417Y mutations were not sufficient to cause inviability or decrease MB-10 affinity for Tim44, whereas mutations H292A, W316A, W316Y, and W417A rendered Tim44 nonfunctional.

To determine whether MB-10 treatment altered interactions of a substrate during translocation, we performed cross-linking of methotrexate-arrested cyt\textsubscript{b}(167)A63P-DHFR using 200 μM disuccinimidyl suberate (DSS), because the cross-linking pattern to TIM23 components has been well defined (36, 37). In WT mitochondria, methotrexate-arrested cyt\textsubscript{b}(167)A63P-DHFR was cross-linked to several proteins by DSS (Fig. 7A, lane 2), indicating that the precursor was present in the translocation channel. In the presence of 100 μM MB-10, a cross-linked product at ~90 kDa was strongly decreased (Fig. 7A, lane 3). According to published experiments, the missing cross-link is the Tim44 adduct (36–38). We confirmed this by comparison with cross-linking in mitochondria purified from the tim44-8 mutant, which also lacks a cross-link to Tim44 (22). In tim44-8 mitochondria incubated at 37 °C, the cross-linked product was not observed in both the presence and the absence of MB-10 (Fig. 7A, lanes 4 and 5), supporting that the ~90-kDa cross-linked product is Tim44. In addition, the cross-linked products, representing precursor bound to mtHsp70, at ~115–130 kDa were increased in abundance, denoting that MB-10 addition may cause an arrest or shift of the interaction of the precursor from Tim44 to mtHsp70.

We also tested the binding between Tim44 and Hsp70 in the presence of MB-10. In WT mitochondria, Tim44 and Hsp70
bound tightly under low ATP conditions, but the addition of ATP and Mg$^{2+}$ subsequently stimulated release of Hsp70 from Tim44 (Fig. 7B), as has been reported previously (39). In contrast, MB-10 addition specifically inhibited Tim44 binding to Hsp70 in low ATP conditions (Fig. 7B). Tim44-Pam16 and Tim44-Pam18 interactions were not disrupted by MB-10 (Fig. 7C). A strain was generated in which a histidine tag was integrated at the 3’ end of TIM44, generating Tim44-His protein for pulldown experiments with Ni$^{2+}$-agarose (40). Mitochondria were incubated in 1% DMSO or 100 μM MB-10, followed by lysis in 1% digitonin. Tim44-His$_{10}$ was purified with Ni$^{2+}$ agarose, and immunoblot analysis was performed with antibodies against Tim44, Pam16, Pam18, and Tom70. 50 μg of input (T) and flow through (FT) and 300 μg of eluate (E) were loaded. D, schematic showing the TIM23 translocon and associated PAM complex in the absence of MB-10 (left panel), MB-10 addition blocks interactions between Tim44 and the precursor and decreases interactions between Hsp70 and the precursor while blocking translocation into the matrix (right panel, marked with an X). STD, standard.

**FIGURE 7.** MB-10 inhibits Tim44 binding to the precursor and to Hsp70, but not other components of the PAM complex. A, radiolabeled cyt b$_2$ (167)A63P-DHFR was imported into mitochondria isolated from WT or the tim44-8 mutant in the presence of 1 μM MTX. Reactions were treated with cross-linker 200 μM DSS. An asterisk indicates the Tim44-cyt b$_2$ (167)A63P-DHFR cross-link that is absent in mitochondria from the tim44-8 mutant strain (22). p, precursor; i, intermediate. B, 20 μg of mitochondrial lysate was treated with 1% DMSO or 100 μM MB-10 and immunoprecipitated with anti-mitochondrial Hsp70 antibody in the absence of or presence of 5 mM MgCl$_2$ and 1 mM ATP (+ ATP/Mg). Tim44 was detected with anti-Tim44 antibody, and mtHsp70 was visualized by Ponceau S staining. An asterisk indicates the IgG heavy chain. C, mitochondria (400 μg) from a strain in which Tim44 contained a C-terminal His$_{10}$ tag and a WT control were treated with 1% DMSO or 100 μM MB-10, followed by lysis in 1% digitonin. Tim44-His$_{10}$ was purified with Ni$^{2+}$ agarose, and immunoblot analysis was performed with antibodies against Tim44, Pam16, Pam18, and Tom70. 50 μg of input (T) and flow through (FT) and 300 μg of eluate (E) were loaded. D, schematic showing the TIM23 translocon and associated PAM complex in the absence of MB-10 (left panel), MB-10 addition blocks interactions between Tim44 and the precursor and decreases interactions between Hsp70 and the precursor while blocking translocation into the matrix (right panel, marked with an X). STD, standard.

**MB-10 Inhibits Protein Import into Mammalian Mitochondria**—The long term goal of our chemical screens is to develop small molecule modulators that also work in other systems such as cultured cells and zebrafish. Therefore, we switched experimental systems to cultured mammalian cells. HeLa cells were treated with increasing concentrations of MB-10 for 24 h, and cell viability was evaluated with a 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay. MB-10 treatment reduced viability of HeLa cells in a dose-dependent manner with an MIC$_{50}$ of 17.2 μM (Fig. 8A). Upon microscopic observation using an antibody against cyt c to mark mitochondria, mitochondrial morphology was normal in the presence of 5–10 μM MB-10 (below the MIC$_{50}$) (Fig. 8, D and E). However, the addition of 20 μM MB-10 (just above the MIC$_{50}$) caused the mitochondrial network to fragment with cyt c release to the cytosol in 40% of the cells (Fig. 8, F and G). As a
control, 20 μM CCCP induced mitochondrial fragmentation but did not induce cyt c release in HeLa cells, and treatment with 1% DMSO did not perturb the mitochondrial network under our experimental conditions (Fig. 8, B and C).

We then tested whether MB-10 inhibited protein import into mitochondria isolated from HeLa cells. In the presence of MB-10, import of Su9-DHFR was inhibited (Fig. 8H). Notably, the precursor and intermediate forms of Su9-DHFR were also

**FIGURE 8. MB-10 inhibits protein import into mammalian mitochondria.** A, cell viability of HeLa cells in the presence of MB-10 was determined using an MTT cell viability assay. HeLa cells were treated for 24 h with MB-10 at the indicated concentrations, and then viability was measured. 100% was defined as the signal from cells treated with 1% DMSO. Average percentage of survival ± S.D. of n = 3 trials. B–F, HeLa cells were treated with 1% DMSO (B), 20 μM CCCP (C), or MB-10 (D–F) for 24 h followed by immunostaining with anti-cyt c antibody. Note that 20 μM MB-10 is just above the MIC<sub>50</sub> in mammalian cells. F, diffuse cyt c staining is indicative of release from mitochondria by apoptosis. Scale bar, 20 μm. G, quantitation of cyt c release in cells from experiments B–F. 200 cells were quantitated. H, an import assay was performed with Su9-DHFR into mitochondria isolated from HeLa cells as described in Fig. 3 in the presence of 50 μM MB-10 or the vehicle control (1% DMSO). I, as in H, import of Su9-DHFR was quantitated in the presence of the indicated concentrations of MB-10. J and K, precursors yeast AAC and Tom40 were also imported. The asterisk indicates a truncation product of Tom40 translation that is imported. L and M, HeLa cells (L) and SH-SY5Y cells (M) were treated with 0.2% DMSO or MB-10 for 24 h. A total cell lysate was analyzed by immunoblotting with antibodies against SOD2, mitochondrial Hsp70, and TOMMM40 antibodies.
detected as in yeast mitochondria (Fig. 2D), supporting that MB-10 behaves similarly in yeast and mammalian mitochondria. Furthermore, MB-10 inhibited protein import more effectively in mammalian mitochondria than in yeast mitochondria. An approximate 70% reduction of Su9-DHFR import was achieved with 25 μM MB-10 (Fig. 8I). Potential reasons for the increased sensitivity of mammalian cells to MB-10 may be that protein import is more dependent on TIMM44 in mammalian cells or that MB-10 has higher affinity to mammalian TIMM44 than yeast Tim44; the crystallization studies support that the structures of yeast and mammalian Tim44 are highly conserved (31), so MB-10 may bind more tightly to mammalian TIM44. Consistent with the results in yeast mitochondria, MB-10 did not inhibit the import of AAC and Tom40 that are imported independently of the TIM23 complex (Fig. 8, J and K). Therefore, we conclude that MB-10 inhibited Tim44 in both yeast and mammalian mitochondria. In addition, loss of TIMM44 activity caused cell death by apoptosis.

**MB-10 Inhibits Biogenesis of SOD2 in Mammalian Culture Cells**—Previous studies show that TIMM44 is up-regulated in diabetic mouse kidneys, and siRNA-mediated knockdown of TIMM44 in a variety of cells decreases the abundance of SOD2 and glutathione peroxidase (11, 41). We therefore measured SOD2 levels in the presence of MB-10 in HeLa cells and SH-SY5Y neuroblastoma cells (42). Sensitivity of SH-SY5Y cells to MB-10 was evaluated by the MTT assay and was similar to that of HeLa cells (data not shown). Upon treatment of 5 and 10 μM MB-10 to cells for 24 h, the abundance of SOD2 was markedly decreased, whereas the abundance of matrix protein mtHsp70 and outer membrane protein TOMM40 was not decreased (Fig. 8, L and M). Thus, MB-10 treatment recapitulates decreased SOD2 levels when TIMM44 levels are decreased by RNAi treatment.

**MB-10 Treatment Impairs Cardiac Development and Induces Apoptosis in Zebrafish Embryos**—Mutations in DNAJC19, a component of the PAM complex, lead to dilated cardiomyopathy with ataxia syndrome, an autosomal recessive Barth syndrome-like condition in patients (43). We applied MB-10 to zebrafish embryos to determine how impaired TIMM44 altered development, particularly focusing on cardiac tissue. A transgenic zebrafish line in which DsRed is targeted to mitochondria under control of the heart specific cardiac myosin light chain promoter cmlc2 was used (13). Embryos were placed in either 1% DMSO, MB-10, MB-10.2, or analog 4 as indicated (Fig. 9) at 3 hpf and allowed to develop until 72 hpf. Embryos incubated with 10 μM MB-10 displayed dorsal body curvature and impaired heart development with excessive pericardiac effusion (Fig. 9) that is characteristic of zebrafish with dilated cardiomyopathy (44, 45). MB-10-treated embryos also showed an increase in apoptotic cells as visualized by acridine orange staining (Fig. 9, highlighted by arrowheads), consistent with the increased apoptosis observed in MB-10-treated HeLa cells (Fig.
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8, D–G). In addition, embryos incubated with MB-10.2 had developmental defects similar to MB-10 (Fig. 9C), whereas embryos incubated with analog 4 looked similar to the zebrafish treated with DMSO (Fig. 9D). Thus, MB-10 is an effective tool for characterizing TIMM44 function in cultured cells and zebrafish as well as yeast.

Discussion

We completed a screen to identify inhibitors of the general import pathway by adapting a genetic screen in yeast (14). Because this assay selects for a gain in growth, false-positive compounds that are generally lethal are omitted. Metrics for evaluating and validating a robust high throughput include a low hit rate typically less than 0.14% and a screening window coefficient (referred to as the Z’ factor) greater than 0.5 (46). Our screening approach was strong with a high Z’ factor (>0.8) and low hit rate (0.02%). Another benefit of this screening approach is that a WT yeast strain, instead of mutants (12), was used. In addition, as shown with previous inhibitors MB-1 and MB-6 (12, 13), MB-10 inhibits import in both yeast and mammalian mitochondria. The SAR studies in the protein import assays (Fig. 3) and zebrafish (Fig. 9) support that MB-10 seems specific for targeting Tim44. Indeed, MB-10 and MB-10.2 specifically induced cardiac deficiency in developing zebrafish embryos and inhibited import. In contrast, analog 4 did not alter zebrafish development, although the compound shared a similar structure to MB-10 and MB-10.2 (Fig. 9). Thus, these probes are valuable for study in a broad range of model systems.

A multifaceted strategy illustrates that MB-10 likely targets Tim44, but interactions at the interface with other components of the PAM and TIM23 complex cannot be ruled out completely. With a genetic approach, the tim23-2 mutant was more sensitive to MB-10 than the tim10-1 mutant. A battery of import assays using substrates of each translocon (TOM, TIM23, and MIA (mitochondrial intermembrane space assembly)) narrowed the candidate to five or six factors of the PAM complex. Subsequent detailed studies including cross-linking to the cyt b5(167)-DHFR and protease protection corroborated that MB-10 likely targeted Tim44. Whereas the direct approach of incorporating MB-10 into crystallography studies was not successful, a genetic approach identified Tim44 mutants that were not sensitive to MB-10. In conjunction with docking and MD simulations, MB-10 binds in a pocket in the C-terminal region that is on the opposite face from a large groove that may interact with the membrane (31). The N-terminal region of Tim44 interacts with motor partners Hsp70, Mge1, Pam18, and Pam16 (47), but our study shows that the C-terminal region is also essential for Tim44 function. Indeed, the C-terminal region is highly conserved, mutations at invariable residues resulted in a dominant-negative phenotype, and MB-10 addition blocked interactions of Tim44 with the imported precursor and Hsp70. As shown in Fig. 7D, we propose that MB-10 blocks binding of the C-terminal domain of Tim44 and the precursor, thereby blocking protein translocation. The cross-linking studies indicate that binding of precursor to Hsp70 increases, but this intermediate is not productive for protein import. Based on our pulldown studies, MB-10 does not disrupt interactions among components of the TIM23 translocon and PAM complex. Our studies thus agree with those of Mokranjac and co-workers (47) in which they reported that the C-terminal domain interacted with the translocating protein. Thus, MB-10 is a specific probe to block interactions of the translocating precursor with the PAM complex.

From the import studies with substrates that have a stop transfer motif, MB-10 at 100 μM inhibited the import of precursors sorted to the intermembrane space (Fig. 3). This import inhibition is similar to the phenotype of tim44 temperature-sensitive mutants reported previously (22, 48). Recently, the role of the import motor in the translocation of precursors that are laterally sorted (i.e. cyt b5(167)-DHFR and cyt c1) has been under debate (49). The “modular” model proposes that the TIM23 complex exists in two forms: one with Tim21 that lacks the import motor and mediates lateral insertion and the second one with the import motor that lacks Tim21 and mediates transport into the matrix (50, 51). The “single-entity” model proposes that all essential subunits of the translocon function as one complex that may be actively remodeled based on targeting sequences of the translocating substrate (49, 52). Given that MB-10 inhibits the import of cyt b5(167)-DHFR and cyt c1, our studies are tilted to support the “single-entity” model, because Tim44 of the motor is required for the import of laterally sorted precursors. MB-10 will be a useful tool to continue mechanistic studies about how the dynamic TIM23 translocon mediates protein import.

Experimental Procedures

High Throughput Screening—Screening was performed using the WT[Su9-URA3] strain that grown in SD medium supplemented with uracil and without leucine. Yeast were thoroughly washed with sterile H2O twice to remove uracil and diluted in SD medium lacking uracil to an A600 of 0.05. The cell suspension was kept on ice throughout the screening run. A Titrtek multidrop instrument was used to dispense 50 μl of cell suspension into column 2–22 of each clear 384-well plate (Greiner Bio-One). The Biomek FX (Beckman Coulter) was used to pin transfer 0.5 μl of compounds from the 1 mm stock or DMSO to respective wells. The approximate screening concentration of the small molecules was 10 μM. As controls, column 2 and 23 consisted of the WT[Su9-URA3] and tim23-2[Su9-URA3] strains, respectively, supplemented with the vehicle 1% DMSO. After completing compound transfer, all plates were incubated at 30 °C in a humidified incubator for 24 h. Each plate was shaken in a Beckman orbital shaker to resuspend the settled cells, and the A600 in each well was read by a Wallac Victor plate reader (PerkinElmer Life Sciences). The compounds that augmented growth of the WT[Su9-URA3] strain by more than 30% of that of the tim23-2[Su9-URA3] strain were identified and cherry-picked into one plate for rescreening. Hit compounds that conferred growth in the repeated assay were reordered from Chembridge for follow-up analysis. Purchased compounds that conferred growth to the WT[Su9-URA3] strain were chosen for additional analysis (Fig. 3F).

Molecular Dynamics and Docking Simulations—Starting from the crystal structure of yeast Tim44 (PDB code 2FXT) for the MD run, we constructed the wild-type protein and four mutants (T290S, I297V, V298A, and F284L). The structure was
aligned with PDB code 3QK9 for better visualization. The structure of MB-10 was generated using Vega ZZ. The AMBER 99SB force field was used for the protein (53, 54). The AMBER12 package was used for standard setup, and the GPU acceleration was used to run the MD simulations. After the energy minimization, the systems were solvated using a box of TIP3P water of at least 12 Å around the protein by the leap module in AMBER12. The systems were composed of ~43,400 atoms. All production runs were performed for 100 ns at 298 K, and the temperature was maintained at 298 K by Langevin dynamics (55). The particle mesh Ewald was used for the long range electrostatic interactions, whereas the van der Waals interactions were cut off using amber default 8.0 Å. A time step of 2.0 fs was used, and MD frames were saved every 1 ps. Molecular docking of the compound was performed using Autodock (56). Briefly, multiple frames selected from the MD simulation for wild-type Tim44 were used to dock the ligand into the binding site. Residues 315, 316, 404, 415, and 417 were kept flexible during docking. The Lamarckian genetic algorithm was used to search for minimum energy ligand conformations and orientations. A total of 20 unique docking results were generated and analyzed, and the lowest energy conformation that placed MB-10 in the binding site was used for Fig. 6B. All three-dimensional molecular structures were generated using VMD (57). Computational details for the simulations are provided in the supplemental “Experimental Procedures.”

General Assays with Mitochondria—Detailed methods are provided in the supplemental “Experimental Procedures.”

Author Contributions—N. M. conceived and designed the study; acquired biological, biochemical, and biophysical data; analyzed and interpreted the data; and drafted or revised the article. Z. T. conceived and performed molecular modeling and docking; acquired biochemical and biophysical data, and analyzed and interpreted the data. M. A. C. acquired biological, biochemical, and biophysical data; analyzed and interpreted the data; and drafted or revised the article. M. E. J. acquired biological data, analyzed and interpreted the data, and drafted or revised the article. C. J. D. analyzed and interpreted the data and drafted or revised the article. S. A. H. conceived and designed the study, acquired biological, biochemical, and biophysical data; analyzed and interpreted the data; and drafted or revised the article. C. M. K. supervised the project, conceived and designed the study, analyzed and interpreted the data, and drafted or revised the article.

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