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Restored Physiology in Protein-Deficient Yeast by a Small Molecule Channel

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Supporting Information

ABSTRACT: Deficiencies of protein ion channels underlie many currently incurable human diseases. Robust networks of pumps and channels are usually responsible for the directional movement of specific ions in organisms ranging from microbes to humans. We thus questioned whether minimally selective small molecule mimics of missing protein channels might be capable of collaborating with the corresponding protein ion pumps to restore physiology. Here we report vigorous and sustainable restoration of yeast cell growth by replacing missing protein ion transporters with imperfect small molecule mimics. We further provide evidence that this tolerance for imperfect mimicry is attributable to collaboration between the channel-forming small molecule and protein ion pumps. These results illuminate a mechanistic framework for pursuing small molecule replacements for deficient protein ion channels that underlie a range of challenging human diseases.

There are many currently incurable human diseases that are caused by missing protein ion channels, including cystic fibrosis, Bartter syndrome, Dravet syndrome, and Dent's disease.[1,2] Like many other human diseases caused by missing proteins, these diseases are difficult to treat, and new approaches are needed. Some small molecules can perform ion channel-like functions,[3−11] suggesting the possibility of replacing missing protein ion channels with small molecule mimics. Closely replicating the functions of ion selective and tightly regulated protein channels with small molecules is challenging. However, robust protein networks comprising pumps and channels drive targeted ions in targeted directions throughout the spectrum of living systems.[12] We thus questioned whether relatively unselective and unregulated small molecule mimics of missing protein channels might be capable of collaborating with the corresponding protein ion pumps to restore physiology.

Yeast represents an excellent model system for studying eukaryotic physiology.[13] Moreover, deficiencies of specific protein ion transporters in yeast are known to lead to dramatic no growth phenotypes, thus providing a unique opportunity for using cell growth as a readout for physiology restoration. In yeast, ATP-driven V-ATPase and Pma1 proton pumps in the vacuolar and plasma membranes, respectively, collaborate with passive Trk potassium transporters in the plasma membrane to achieve intracellular movement of potassium required for cell growth (Figure 1a, left).[14] Loss of Trk transporters impairs this uptake of environmental potassium and results in a no growth phenotype (Figure 1a, middle).[15−17] Because the primary drivers of ion movement, the corresponding ATP-driven pumps, are still active in such yeast, we hypothesized that a small molecule ion channel permeable to potassium could collaborate with V-ATPase and Pma1 to restore cell growth (Figure 1a, right).

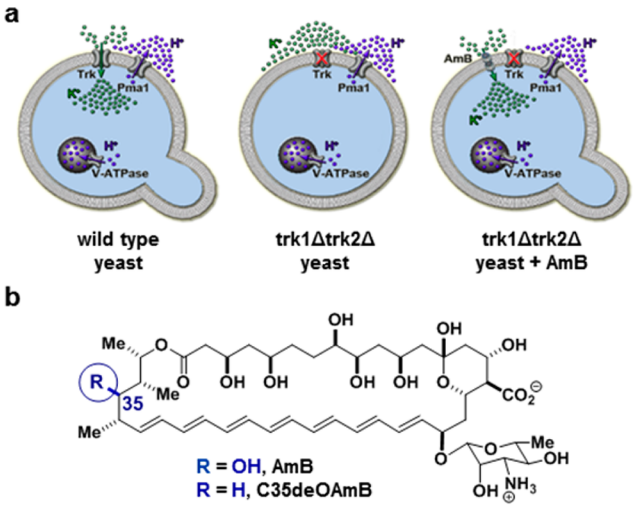

Figure 1. (a) Prospect of replacing missing protein ion transporters with small molecule mimics. (b) Chemical structures of the archetypical ion channel-forming small molecule amphotericin B and its single atom-deficient and channel-inactivated derivative C35-deoxy amphotericin B (C35deOAmB).

The ion channel-forming natural product amphotericin B (Figure 1b) was identified as a small molecule that could enable testing of this hypothesis.[3] AmB can permeabilize yeast cells to potassium and other ions.[18,19] AmB is also highly toxic to yeast, and this toxicity was thought to be inextricably linked to its membrane permeabilization. However, we found a synthesized derivative of AmB lacking a single oxygen atom at C35 (C35deOAmB) (Figure 1b) does not form ion channels and yet still maintains potent fungicidal activity.[20] Further studies revealed that AmB primarily kills yeast by binding and extracting sterols from membranes and is only cytotoxic when the amount of AmB exceeds that of ergosterol.[21,22]

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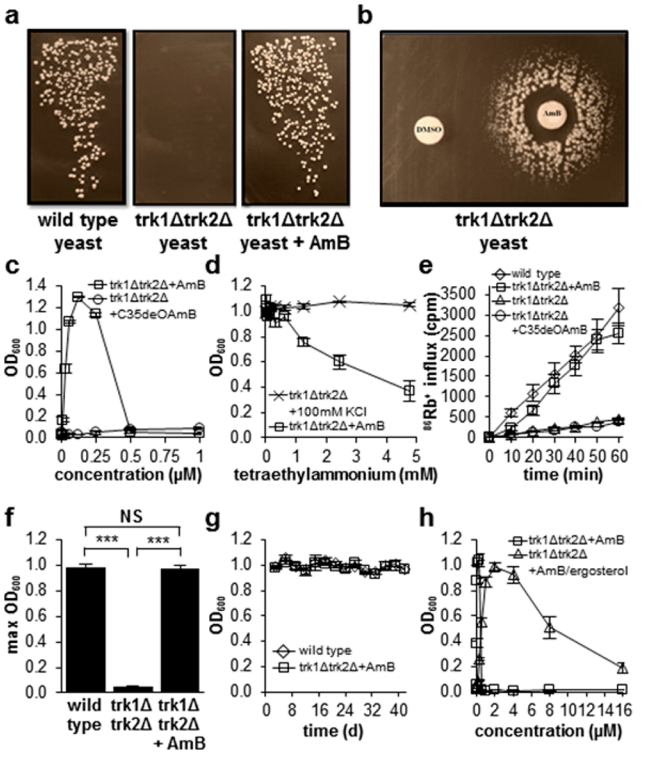

Figure 2. (a) Restoration of yeast cell growth under normal potassium conditions (10 mM) with a small molecule mimic of missing protein potassium transporters. (b) Disc diffusion with AmB on a plate of trk1Δtrk2Δ cells. (c) AmB restores cell growth at concentrations below its minimum inhibitory concentration, while C35deOAmB does not restore growth. (d) Tetraethylammonium diminishes AmB-mediated growth restoration in trk1Δtrk2Δ cells but has no effect on trk1Δtrk2Δ cells grown under permissive conditions (100 mM KCl). (e) AmB restores uptake of extracellular $^{86}Rb^{+}$, a tracer for K^{+}, in trk1Δtrk2Δ cells, but C35deOAmB does not. (f) Vigorous restoration of cell growth is observed upon treating trk1Δtrk2Δ cells with AmB. (g) Similar to wild type cells, AmB-rescued trk1Δtrk2Δ cells show sustained growth over a period of >40 days. (h) A preformed AmB-ergosterol complex dramatically increases the range of concentrations over which physiology is restored. NS, not significant. $***P \leq 0.0001$. Graphs depict means ± SEM.

This all suggested the channel activity of AmB might be separated from its cytocidal activity by simply adding this compound at low concentrations. Moreover, AmB and C35deOAmB, which differ via a single atom, represent a unique pair of probes for determining the impact of small molecule-mediated ion channel activity on organismal physiology.

We thus tested the hypothesis that AmB could restore cell growth in potassium transporter-deficient yeast with a modified functional complementation experiment.[23] Consistent with prior reports,[15,16] growth was observed when wild type *Saccharomyces cerevisiae* were streaked onto agar plates containing normal concentrations of potassium (10 mM) (Figure 2a, left), and no growth was observed for the potassium transporter-deficient strain (trk1Δtrk2Δ) under the same conditions (Figure 2a, middle). Strikingly, the addition of a low concentration of AmB (125 nM) to an otherwise identical agar plate vigorously restored growth of the trk1Δtrk2Δ mutant (Figure 2a, right).

A series of additional experiments confirmed the observed restoration of cell growth is caused by the small molecule-based

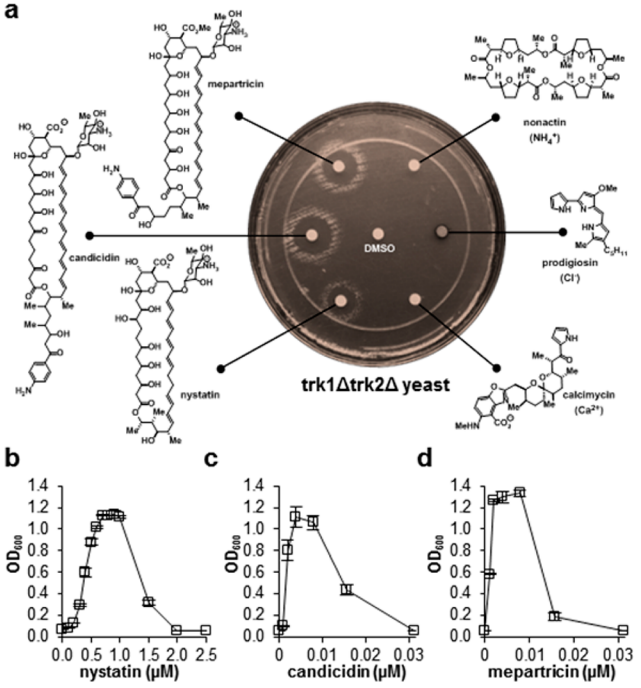

Figure 3. (a) Series of potassium-transporting polyene macrolide natural products restore vigorous cell growth in trk1Δtrk2Δ yeast, whereas no growth is observed upon treating with other small molecules that selectively transport other ions. (b–d) Nystatin, candicidin, and mepartricin were similarly able to restore physiology in a liquid broth dilution assay over a variety of concentrations. Graphs depict means ± SEM.

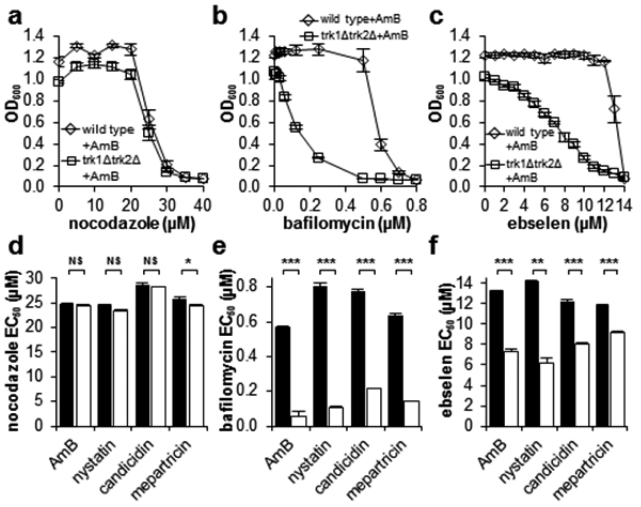

Figure 4. (a) AmB-treated wild type *S. cerevisiae* and trk1Δtrk2Δ cells are equally sensitive to the off-pathway microtubule inhibitor nocodazole. (b) AmB-rescued trk1Δtrk2Δ cells are substantially more sensitive to the V-ATPase inhibitor bafilomycin compared to wild type cells. (c) AmB-rescued trk1Δtrk2Δ cells are substantially more sensitive to the Pma1 inhibitor ebselen than wild type cells. (d–f) EC_{50} values for various inhibitors of cell growth against wild type (black bars) and trk1Δtrk2Δ cells (white bars) treated with optimum rescue concentrations of potassium-transporting polyene macrolide natural products AmB, nystatin, candicidin, and mepartricin. NS, not significant. $*P \leq 0.05$. $**P \leq 0.001$. $***P \leq 0.0001$. Graphs depict means ± SEM.

ion channel activity. A disc diffusion assay visually revealed the predicted dependence of this growth rescue on the

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concentration of AmB (Figure 2b). To quantify this concentration dependence and eliminate the potentially complicating issue of plating efficiency,[24] we also measured trk1Δtrk2Δ yeast cell growth in a broth dilution assay using media containing a normal concentration of potassium (10 mM) (Figure 2c). Consistent with the disc diffusion results, no cell growth was observed in the absence of AmB, a dose-dependent increase of growth was observed at intermediate concentrations, and no growth was observed at or above the minimum inhibitory concentration of this antifungal agent. Further ruling out any type of generic hormetic effects,[25] no growth stimulatory effects were observed when wild type cells or trk1Δtrk2Δ cells grown under permissive conditions were treated with AmB (Figure S1). AmB at 125 nM also did not cause any toxicity in these experiments.

To directly probe the importance of the ion channel activity of AmB, we also tested the single atom-deficient variant C35deOAmB, which does not form ion channels (Figure 1b).[20] This derivative failed to restore growth in trk1Δtrk2Δ cells at any tested concentration (Figure 2c). In a complementary experiment, we utilized the sterically bulky tetraethylammonium cation to block the AmB-based ion channel.[26] This cation inhibited the functional complementation observed with AmB in a dose-dependent manner without causing general toxicity (Figure 2d). We also monitored uptake of radioactive $^{86}Rb^+$ as a reporter of transmembrane potassium movement (Figure 2e).[27] $^{86}Rb^+$ uptake was observed in wild type yeast but not in the trk1Δtrk2Δ mutant, and no uptake was observed when the trk1Δtrk2Δ mutant was treated with the channel-inactivated derivative C35deOAmB. In contrast, $^{86}Rb^+$ uptake was restored when trk1Δtrk2Δ cells were treated with AmB.

We further quantified the vigor and sustainability of this AmB ion channel-mediated restoration of yeast cell growth. AmB-treated trk1Δtrk2Δ cells reached a maximum cell density that matched that of the wild type (Figure 2f), and the doubling time for AmB-rescued trk1Δtrk2Δ cells was only 1.7 times longer (Figure S2). We also observed equivalent levels of cell viability in wild type yeast, wild type yeast treated with 125 nM AmB, and trk1Δtrk2Δ cells rescued with 125 nM AmB (Figure S3). To probe the sustainability of this rescue effect, we reiterated the max cell density and doubling time experiments for over a month. Like wild type cells, the AmB-rescued trk1Δtrk2Δ cells showed sustained vigorous cell growth throughout this entire period of time (Figure 2g). Removing AmB from the media at any point resulted in rapid loss of growth for the trk1Δtrk2Δ cells. We further confirmed the mechanism-based hypothesis that a preformed AmB-ergosterol complex should retain the capacity to permeabilize yeast cells (Figure S4) but show substantially decreased cell killing.[21] This preformed complex dramatically extended the range of concentrations over which rescue is observed (Figure 2h).

To probe the scope and limitations of this tolerance for imperfect mimicry of missing proteins with small molecules, a series of additional ion transporting natural products were evaluated. Vigorous restoration of trk1Δtrk2Δ cell growth was observed with other small molecules that form potassium ion channels, including nystatin, candicidin, and mepartricin, but not with those that selectively transport NH_4^+ (nonactin), Cl^- (prodigiosin), and Ca^{2+} (calcimycin) (Figure 3). Interestingly, the potassium ion carrier valinomycin[28] is unable to restore cell growth (Figure S5).

Finally, we tested the mechanistic hypothesis that these potassium channel-forming small molecules restore physiology by collaborating with the V-ATPase and Pma1 proton pumps. Such a model predicts selective sensitivity of the small molecule-rescued mutants to chemical inhibition of these pumps. As a negative control, AmB-treated wild type and AmB-rescued trk1Δtrk2Δ cells were equally sensitive to nocodazole, an off-pathway inhibitor of microtubule dynamics (Figure 4a). In contrast, AmB-rescued trk1Δtrk2Δ cells were exceptionally sensitive to inhibition of V-ATPase with bafilomycin (Figure 4b) and Pma1 with ebselen (Figure 4c). Similar results were observed with nystatin-, candicidin-, and mepartricin-rescued trk1Δtrk2Δ cells (Figure 4d–f).

Thus, imperfect small molecule mimics of missing protein ion transporters can restore physiology in yeast, and evidence supports that this phenomenon is attributable to functional collaboration between small molecule channels and protein ion pumps. A common channels and pumps architecture is responsible for directional ion movement in organisms from yeast to humans,[12] suggesting that the same type of functional collaboration observed herein might enable small molecule surrogates for missing protein ion channels to impact on human disease.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05765.

Materials, methods, and supplementary figures (PDF)

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Notes

The authors declare no competing financial interest.

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