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Rapid Inhibitor Discovery by Exploiting Synthetic Lethality

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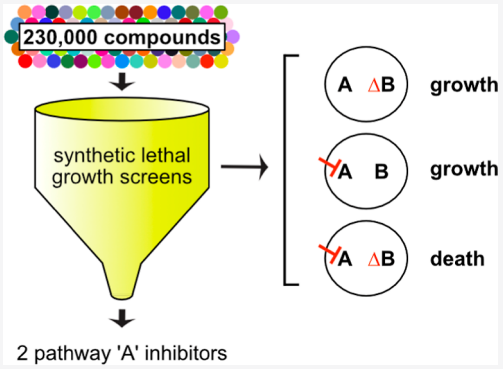
ABSTRACT: Synthetic lethality occurs when inactivation of two genes is lethal but inactivation of either single gene is not. This phenomenon provides an opportunity for efficient compound discovery. Using differential growth screens, one can identify biologically active compounds that selectively inhibit proteins within the synthetic lethal network of any inactivated gene. Here, based purely on synthetic lethalities, we identified two compounds as the only possible inhibitors of *Staphylococcus aureus* lipoteichoic acid (LTA) biosynthesis from a screen of ∼230,000 compounds. Both compounds proved to inhibit the glycosyltransferase UgtP, which assembles the LTA glycolipid anchor. UgtP is required for β-lactam resistance in methicillin-resistant *S. aureus* (MRSA), and the inhibitors restored sensitivity to oxacillin in a highly resistant *S. aureus* strain. As no other compounds were pursued as possible LTA glycolipid assembly inhibitors, this work demonstrates the extraordinary efficiency of screens that exploit synthetic lethality to discover compounds that target specified pathways. The general approach should be applicable not only to other bacteria but also to eukaryotic cells.



INTRODUCTION

The bacterial cell envelope is a complex structure that serves as the interface between the organism and its environment.[1,2] Maintaining cell envelope integrity is crucially important for bacterial survival. Defects in cell envelope assembly often result in attenuated pathogenesis and increased susceptibility to antibiotics. In Gram-positive organisms such as *Staphylococcus aureus*, teichoic acids are vital components of the cell envelope.[3,4] Teichoic acids are anionic polymers that come in two major types: wall teichoic acid (WTA), which is attached to the peptidoglycan cell wall, and lipoteichoic acid (LTA), which is anchored in the cell membrane. Proper assembly of LTA and WTA is required to control cell growth and division and for pathogenicity and β-lactam resistance.[5−12] Both these pathways are therefore proposed targets for compounds that resensitize methicillin-resistant *S. aureus* (MRSA) to β-lactam drugs. We report here a highly efficient phenotypic screening pipeline to discover biologically active inhibitors of these and other pathways important for bacterial cell envelope integrity. The strategy outlined here can be adapted to find compounds that exploit synthetic lethality to inhibit growth of other cells, including cancer cells.

Traditionally, high-throughput small molecule screens are divided into in vitro biochemical screen designed to identify compounds that alter activity of a specified target and screens that identify compounds that elicit a specified phenotype, for example, growth inhibition, in whole cells. However, the former often return compounds that lack biological activity, and the latter can return compounds that act through nonspecific mechanisms of action such as metal chelation or promiscuous binding. Following up on low-quality hits from either type of screens can be extraordinarily time-consuming. Seeking a more efficient approach to identify inhibitors for pathways of interest, we have developed a platform that uses differential growth screens against a wildtype strain and a mutant strain of *S. aureus* to return biologically active compounds that target proteins in a specified region of biological space. Our platform relies on synthetic lethality, the term for the lethal phenotype that can result from disrupting two individually dispensable cellular processes. Synthetic lethal relationships often exist between redundant proteins that perform the same essential reaction, and these are straightforward. Pathways that assemble different molecules or mediate different cellular processes can also be synthetically lethal if they contribute to the same essential function. For example, cell envelope integrity is crucial for bacterial viability, and numerous proteins and pathways contribute to that integrity through different mechanisms. We capitalized on the often

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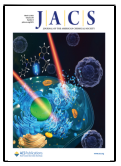

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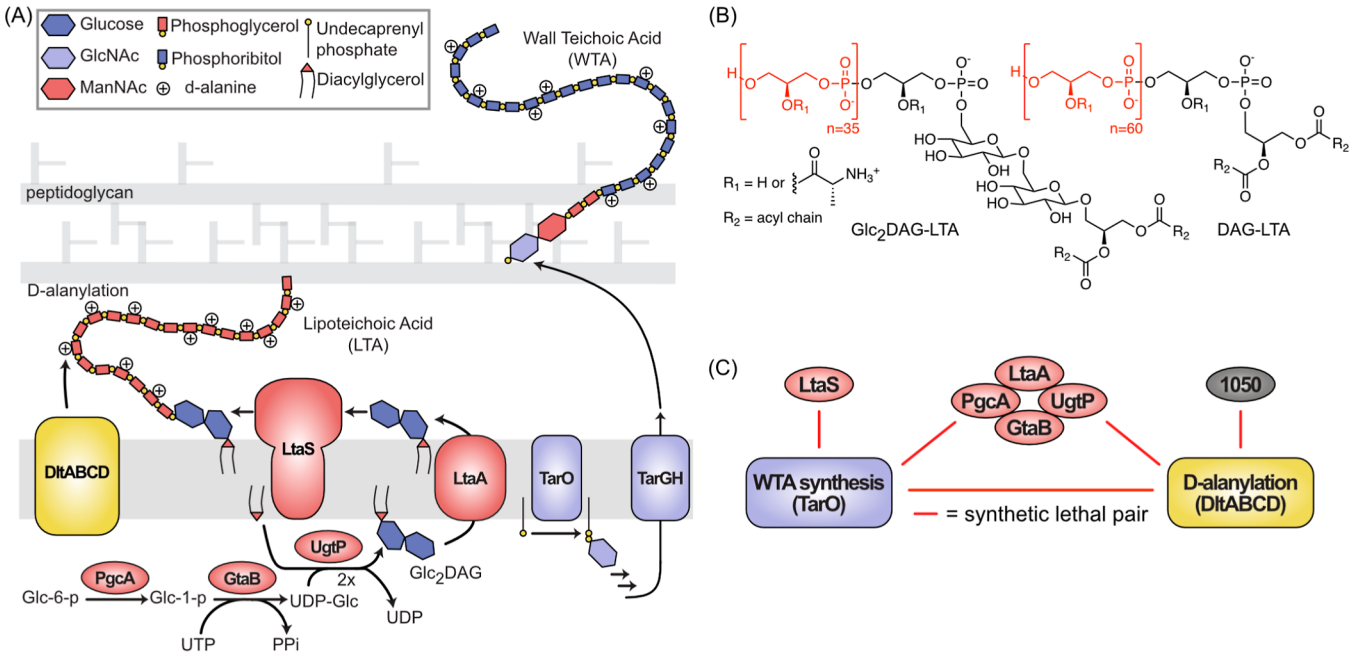

Figure 1. LTA assembled on Glc_2DAG plays a critical role in *S. aureus* cell envelope integrity. (A) Schematic showing assembly of Glc_2DAG-LTA having D-alanine modifications. A simplified pathway for WTA synthesis is also shown and highlights the role of TarO in catalyzing the first step. (B) Chemical structures of Glc_2DAG-LTA and DAG-LTA showing that the polymers have different numbers of phosphoglycerol repeats. (C) Selected synthetic lethal relationships between LTA proteins (red), the WTA pathway (blue), and the D-alanylation pathway (yellow) that were exploited for compound discovery in this work.

dense synthetic lethal networks that exist among cell envelope genes by screening a small molecule library against wildtype *S. aureus* and a cell envelope mutant deficient in WTA synthesis (Δ*tarO*; see Figure 1A). The LTA biosynthesis pathway was among the pathways for which we expected to find inhibitors.

The LTA biosynthesis pathway comprises five proteins required for polymer assembly (Figure 1A). The first two enzymes in the pathway, PgcA and GtaB, make UDP-glucose; the next, UgtP, attaches two glucose units to diacylglycerol (DAG) to make Glc_2DAG.[8,13,14] Glc_2DAG is then flipped by LtaA[15] to the cell surface, where LTA synthase (LtaS) uses it as the glycolipid anchor on which to build the LTA polymer (Figure 1A).[16−18] The polymer is composed of glycerol phosphate repeats derived from phosphatidylglycerol. These repeats are modified with D-alanine esters,[19,20] which modulate polymer charge and provide resistance to the innate immune response.[21−24] Deleting any of the genes upstream of LtaS (*pgcA*, *gtaB*, *ugtP*, or *ltaA*) does not block LTA synthesis but instead prevents its assembly on the Glc_2DAG glycolipid anchor. In these knockout strains, LtaS uses phosphatidylglycerol as an alternative membrane anchor,[9,14,25] and the LTA assembled on this anchor is abnormally long (Figure 1B).[8,17]

Cells defective in LTA assembly are often severely impaired. Deleting *ltaS*, which removes the polymer completely, produces fragile cells that cannot grow except under osmoprotective conditions[26] or unless they acquire a suppressor mutation that somehow compensates for the lack of LTA.[27−29] Cells that make LTA on phosphatidylglycerol instead of Glc_2DAG are able to grow without suppressor mutations but have numerous defects. They grow to an unusually large size, have dysregulated cell division, are substantially less pathogenic than wildtype *S. aureus*, and are more susceptible to antibiotics than cells making normal LTA.[8,9,11,14] These cells are also dependent for growth on other cell envelope pathways that are dispensable in wildtype bacteria (Figure 1C). For example, it is not possible to simultaneously delete genes required for LTA glycolipid assembly and genes required for D-alanine modification of teichoic acids.[9,30,31] Glycolipid-deficient *S. aureus* cells also die if WTA biosynthesis is blocked.[30] Therefore, we hypothesized that it should be possible to exploit synthetic lethal interactions among cell envelope pathways to rapidly identify compounds that target LTA biosynthesis. Here, we describe how we used synthetic lethal growth screens to identify two potent LTA pathway inhibitors that block UgtP and resensitize MRSA to oxacillin. In a striking example of how efficient synthetic lethal growth screens can be, these two compounds were the only screening hits we pursued as possible LTA pathway inhibitors. In addition to serving as useful probes, the compounds provide rare examples of cell-permeable inhibitors that target a glycosyltransferase.[32,33]

RESULTS

Mutant Profiling Identifies Possible LTA Pathway Inhibitors. We conducted a high-throughput screen of ~230,000 small molecules to identify compounds that differentially inhibited growth of wildtype *S. aureus* and a mutant incapable of producing WTA (Δ*tarO S. aureus*). This screen produced 68 hits that prevented growth of the Δ*tarO* mutant but did not affect wildtype growth (Figure 2A, left panel).[34,35] These hits were expected to inhibit targets in Δ*tarO*'s synthetic lethal network, which we previously defined by probing a *S. aureus* transposon library with tunicamycin, a highly selective TarO inhibitor.[30,31] When WTA synthesis is prevented, all components of the LTA pathway, the D-alanylation pathway (Figure 1C), and multiple other proteins involved in cell envelope biogenesis and regulation become essential.

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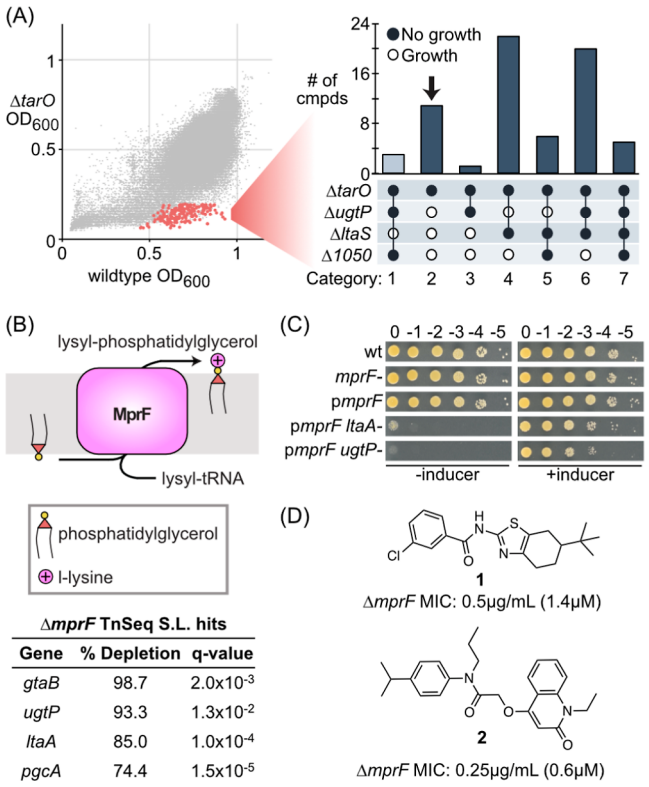

ΔmprF TnSeq S.L. hits

Gene	% Depletion	q-value
gtaB	98.7	2.0×10^{-3}
ugtP	93.3	1.3×10^{-2}
ltaA	85.0	1.0×10^{-4}
pgcA	74.4	1.5×10^{-5}

Figure 2. Two potential LTA pathway inhibitors were identified from a ∼230,000 compound screen using only synthetic lethal growth assays. (A) A differential growth screen of wildtype and Δ*tarO S. aureus* (left panel) identified 68 hits that were classified into categories based on growth profiles against a four-strain mutant panel (right panel). Category 1 (light gray) contained D-alanylation inhibitors.[35] Category 2 (arrow), the only category containing compounds that do not inhibit growth of mutants with disruptions in the LTA pathway, contained the possible LTA pathway inhibitors. (B) MprF synthesizes lysyl-phosphatidyglycerol intracellularly and translocates it to the extracellular surface of the membrane. A TnSeq experiment in a Δ*mprF* background showed depletion of transposon insertion reads in LTA pathway genes. (C) Spot dilution assays confirm that strains lacking LTA pathway genes *ugtP* or *ltaA* grow only if MprF is expressed. (D) Structures of the two compounds from category 2 that prevent growth of Δ*mprF*, with minimum inhibitory concentrations (MICs) against this strain.

We sought a strategy to bin the hits from our high-throughput screen so that we could quickly identify the compounds that inhibited a pathway of immediate interest. We therefore grouped the compounds into categories based on their ability to inhibit the growth of a small panel of mutant strains. In addition to Δ*tarO*, our original test strain, we included three additional *S. aureus* mutants, Δ*ugtP*, Δ*ltaS*, and Δ*1050*. The first two of these mutants disrupt steps in the LTA pathway, either glycolipid anchor synthesis (Δ*ugtP*) or polymer synthesis (Δ*ltaS*); the last (Δ*1050*) deletes a gene of unknown function that is important for cell envelope integrity.[36,37] We originally chose this test panel because it was diagnostic for D-alanylation (Dlt) pathway inhibitors.[35] From a previous discovery of a Dlt pathway inhibitor, we expected any new compounds that prevented LTA D-alanylation to kill Δ*tarO*, Δ*ugtP*, and Δ*1050* but not Δ*ltaS*.[30] Identifying these compounds immediately allowed us to focus our attention on identifying inhibitors for other pathways. Based on their growth profiles against the four-strain panel, the 68 hit compounds were grouped into seven categories (Figure 2A, right panel). Category 1, which included the putative Dlt pathway inhibitors, contained three compounds representing two distinct chemotypes. Both chemotypes proved to inhibit LTA D-alanylation.[35,38]

We were left with six other categories of compounds with other targets. Because the compounds within each category have targets that are distinct from the targets of compounds in all other categories, we can prioritize hits based not only on structure and potency but also on possible mechanisms of action. Our goal was to identify LTA pathway inhibitors among the remaining 65 hits. We reasoned that LTA pathway inhibitors would not inhibit growth of strains in which the LTA pathway was already disrupted. If this reasoning was correct, then any LTA pathway inhibitors could only be found among the category 2 compounds because only these compounds did not inhibit growth of the two strains having defects in the LTA pathway. Category 2 contained 11 compounds (Figure S1).

Synthetic Lethality with an Enzyme that Modifies Phosphatidylglycerol Identifies Two Inhibitors that Block Assembly of LTA on Glc_2DAG. We needed a rapid strategy to determine if any of the 11 compounds in category 2 acted on proteins in the LTA pathway. With so few compounds to test, we could have proceeded immediately to experiments examining whether any of them caused changes in LTA length. However, a more elegant, and ultimately more rapid, approach would be to identify all possible target pathway inhibitors using only synthetic lethal growth screens. Although there is not yet a complete synthetic lethal network for *S. aureus*, we fortuitously found a diagnostic synthetic lethal relationship for LTA pathway inhibitors in the course of a separate investigation. We were investigating a lipid-modifying enzyme called MprF (for multiple peptide resistance factor), which synthesizes lysyl-phosphatidylglycerol on the inner leaflet of the cytoplasmic membrane and then translocates it to the cell surface. MprF is important in resistance to cationic antimicrobial peptides.[39] A genome-wide transposon screen we carried out showed that all four genes required for assembly of LTA on Glc_2DAG became essential in a Δ*mprF* background (Figures 2B and S2). We therefore tested if strains lacking *ugtP* or *ltaA* could grow if *mprF* expression was prevented. The knockouts were only viable when expression of *mprF* was induced, confirming the synthetic lethal relationship identified in the genome-wide screen (Figure 2C). Therefore, Glc_2DAG is required as the membrane anchor for LTA assembly when lysyl-phosphatidylglycerol is not made. Because LTA pathway components were the only synthetic lethal partners we identified for *mprF* in the genome-wide screen, we concluded that compound lethality against Δ*mprF* would identify inhibitors of this pathway. When we tested the ability of the 11 compounds in category 2 to prevent growth of Δ*mprF*, we found two possible LTA pathway inhibitors (**1** and **2**, Figures 2D and S4).

Cellular Biochemistry and Overexpression Implicate UgtP as the Target of 1 and 2. To test if **1** and **2** affect LTA synthesis, we examined LTA production in their presence. Mutants deficient in glycolipid biosynthesis or export make abnormally long LTAs, and thus, we expected compounds that inhibit LTA assembly on Glc_2DAG to result in similarly long LTAs.[8,17] Indeed, we observed a dose-dependent increase in LTA length upon treatment with either **1** or **2**, starting at compound concentrations similar to those that inhibited

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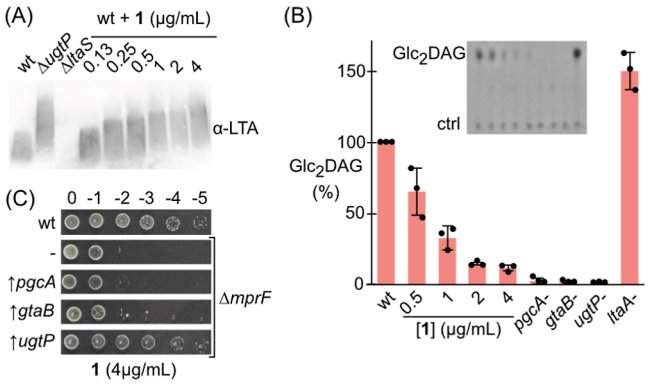

Figure 3. Compound **1** blocks Glc_2DAG synthesis, and overexpression analysis nominated the Glc_2DAG synthase, UgtP, as the target of **1**. (A) Representative western blot showing dose-dependent length changes in LTA upon treatment of wildtype *S. aureus* with **1**. See Figure S5A for data on **2**. (B) Bar graph showing normalized Glc_2DAG levels from compound-treated wildtype *S. aureus* and LTA pathway mutants (n = 3 with individual data points shown; error bars = mean + SD). Inset shows a representative TLC image with the lanes from left to right in the same order as that of the bar graph (ctrl indicates the spike-in control used for quantification; see the Supporting Information Materials and Methods section). See Figure S5B for data on **2**. (C) Spot dilution assays testing if overexpression of the genes involved in Glc_2DAG synthesis from a multi-copy plasmid permits growth of Δ*mprF* on **1**. Only *ugtP* overexpression rescued growth. See Figure S5C for data on **2**.

growth of Δ*mprF* (Figures 3A and S5A). These results suggested that **1** and **2** inhibit one of the four proteins upstream of LtaS in the LTA synthesis pathway, that is, PgcA, GtaB, UgtP, or LtaA.

We sought to identify the step(s) in the LTA pathway that **1** and **2** block. To do so, we first used a thin-layer chromatography (TLC) assay to assess Glc_2DAG amounts in *S. aureus*.[8,17] Treatment with either **1** or **2** caused a dose-dependent reduction in Glc_2DAG recovered from wildtype cells (Figures 3B and S5B). Because Glc_2DAG would form but would not be exported if LtaA was inhibited, this experiment narrowed the possible targets to PgcA, GtaB, and UgtP.

Target overexpression often decreases susceptibility to a compound, so we next expressed each of the three possible targets from a multi-copy plasmid in a Δ*mprF* background and tested for susceptibility to **1** and **2**. Expression of *ugtP*, but not *pgcA* or *gtaB*, resulted in decreased susceptibility to both **1** and **2**, as indicated by improved growth on plates containing the compound (Figures 3C and S5C). These results suggested that the glycosyltransferase UgtP was the target of both compounds.

Expression of an Intrinsically Resistant Ortholog of UgtP Confers Resistance to 1 and 2. One strategy to confirm the target of a compound is to test if expression of an intrinsically resistant ortholog of the proposed target confers resistance. *Bacillus subtilis* UgtP has the same enzymatic function as *S. aureus* UgtP but shares only 36% sequence identity. Given the extensive amino acid changes compared with *S. aureus* UgtP, we reasoned that there was a high probability that the *B. subtilis* enzyme would be resistant to our compounds. We expressed either *S. aureus ugtP* or *B. subtilis ugtP* from a multi-copy plasmid in a Δ*ugtP* background and assessed production of the LTA glycolipid anchor in the presence of increasing concentrations of **1** or **2**. Glc_2DAG production was blocked in a dose-dependent manner in strains expressing *S. aureus ugtP*. However, strains expressing *B. subtilis ugtP* produced abundant Glc_2DAG in the presence of both compounds (Figures 4A and S5D). Therefore, the *B. subtilis* enzyme can complement the loss of *S. aureus* UgtP, and it is intrinsically resistant to both **1** and **2**. We thus predicted that expressing *B. subtilis ugtP* in Δ*mprF* cells would permit growth in the presence of compounds **1** and **2**. Indeed, Δ*mprF* cells expressing *B. subtilis ugtP* from a genomic locus survived in the presence of the compounds, whereas cells expressing *S. aureus ugtP* did not (Figure 4B). These data confirmed *S. aureus* UgtP as the target of compounds **1** and **2**.

Resistance Mutations in *S. aureus* UgtP Identify the Binding Sites of Compounds 1 and 2. Mutants having single amino acid substitutions that confer resistance to a compound can provide insights into where a compound binds its target. We reasoned that it should be possible to identify mutations in *S. aureus* UgtP by selecting for resistance to our UgtP inhibitors in a susceptible mutant background. However, we suspected that it might be difficult to identify target mutants by whole-genome sequencing if a substantial fraction of the mutants contained suppressor mutations elsewhere in the genome. In our experience, extragenic suppressors can arise at much higher frequency than target mutants when knockout strains are plated on a synthetically lethal compound.[30] We therefore needed a strategy to sort target mutants from other mutants (Figure 4C). One possible sorting strategy would be to test resistant mutants for their ability to grow in the presence of the test compound used in the initial selection (here, compound **1** or **2**) plus another compound that is lethal to *S. aureus* when combined with the test compound. For this strategy to work, the "sorting compound" must not inhibit growth of the susceptible strain used to raise resistant mutants to **1** and **2**. If these conditions are met, cells with target mutations that render the test compound inactive will grow on the compound combination, but cells with other mutations will not (Figure 4C).

We previously identified a compound, **3**, that inhibits growth of Δ*ugtP* but not wildtype *S. aureus* (Figure 4C; see the Supporting Information Materials and Methods section). This compound is therefore lethal in combination with either **1** or **2**. Although the target of **3** is still unknown, we have established that it does not prevent growth of Δ*tarO*, a strain that is highly susceptible to **1** and **2**. Therefore, **3** meets our criteria for a useful compound to sort target mutants from other mutants after selection of mutants resistant to **1** or **2** in a Δ*tarO* strain.

We selected resistant mutants by plating Δ*tarO* cells on agar containing **1** or **2**. Mutants arose at a rate of roughly one in a million. We next tested approximately 50 resistant mutants raised against each compound against a combination of test compound (**1** or **2**) and compound **3** (Figure 4C). About 10% of the mutants survived the compound combinations and were therefore expected to be enriched for target mutants. Targeted sequencing showed that all the surviving mutants had mutations in or just upstream of *ugtP* (Figure S9). For compound **1**, we found missense mutations in P113 (P113S) and F75 (F75V). For **2**, we found only F75V and F75L. To confirm that these mutations conferred resistance, we expressed *S. aureus* UgtP variants having either the P113S or F75V substitutions in a Δ*mprF* background and tested for compound lethality. While cells expressing wildtype UgtP in this background could not grow on the compound, cells expressing either the P113S or the F75V UgtP variants grew on compound **1** and cells expressing the F75V variant grew on

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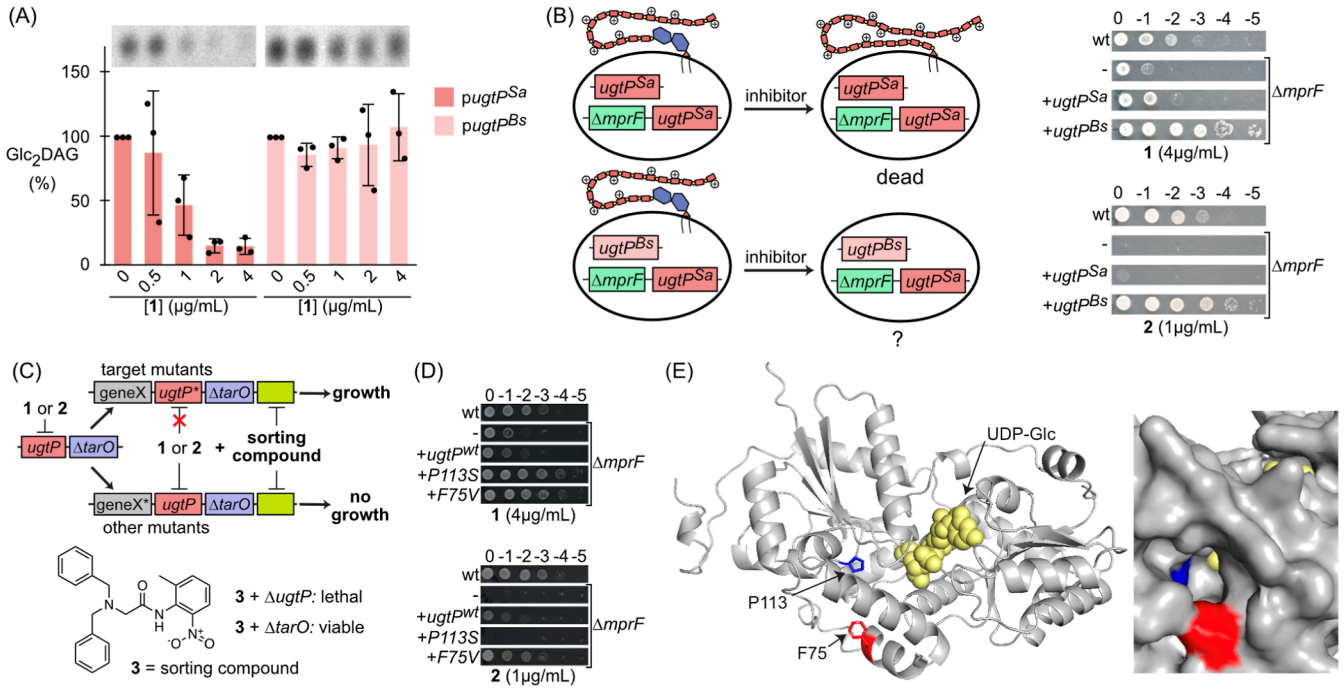

Figure 4. UgtP is the target of both **1** and **2**. (A) Bar graph showing normalized Glc_2DAG levels in the presence of **1** when *S. aureus ugtP* (red bars) or *B. subtilis ugtP* (pink bars) is expressed from a plasmid in a Δ*ugtP* background ($n = 3$ with individual data points shown; error bars = mean + SD, representative inset). See Figure S5D for data on **2**. (B) Schematic (left panel) depicting cells tested in spot dilution assays (right panel). Chromosomal expression of *B. subtilis ugtP*, but not *S. aureus ugtP*, from an ectopic locus permits growth of Δ*mprF* cells on **1** or **2**. (C) Schematic depicting selection and sorting of target mutants resistant to **1** or **2**. Mutants were selected by plating Δ*tarO* on **1** or **2**. ~50 mutants resistant to each compound were then grown on either **1** or **2** and compound **3**, which inhibits growth of Δ*ugtP* but not Δ*tarO* strains. Targeted sequencing of the surviving mutants showed that all contained mutations in or upstream of *ugtP* (see Figure S9). (D) Spot dilution assays show that point mutations in *ugtP* that change a single amino acid permit growth of Δ*mprF* on **1** or **2**. (E) Left panel: Cartoon representation of the AlphaFold model for *S. aureus* UgtP (gray)[41,42] showing UDP-Glc (yellow spheres) in the active site. The locations of the residues that confer resistance when altered (F75 and P113) are shown in red and blue. Right panel: Close-up view of surface representation of a hydrophobic tunnel that leads to the active site cleft. F75 and P113 flank this tunnel.

compound **2** (Figure 4D). These results suggest that the binding sites in UgtP for these compounds overlap.

Resistance Mutations Map to a Hydrophobic Tunnel Leading to the Active Site. UgtP belongs to a major superfamily of glycosyltransferases (GT-B) that includes the peptidoglycan biosynthesis enzyme MurG[40] and other membrane-associated glycosyltransferases involved in cell envelope biogenesis. An AlphaFold model of UgtP (Figure 4E, left panel)[41,42] shows the features characteristic of all GT-B family glycosyltransferases.[43,44] The enzymes have a two-lobed structure in which both lobes have Rossman folds; the reaction takes place in the cleft between the lobes. The nucleotide–sugar donor substrate is anchored via characteristic contacts in the C-terminal lobe with the sugar extending into the active site cleft. Although there is considerable variation in how acceptor substrates bind to GT-B family members, contacts to the N-terminal lobe are often important.[43] The amino acids that change to confer resistance to **1** and **2**, F75 and P113, are located in the N-terminal lobe of UgtP at the mouth of (F75), or within (P113), a tunnel lined with hydrophobic residues that leads to the active site (Figure 4E, right panel). We speculate that DAG enters the active site from the membrane through this greasy tunnel and that compounds **1** and **2** block the tunnel to prevent DAG access to the active site.

UgtP Inhibitors Are Lethal with Inhibitors of Other Cell Envelope Pathways and Sensitize MRSA to β-Lactams. One motivation for seeking LTA pathway inhibitors is that they could be useful as components of therapeutic compound combinations. We conducted a limited exploration of commercially available analogues of **1** and **2** to assess whether simple changes would improve activity (Figure S10). All tested changes to **2** resulted in increased minimum inhibitory concentrations (MICs) against Δ*mprF*; however, two analogues of **1** showed an 8- to 16-fold decrease in the MIC (**4** and **5**, Figures 5A, S10 and S11). These compounds contain a stereocenter and are only available commercially as racemic mixtures, so we separated **4** into its two enantiomers via supercritical fluid chromatography (Figure S12). The enantiomer that eluted second was 4- to 8-fold more potent, but both had activity against Δ*mprF*. Therefore, in the following studies that explore compounds in synergistic combinations, we used **2** and the racemic mixture of **4**.

We have identified multiple D-alanylation pathway inhibitors from our high-throughput screen (Figure 2A). Because these compounds are lethal to Δ*ugtP*, we would expect them to be lethal in combination with UgtP inhibitors.[30,35] We would also expect UgtP inhibitors to potentiate β-lactam activity in MRSA because we previously showed that *ugtP* is required for high level resistance to oxacillin.[9] We therefore sought to test our UgtP inhibitors in combination with other compounds. As predicted, combining either **2** or **4** with either of the two structurally distinct D-alanylation inhibitors prevented growth of the MRSA strain COL (Figure S13), and both UgtP inhibitors strongly potentiated the activity of oxacillin (Figure 5B). Only 0.5 μg/mL of **4** or 1 μg/mL of **2** decreased the concentration of oxacillin required to inhibit the growth of

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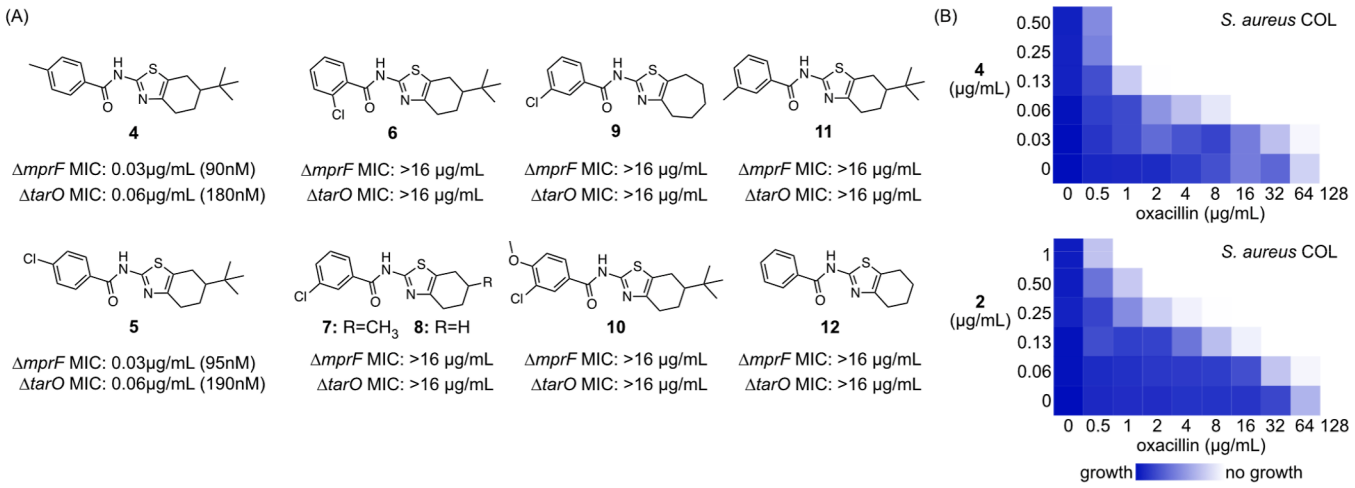

Figure 5. UgtP inhibitors restore β-lactam sensitivity in MRSA. (A) Structures and Δ*mprF* and Δ*tarO* MICs of a small survey of commercially available analogues of **1**. The survey identified **4** and **5** as more potent analogues. (B) Checkerboard assay showing decreasing oxacillin MIC for the MRSA strain COL in the presence of increasing concentrations of **4** or **2**. Individual checkers are colored based on percentage growth relative to that of the untreated; values are a mean of three replicates. **4** and **2** are not lethal to wildtype *S. aureus* up to at least 16 μg/mL.

COL from 128 to 1 μg/mL. UgtP inhibitors may therefore have potential for use in compound combinations to treat infections. We expect that additional inhibitor scaffolds can be rapidly found using a screening pipeline similar to that outlined here.

DISCUSSION

We have demonstrated a remarkably efficient screening pipeline for identifying compounds with targets in a specified region of biological space. This pipeline uses simple growth inhibition screens that exploit synthetic lethality to (i) identify a collection of primary hits in that specified region of space, (ii) classify these hits into groups having distinct sets of targets, and (iii) find the compounds within a group that target a specific pathway of interest. We showed that this screening pipeline can identify compounds that affect a designated target pathway with perfect efficiency, meaning that all candidates predicted to target that pathway indeed do so. Although we focused here on finding compounds that affect pathways important in *S. aureus* cell envelope integrity, this screening approach is not limited to the cell envelope, to *S. aureus*, or even to bacteria. We believe similarly efficient synthetic lethal screening pipelines can be designed for any cells for which some knowledge of synthetic lethal networks is available.

High-throughput screens have been widely used for over 2 decades to discover drug leads and chemical probes. There has been considerable discussion about the advantages and disadvantages of target-based versus phenotypic screens. Two key advantages of phenotypic screens are that biological activity is guaranteed and the target space is broad. In contrast, target-based screens do not guarantee biological activity and typically focus on one target, which limits opportunities for success. Despite their advantages, growth-based phenotypic screens performed using only one strain, cell line, or condition also have limitations. In addition to returning a large number of "nuisance compounds" with nonspecific or toxic mechanisms of action, these screens will not yield inhibitors of targets that are not essential for growth in the laboratory. Over 90% of proteins in cells fall into this category.[45−49] However, a substantial fraction of these proteins are required under other conditions—for example, for growth in a host organism—that may make them valuable therapeutic targets.[50] The limitations of unbiased phenotypic screens led us to explore "pathway-directed" screens, which are screens designed to identify compounds that differentially affect growth of either a wildtype strain or a mutant strain. The vast majority of nuisance compounds are filtered out because they kill both strains. Moreover, the hits are biased toward the region of biological space determined by the mutant chosen for the screen.[30,34,51,52] Reducing the target space in this manner increases the probability that inhibitors with desired mechanisms of action will be discovered.

In the differential growth screen described here, we used a wildtype strain and a strain lacking WTA. We expected to find compounds that affect cell envelope targets required for growth in the absence of WTA, and we identified 68 hits from a screen of ∼230,000 compounds. This number of hits is sufficiently low that one could test all the compounds for activity in a biochemical assay. However, for many cell envelope targets, biochemical assays are challenging to develop because the substrates are difficult to obtain and the proteins operate at a membrane interface. We realized that by grouping the hit compounds into categories with different mechanisms of action, we could prioritize compounds in a particular category rather than testing all 68 of them.

We expected the 68 hit compounds to affect a range of different cell envelope-related targets with distinct sets of synthetic lethal interactions. Having complete knowledge of all synthetic lethal interactions in *S. aureus* would make it possible to design a mutant panel that would optimally group the compounds into categories that ensured the most efficient follow-up. Because we would know all the possible targets for the compounds in any given category, we could design simple follow-up growth screens to identify inhibitors of those targets. However, complete information about synthetic lethal relationships in *S. aureus* is not yet available. Indeed, it has been acquired for only a few organisms,[53−56] although recent advances in genomic technologies promise to make it easier to fully map these relationships.

We designed the mutant panel used to classify hits based on the partial knowledge of synthetic lethal networks that we had gleaned from a limited number of genome-wide screens. One

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can map synthetic lethal interactions very rapidly using TnSeq[30,31] by probing a transposon library with a small molecule inhibitor. When the work described here commenced, we had small molecule inhibitors for only two cell envelope pathways. One compound inhibited TarO to prevent WTA biosynthesis and the other inhibited DltB to prevent D-alanylation.[30,31] Because the WTA pathway is synthetically lethal with a large number of other proteins and pathways, our primary screen used a Δ*tarO* mutant. We designed our four-strain mutant panel to immediately identify any new D-alanylation inhibitors,[35] which minimized the time we spent following up on compounds for targets we could already inhibit. We were able to group our 68 primary hits into seven categories based on their growth profiles.[35] One category contained predicted D-alanylation inhibitors, and all were shown to target this pathway.[35,38] This example demonstrated the power of synthetic lethal screens for identifying new compounds rapidly given an existing inhibitor to guide discovery of new chemotypes. Here, we wanted to show that a synthetic lethal screening approach could be used to find inhibitors for a pathway where there were no existing inhibitors.

We were particularly interested in compounds that blocked assembly of the LTA glycolipid anchor, which is important in physiology, pathogenesis, and antibiotic resistance.[8,9,11,14] Our four-strain screening panel contained two LTA pathway mutants, and LTA glycolipid synthesis inhibitors would not be expected to prevent growth of these strains. Only one category of compounds did not affect the growth of the LTA pathway mutants. Therefore, any possible LTA pathway inhibitors had to be in this category. We were able identify two candidate LTA inhibitors based on their ability to inhibit growth of a mutant we serendipitously found to be diagnostic for a block in LTA glycolipid assembly. Both compounds proved to inhibit the glycosyltransferase UgtP.

We have now shown for two different pathways that we can find biologically active inhibitors using only simple growth screens without following up on any false leads. This level of efficiency in inhibitor discovery cannot be surpassed. It is true that implementing this type of screening approach requires some knowledge of synthetic lethal relationships and acquiring this knowledge might appear to be a substantial barrier. However, TnSeq and CRISPR screens have made obtaining information on synthetic lethal networks straightforward. Genome-wide screens performed with small molecules are particularly convenient, as we have shown in mapping synthetic lethal networks for the *S. aureus* WTA and D-alanylation pathways. If suitable compounds are not available to perturb a pathway, similar screens using genetic knockdowns or knockouts can be used. Indeed, for the work described here, we used results from a fortuitously timed TnSeq screen that identified Δ*mprF* as a diagnostic strain for LTA pathway inhibitors. We expect that it will become easier to design synthetic lethal screening pipelines for efficient inhibitor discovery as functional genomics delivers on its promise to elucidate genetic networks in different microorganisms and cell types. In a general scenario, a primary screen would be carried out using strains, cell lines, or conditions predicted to return hits for the greatest number of pathways. Using small screening panels in follow-up work, the primary hits would then be grouped into categories where the possible targets for each category are known. Hits for different target pathways within a category would be differentiated using further growth tests, similar to our diagnostic use of the Δ*mprF* mutant to identify LTA pathway inhibitors. Using this approach, it should be possible to quickly discover inhibitors for multiple targets from any given primary screen.

It is worth pointing out here that differential growth screens that exploit synthetic lethality have been leveraged previously in screening for anticancer compounds. Cancer cells have genetic dependencies not found in normal cells, and there are a few examples in which a genetic dependency in a cancer cell has been exploited to identify compounds that kill the cancer cell but not a normal cell.[57] In some cases, the targets for compounds that leverage synthetic lethality are known; however, there are also cases where the targets for compounds found to kill cancer cells in a growth screen have not been identified. Systematic approaches to fully elucidate the synthetic lethal networks unique to cancer cells will make it possible to design strategies that group hit compounds from any large synthetic lethal growth screen into categories with different mechanisms of action. This would accelerate target identification.

We want to make one further point about our experience with hits from pathway-directed screens. We have so far found inhibitors and targets for three different pathways using this screening approach. Although there are multiple possible targets within each pathway, we have only found compounds that hit one of the possible targets.[30,34,35,58,59] Because we found more than one chemotype for each of these targets, we have concluded that some targets in a pathway are easier to inhibit and achieve a biological effect than others. It is possible that the types of compounds included in a typical screening library are a better match for some targets than others, but other factors are also likely at play. Target accessibility is one factor that may be important. We note that the targets in two of the pathways we have hit are polytopic membrane proteins, and resistant mutant analyses show that the inhibitor binding pockets are at least partially exposed to the cell surface.[30,34,35,58] UgtP, however, is an intracellular protein, albeit membrane-associated. Therefore, we speculate that the preferred targets within a pathway may be wholly or partially rate-limiting, making it unnecessary to achieve full inhibition to observe a biological effect. UgtP catalyzes two different glycosylation reactions and may need to dissociate from the membrane to release Glc_2DAG. It would not be surprising if UgtP was a slow step in the LTA pathway. Whatever the explanation, the observation that some targets in a pathway are better for inhibitor discovery than others provides a compelling reason to use pathway-directed phenotypic screens rather than biochemical screens. Biochemical screens are often designed for the targets in a pathway that are the easiest to assay, and these may not be the optimal targets.

In closing, we simply want to emphasize that the results described in this article provide a roadmap for highly efficient discovery of biologically active inhibitors. We urge chemists, biologists, and computer scientists to collaborate in leveraging functional genomics and information about synthetic lethal networks to design optimal screening pipelines for rapid discovery of compounds and targets in desired regions of biological space.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c12697.

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Materials and methods, strain and plasmid tables, structures and liquid MICs for category 2 compounds and analogues of **1** and **2**, Δ*mprF* transposon sequencing data visualization, biological replicates for all spot diltutions and Western blots, target identification data for compound **2**, point mutations that confer resistance to **1** and **2**, supercritical fluid chromatography for compound **4**, UgtP and Dlt inhibitor combination treatments (PDF)

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Notes

The authors declare no competing financial interest.

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