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Imidazoles Induce Reactive Oxygen Species in *Mycobacterium tuberculosis* Which Is Not Associated with Cell Death

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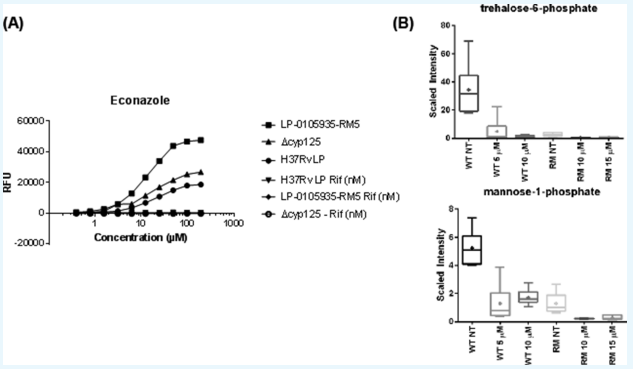
Supporting Information

ABSTRACT: Azoles are a class of antimicrobial drugs used clinically to treat yeast and fungal infections. Against pathogenic yeast and fungi, azoles act by inhibiting the activity of the cytochrome P450 Cyp51, which is involved in the synthesis of a critical component of the yeast and fungal cell membrane. Azoles have antibacterial activity, including against mycobacteria, but the basis for this activity is not well-understood. We demonstrated that imidazoles are bactericidal to *Mycobacterium tuberculosis*. A marked increase in reactive oxygen species (ROS) was observed within imidazole-treated *M. tuberculosis*. The generation of ROS did not appear to be related to the mechanism of killing of imidazoles, as the addition of antioxidants or altered expression of detoxifying enzymes had no effect on growth. We examined the metabolic changes induced by econazole treatment in both wild-type and econazole-resistant mutant strains of *M. tuberculosis*. Econazole treatment induced changes in carbohydrates, amino acids, and energy metabolism in both strains. Notably, the untreated mutant strain had a metabolic profile similar to the wild-type drug-treated cells, suggesting that adaptation to similar stresses may play a role in econazole resistance.



INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, now ranks as a leading cause of death worldwide.[1] In 2014, 9.6 million people were newly diagnosed with TB and 1.5 million people died.[1] Globally, 3.3% of new cases and 20% of those previously treated had multidrug resistant TB.[1] In addition to the current active burden of diseases, roughly one-third of the world's population is latently infected with TB, with 5−10% of this population expected to develop active TB at some point in their lifetime.[2] Given the current and estimated future incidence of TB, there is a pressing need to develop new TB drugs and to understand the biology of this organism in response to the drug treatment.

Bioinformatic analysis of the *M. tuberculosis* genome sequence revealed a surprisingly large number of cytochrome P450 mono-oxygenases (P450s).[3] *M. tuberculosis* has 20 P450s, whereas previously sequenced bacterial genomes had indicated that these enzymes were rare in bacteria. Interestingly, one of the *M. tuberculosis* P450s is found to be a homolog of the eukaryotic 14α-sterol demethylases (CYP51), the target of the imidazole class of drugs commonly used in the treatment of fungal infections. CYP51 is involved in the synthesis of ergosterol, a critical component in the cell wall of yeast and fungi.[4] Imidazoles bind to the *M. tuberculosis* CYP51,[5] as well as several other P450s, notably CYP121,[6] CYP130,[7] CYP125,[8] and CYP144.[9] Imidazoles have antimycobacterial activity both in vitro and in vivo,[10] with activity against persistent bacteria.[11]

Imidazole resistance in fungi is mediated by increased levels of the cellular target, decreased affinity of the target for imidazole binding, upregulation of genes controlling drug efflux, and alterations in sterol synthesis.[12] In *M. tuberculosis*, mutations in Rv0678 confer resistance to imidazoles.[13,14] Rv0678 is a transcriptional regulator of the *mmpS5-mmpL5* efflux system.[14] Thus, it appears that resistance to imidazoles in *M. tuberculosis* is via increased efflux from the cell rather than alterations in target levels or specificity.

The mode of action of imidazoles in *M. tuberculosis* is still unclear. The binding of imidazoles to multiple P450s, none of which are essential for bacterial growth in vitro, suggests that these enzymes may not be the actual targets but instead act to dilute the amount of drugs available within the cell to interact with their true target(s). Another possibility is that there is no single target and that imidazoles have a general toxic effect to *M. tuberculosis*.

In this study, we show that econazole is bactericidal to *M. tuberculosis*. We demonstrate that econazole exposure leads to a

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rapid and transient increase in reactive oxygen species (ROS) within *M. tuberculosis*, but this effect does not appear to contribute to the bactericidal activity. We also profile the metabolome of wild-type and econazole-resistant bacteria, which suggest that econazole treatment leads to global metabolic adaptations. These appear to be primarily a mechanism to respond to the drug action because the (untreated) resistant mutant resembled wild-type bacteria after exposure to econazole.

RESULTS

Econazole is Bactericidal to Replicating *M. tuberculosis*. We are interested in understanding the mode of action of imidazoles in *M. tuberculosis*. Imidazoles inhibit the growth of *M. tuberculosis* in vitro,[10,11] but it is not clear whether this translates into bacterial killing. We determined whether econazole was able to kill *M. tuberculosis* under normal aerobic culture conditions. We exposed *M. tuberculosis* H37Rv to econazole concentrations (from 5 to 200 μM) over 28 days and monitored viability by colony counts (Figure 1). We saw concentration-dependent kill.

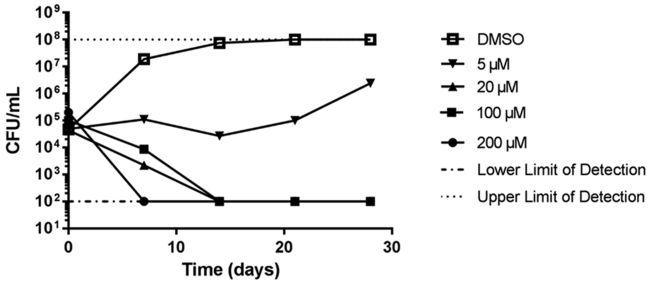

Figure 1. Econazole is bactericidal against *M. tuberculosis*. *M. tuberculosis* H37Rv LP was incubated with varying concentrations of econazole under aerobic growth conditions in standing culture. Colony-forming units (CFUs) were determined over 28 days.

At the highest concentration used (200 μM), there were no viable colonies obtained within 7 days representing >3 logs kill (limit of detection was 100 CFU). Similarly, at 20 and 100 μM, the kill was seen, with sterilization of cultures by day 14. The lowest concentration tested resulted in growth inhibition, with a small increase in CFU over 21 days (~50-fold increase, compared with $>4 \times 10^3$ log increase in the control). Thus, we see that econazole has bactericidal activity against actively growing *M. tuberculosis*.

Imidazoles Induce Reactive Oxygen Species Production in *M. tuberculosis*. The induction of ROS has been suggested as the mechanism by which the bactericidal antibiotics work.[15] The antitubercular agent clofazimine works by inducing oxidative stress via ROS,[16] and *M. tuberculosis* is sensitive to endogenous oxidative stress[17] and to killing by a vitamin C-mediated Fenton reaction.[18,19] In addition, the mechanism of action of miconazole in fungi is by the production of ROS generated by respiration.[20] By contrast, other studies have indicated that ROS is not responsible for the killing of bacteria by antibiotics.[21−24] We took several approaches to determine if this could be how imidazoles kill *M. tuberculosis*. First, we measured the production of ROS in *M. tuberculosis* upon incubation with imidazoles using a fluorescent reporter (Figure S1). We used the reporter 2′,7′-dichlorofluorescin diacetate (DCFDA), which is commonly used to detect ROS or more generally oxidative stress. DCFDA is converted into a fluorescent form after oxidation, which can be mediated by a number of different molecules including hydrogen peroxide and hydroxyl radicals.[25]

We observed a concentration-dependent increase in ROS levels in response to econazole, clotrimazole, ketoconazole, and miconazole (Figure 2). We did not detect an increase in the ROS levels in response to incubation with isoniazid (data not shown) or rifampicin (Figure 2), suggesting that this was a response specific for the imidazole class. Our data for rifampicin are consistent with previous works that showed that in vitro it was not able to induce oxidative stress.[26]

Superoxide Dismutase Does Not Protect *M. tuberculosis* from Killing by Imidazoles. If ROS were responsible for the bactericidal activity of azoles, we would expect that the detoxification of oxygen radicals would be protective against imidazoles. To test this, we generated a strain of *M. tuberculosis* capable of overexpressing the superoxide dismutase SodC (Rv0432). We used an anhydrotetracycline-inducible promoter to control the SodC expression. We determined the minimum inhibitory concentrations (MICs) for econazole, ketoconazole, and miconazole against the wild-type and SodC overexpressing (OE) strains (Table 1) under both inducing (aTc) and noninducing conditions (no aTc) (Table 2).

We noted a small shift in MICs (2−4 fold) between the wild-type and SodC-OE strains, but this shift was seen under both induced and noninduced conditions. The largest shift was seen with clotrimazole. Because the shift, if any, was relatively small, we considered that SodC overexpression was not able to mitigate the effect of azole activity. However, because we do not have a positive control that is known to induce ROS in *M. tuberculosis*, we cannot exclude the possibility that the SodC expression levels were not high enough to effect a difference in ROS defense.

To further examine the relationship between ROS detoxification and imidazole susceptibility, we determined MICs for two strains with *katG* deletion (Table 3). If ROS generation were responsible for killing of *M. tuberculosis* by imidazoles, we might expect that the lack of a functional catalase gene would make the bacteria more sensitive to these drugs. The MIC for the two mutant strains tested was nearly identical to the parental strain for econazole, clotrimazole, ketoconazole, and miconazole. *M. tuberculosis* has a number of other enzymes involved in oxidative defenses, but because KatG normally plays a major role, we would have expected to see a difference.

Induction of ROS Is Not Linked to Imidazole MICs. If ROS generation is responsible for imidazole-mediated killing of *M. tuberculosis*, we reasoned that a strain, which is more sensitive to imidazoles, would have increased the ROS production or at least that the ROS production would be induced at lower concentrations. We tested this using a Δ*cyp125* strain;[28] this strain has an unmarked, in-frame deletion of *cyp125*, and we have previously shown that is more sensitive to growth inhibition by imidazoles.[28] We also used the *cyp125*-complemented strain. We included a strain that is slightly more resistant to imidazoles owing to the increased expression of the MmpL5-MmpS5 efflux system (LP-0105935-RM5; Rv0678 R72P).[13] We determined the MIC for several imidazoles (Table 4).

As expected, the Δ*cyp125* strain was slightly more sensitive, and the Rv0678 mutant was slightly more resistant—approximately 2−3 fold difference between strains. Although

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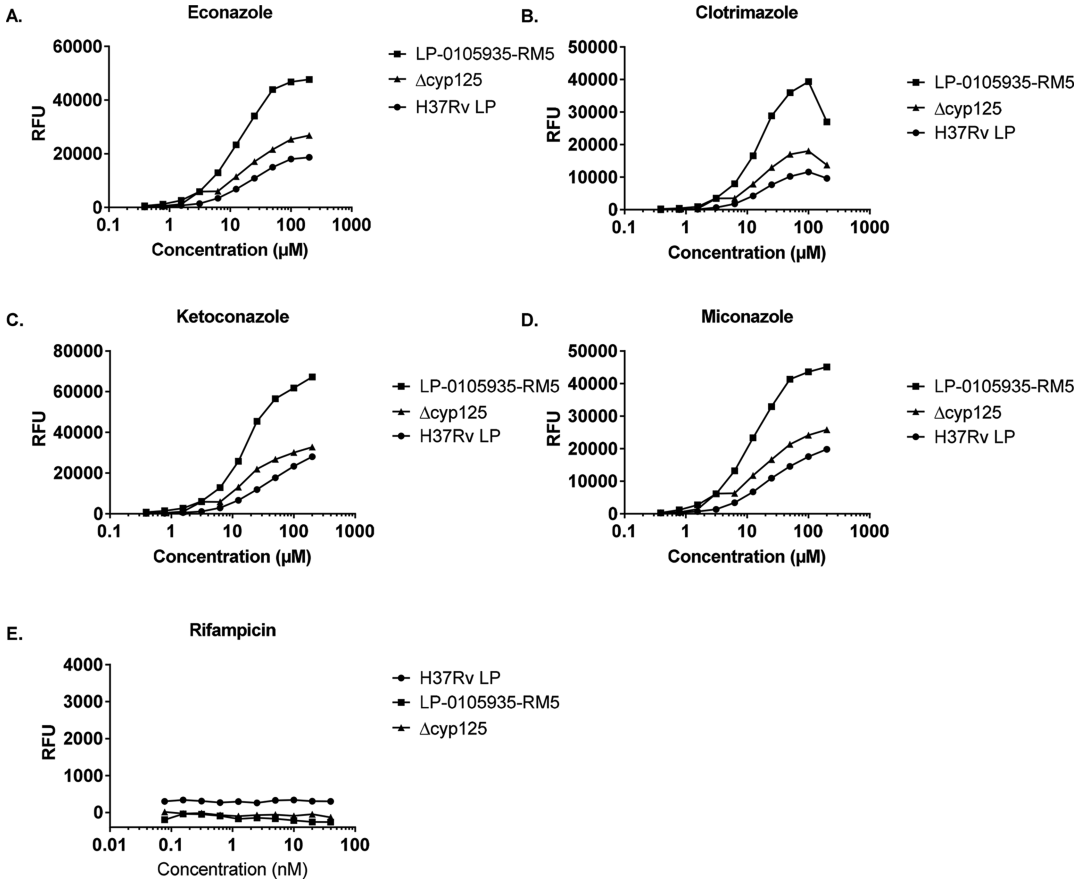

Figure 2. Imidazoles induce ROS in *M. tuberculosis*. *M. tuberculosis* strains were cultured aerobically. Bacteria were loaded with DCFDA and incubated with the indicated concentrations of (A) econazole, (B) clotrimazole, (C) ketoconazole, (D) miconazole, or (E) rifampicin for 90 min before fluorescence was measured. Background fluorescence was subtracted from each value shown. The strains were H37Rv (LP) wild-type, the econazole-resistant mutant LP-0105935-RM5 (Rv0678 R72P), and Δcyp125.

Table 1. *M. tuberculosis* Strains Used in This Study

strain	relevant characteristics	source/reference
H37Rv (LP)	wild type	ATCC 25618[27]
LP-0105935-RM5	Econazole resistant mutant; Rv0678 (R72P)	13
Tame127	deletion in *cyp125* (Rv3545c) in H37Rv (LP)	28
Tame 210	Δ*cyp125* with complementing vector, gm^R in H37Rv (LP)	28
sodC OE	H37Rv (LP) with vector overexpressing Rv0432; hyg^R	this study
H37Rv (MA)	wild type	ATCC 27294[27]
SB-0105927-RM2	H37Rv (MA) *katG* (M105FS); resistant to isoniazid	this study
SB-0105927-RM3	H37Rv (MA) katG (W198*); resistant to isoniazid	this study
Erdman	wild type	29
Δ*icl1/2*	Erdman with deletions in both *icl1* and *icl2*	29
icl1/2 complement	Δ*icl1/2* with complementing vector expressing *icl1*; Km^R	29
icl1 OE	Erdman with vector overexpressing Rv0467; hyg^R	this study

Table 2. Effect of *sodC* Overexpression on Imidazole Activity

	strain[a]			
compound	wild-type	wild-type aTc	*sodC* OE	*sodC* OE + aTc
econazole	15	16	32	28
clotrimazole	17	12	43	51
ketoconazole	40	33	62	47
miconazole	19	17	40	39

[a]Minimum inhibitory concentrations (μM) were determined against each strain cultured in a liquid medium. H37Rv (LP): ATCC 25618 was the parental strain. Anhydrotetracycline (150 ng/mL) was added to induce the expression of *sodC* from the tetracycline-inducible promoter where noted.

Table 3. Effect of *katG* Mutations on Imidazole Activity

	strain[a]		
compound	wild-type	*katG* (M105FS)	*katG* (W198[a])
econazole	42	36	38
clotrimazole	85	86	84
ketoconazole	33	33	43
miconazole	67	51	54

[a]Minimum inhibitory concentrations (μM) were determined against each strain cultured in a liquid medium. H37Rv (MA): ATCC 27294 was the parental strain. Strains with mutations that rendered *katG* inactive were tested.

these differences were small, they were reproducible and we reasoned that we would expect to see differences in ROS generation. ROS generation was measured in the Cyp125 deletion and econazole-resistant strains exposed to the four imidazoles (Figure 2). ROS generation was highest in the

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Table 4. Activity of Imidazole Antifungal Drugs against Recombinant Strains of *M. tuberculosis*[a]

compound	Wild-type	Δ*cyp125*	*cyp125* C′	LP-0105935-RM5
econazole	13	5.5	10	19
clotrimazole	13	9.5	8.0	24
ketoconazole	37	26	44	71
miconazole	15	11	13	24

[a]Minimum inhibitory concentrations (μM) were determined against each strain cultured in a liquid medium. H37Rv (LP): ATCC 25618 was the parental strain. LP-0105935-RM5 is the econazole-resistant strain.

imidazole-resistant strain relative to the wild-type for all drugs in this class (Figure 2). The values for the more sensitive Δcyp125 were slightly higher than those for the wild-type (Figure 2). We calculated half-maximal effective concentration (EC_{50}) values to determine whether there were differences in the concentrations required to elicit an increase in ROS between the three strains (Table 5). The values were similar between all strains, and if anything ROS generation was lower in the Δ*cyp125* strain for ketoconazole (the opposite of that predicted). These results suggest that the generation of ROS is not directly linked to the sensitivity of *M. tuberculosis* to imidazole drugs because we saw no correlation.

Antioxidants Are Not Protective against Imidazole Activity. The induction of isocitrate lyase in response to the treatment with isoniazid, rifampicin, and streptomycin has been reported and linked to antioxidant defense.[30] Icl function was required for defense against all three compounds because an *icl1/2* deletion strain was more sensitive, and this could be reversed by coincubation with an antioxidant.[30] We tested whether imidazoles might have similar activity using the *icl1/2* deletion strain or inclusion of antioxidants in the medium as previously used by others.[30] We determined the MIC for four imidazole drugs against the Erdman, Δ*icl1/2*, *icl1* C′, and *icl1* OE strains (Table 6).

The presence or absence of *icl1* had no effect on the growth of *M. tuberculosis* when incubated with econazole, clotrimazole, ketoconazole, or miconazole. The overexpression of *icl1* had no effect on growth at any of the concentrations of these compounds that we tested. Interestingly, we observed higher levels of ROS generation in the Δ*icl1/2* strain in response to incubation with imidazoles (Figure 3). Because we saw no difference in MIC with these compounds in either strain, this suggests that Icl may play a role in the antioxidant defense but that this effect does not protect *M. tuberculosis* from the action of imidazoles. Given its role in the glyoxylate shunt, it is likely that Icl is required to divert metabolism and prevent ROS accumulation.

Table 6. Effect of *icl1* Expression on Imidazole Activity[a]

compound	Erdman	Δ*icl*1/2	*icl1* C′	*icl1* OE	*icl1* OE (+aTc)
econazole	15	19	15	21	16
clotrimazole	52	53	56	43	41
ketoconazole	25	33	26	52	51
miconazole	29	36	34	24	24

[a]Minimum inhibitory concentrations (μM) were determined against each strain cultured in a liquid medium. Erdman was the parental strain. Anhydrotetracycline (150 ng/mL) was added to induce the expression of *icl1* from the tetracycline-inducible promoter where noted.

We also evaluated the effect of adding antioxidants (Table 7). The addition of 1 mM *N*-acetyl-L-cysteine (NALC), which acts by increasing the cellular pools of antioxidants, did not alter the MIC of econazole, clotrimazole, or ketoconazole. The inclusion of 1 mM Tempol, a superoxide scavenger, or 5 mM thiourea did not lead to an increase in MIC. These results indicate that none of these antioxidants are capable of protecting *M. tuberculosis* from growth inhibition by imidazoles.

ROS Induction Is a Transient Effect. The rapid increase in ROS levels induced by imidazoles initially suggested that it could be a cause of the bactericidal activity of these drugs. However, the lack of effect of superoxide dismutase overexpression, catalase mutations, or antioxidants suggests that this is not the case. We evaluated the longevity of the increased ROS levels observed by incubating *M. tuberculosis* with sublethal concentrations of econazole and by measuring ROS levels over 7 days (Figure 4). We saw that the induction of ROS was short-lived; an increase was observed at 90 min, but this had reduced to background by 24 h of drug exposure. No further induction was seen at 72 or 144 h. These results indicate that the rapid increase in ROS initially observed with econazole treatment is resolved within the first 24 h of exposure, providing further evidence that ROS generation is not responsible for the bactericidal activity of imidazoles in *M. tuberculosis*.

Econazole Induces Global Metabolic Changes in *M. tuberculosis*. To gain better understanding of the global effects of imidazole activity on *M. tuberculosis* at a biochemical level, we undertook metabolomic analysis of the wild-type *M. tuberculosis* and the resistant mutant following econazole treatment. Antibiotic concentrations were selected, which slightly reduced the growth rate (Figure S2). A total of 141 named biochemicals were identified spanning 8 superpathways and 43 subpathways. Table S1 contains the complete data set with normalized metabolite measurements and pathway associations.

A strong signature across the data set involved alterations in pathways supporting the cell wall synthesis. *N*-Acetylglucosamine, a key component of peptidoglycan,[31] was significantly

Table 5. Generation of Reactive Oxygen Species in Strains of *M. tuberculosis*

	strain[a]			
compound	wild-type	Δ*cyp125*	*cyp125* C′	LP-0105935-RM5
econazole	18.2 ± 5.7	19.6 ± 8.2	19.4 ± 5.9	18.4 ± 7.1
clotrimazole	16.6 ± 6.5	19.8 ± 10.6	16.9 ± 6.4	21.1 ± 11.9
ketoconazole	29.7 ± 10.4	17.5 ± 6.9	21.4 ± 7.2	21.2 ± 9.7
miconazole	16.6 ± 5.4	20.3 ± 10.2	15.2 ± 4.3	14.2 ± 4.7

[a]ROS was measured using the fluorescent probe DCFDA. EC_{50} values are the averages of three independent experiments with the standard deviation indicated. LP-0105935-RM5 is the econazole-resistant strain. EC_{50} = effective concentration of 50%, that is, the concentration required to generate 50% of the response.

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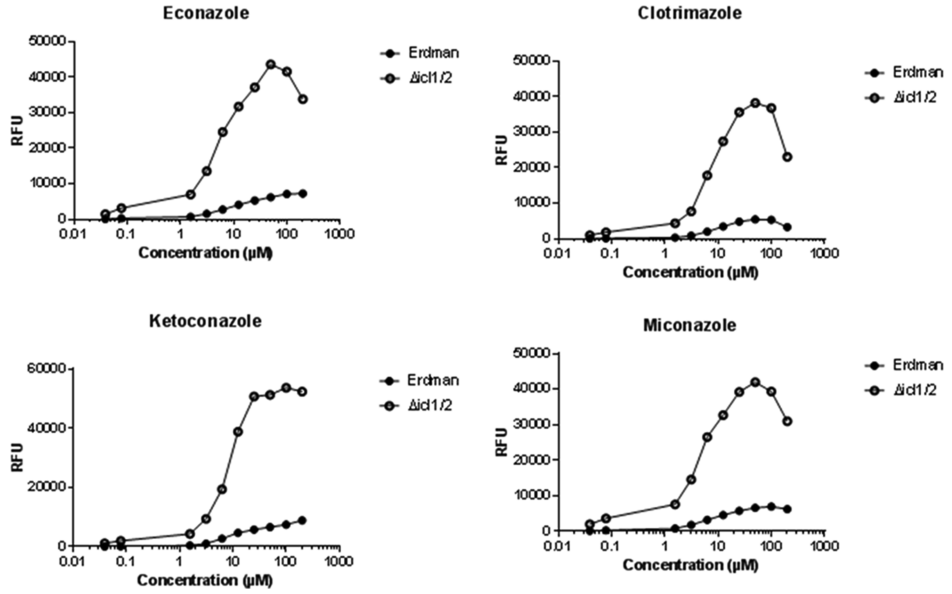

Figure 3. Icl1 protects against ROS induced by imidazoles. *M. tuberculosis* strains were loaded with DCFDA and incubated with concentrations of econazole, clotrimazole, ketoconazole, or miconazole for 90 min before fluorescence was measured. Strains were Erdman wild-type and the double mutant Δ*icl1/2*.

Table 7. Effect of Antioxidants on Imidazole Activity[a]

compound	wild-type	NALC	Tempol	thiourea	catalase
econazole	14	16	8.8	11	9
clotrimazole	9	11	7.6	5.9	4.7
miconazole	15	19	15	9.5	8.6

[a]Minimum inhibitory concentrations (μM) were determined against H37Rv (LP): ATCC 25618. NALC was added to 1 mM, Tempol at 1 mM, thiourea at 5 mM, and catalase at 10 mg/mL.

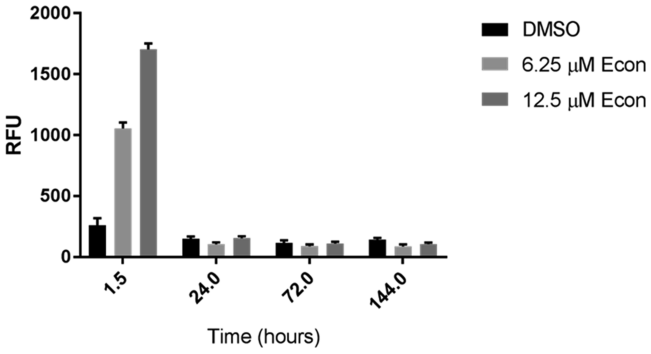

Figure 4. Econazole-mediated induction of ROS is transient. *M. tuberculosis* was exposed to econazole in culture. Bacteria were loaded with DCFDA, and the fluorescence was measured.

decreased with both 5 and 10 μM econazole treatment in the wild-type strain (Figure 5). Similarly, mannose, mannose-1-phosphate, and inositol-1-phosphate (Figure 5) were significantly decreased in the presence of econazole. These are all intermediates in the cell wall biosynthesis for components such as phosphatidylmyoinositol mannosides (PIMs), lipomannan, and lipoarabinomannan.[32] The precursors of these metabolites, such as glucose-6-phosphate, fructose-6-phosphate, glucosamine-6-phosphate, and mannose-6-phosphate, were elevated in the treated wild-type samples (Figure 5). Trehalose-6-phosphate, which is important for the incorporation of mycolic acids into the cell wall and synthesized from glucose-6-phosphate and glucose, was also found to be significantly decreased in econazole-treated cells (Figure 5). Because we saw changes in the cell wall, we looked at the effect of econazole on membrane potential (Figure S3) and mycolic acid production (data not shown).

Comparison of the levels of these metabolites in the untreated resistant strain revealed changes that were similar to those of the treated wild-type strain. Differences included higher levels of glucose-6-phosphate, fructose-6-phosphate, glucosamine-6-phosphate, and mannose-6-phosphate as well as lower levels of *N*-acetylglucosamine, mannose, mannose-1-phosphate, inositol-1-phosphate, and trehalose-6-phosphate (Figure 5). Econazole treatment led to similar changes in the levels of metabolites related to PIM synthesis between the wild-type and resistant mutant strains. By contrast, the levels of glucose-6-phosphate, fructose-6-phosphate, and glucosamine-6-phosphate decreased in the resistant mutant strain with econazole treatment as opposed to the increased levels observed in the wild-type strain (Figure 5). Trehalose also decreased significantly in a dose-dependent manner in the resistant mutant but not in the wild-type strain, in the presence of econazole (Table S1).

In addition to the utilization of glucose through glycolysis, glucose-6-phosphate may also be shunted through the pentose phosphate pathway (PPP) to generate nicotinamide adenine dinucleotide phosphate (NADPH) for lipid biosynthesis, as well as for reducing equivalents and ribose-5-phosphate for nucleotide synthesis. Levels of the PPP intermediate 6-phosphogluconate and 5-phosphoribosyl diphosphate (PRPP), a key metabolite in the nucleotide synthesis generated from ribose-5-phosphate, were significantly higher in the resistant mutant strain as compared with the wild-type (Figure 6). These observations may indicate increased PPP activity in the resistant mutant strain that may influence the availability of NADPH. As 6-phosphogluconate and PRPP were also increased in the wild-type cells exposed to econazole (Figure 6), elevated PPP activity may contribute to the drug resistance phenotype of the resistant mutant strain.

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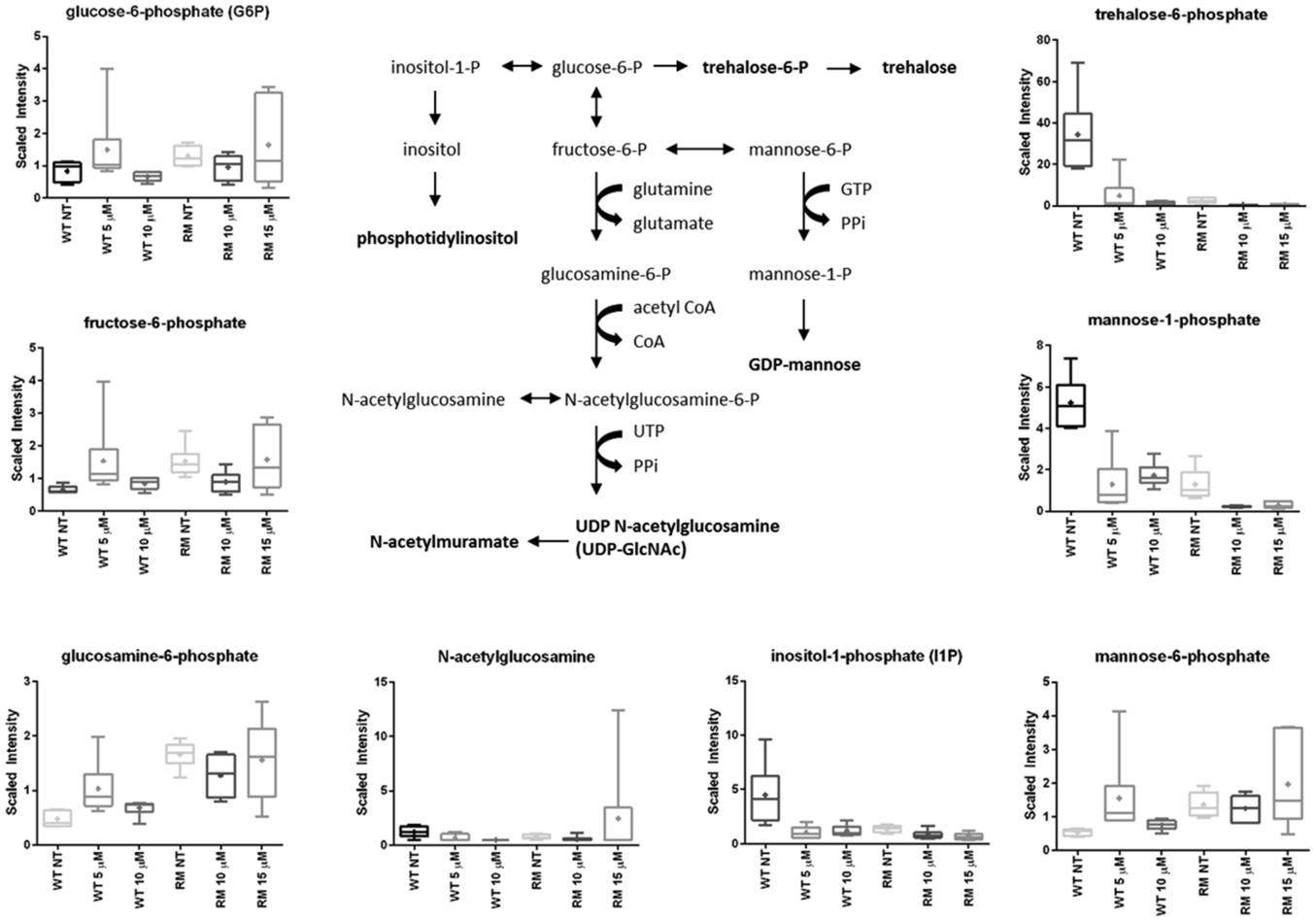

Figure 5. Metabolomic signatures in the wild-type and resistant mutant strains after exposure to econazole: changes in the cell wall metabolites. Box plots show the levels of metabolites present in H37Rv (LP) and LP-0105935-RMS ± econazole. Data are the average of six replicates. Metabolites highlighted here are important for cell wall synthesis through the indicated pathways.

M. tuberculosis can use modified versions of the tricarboxylic acid (TCA) cycle to conserve carbon atoms during the metabolism of acetyl-CoA produced from the glycolysis and/or oxidation of even-numbered fatty acids (via the glyoxylate cycle) and propionyl-CoA produced from the oxidation of odd-chain fatty acids (via the methylcitrate cycle) for energy production. In both the presence and absence of econazole, several differences in metabolite levels suggest differential contribution of these pathways to energy production in the resistant mutant and wild-type strains. Higher levels of citrate, succinate, and malate and lower levels of acetyl-CoA, α-ketoglutarate, and fumarate suggest greater dependence on the glyoxylate cycle in the resistant mutant strain (Figure 7). Similar changes were observed in the wild-type cells grown in the presence of econazole (Figure 7). Drug treatment also appeared to induce utilization of the methylcitrate cycle in both strains as the levels of methylcitrate were below the level of detection in the untreated samples and rose in a dose-dependent manner (Figure 7).

DISCUSSION

Better understanding of the sequences of events leading to cell death from a host of bactericidal antibiotics is necessary for the development of more effective antibacterial therapies. We have demonstrated that econazole is bactericidal to *M. tuberculosis*. We have shown that imidazole drugs induce a rapid increase in ROS in *M. tuberculosis*. A previous work has indicated that the induction of ROS by imidazoles is associated with killing of other organisms such as *Leishmania*[33] and *Staphylococcus aureus*,[34] as well as biofilm-associated and planktonic fungi.[35] In addition, miconazole action against *Candida* and *Saccharomyces* involves the generation of ROS by respiration;[20] and azole exposure results in metabolic remodeling toward respiration and increased mitochondrial activity.[20] All organisms that undergo aerobic respiration are subjected to ROS exposure as a byproduct of normal respiration, including superoxide anions, hydroxyl radicals, and hydrogen peroxides. Redox imbalance occurs when endogenous antioxidants fail to cope with the excessive ROS (both endogenous and exogenous), leading to the development of oxidative stress. If not effectively controlled, the accumulation of hydroxyl radicals can kill cells through its numerous deleterious effects including breakage of nucleic acids, protein carbonylation, and lipid peroxidation.

We determined that both strains of *M. tuberculosis* that are either resistant or sensitive to imidazoles exhibit a concentration-dependent increase in intracellular ROS upon incubation with imidazoles similar to the parental strain. The magnitude of the response had no correlation with the sensitivity of each strain to imidazole drugs, meaning that the most sensitive strain did not display the highest levels as might be expected if ROS were the mode of action of these drugs.

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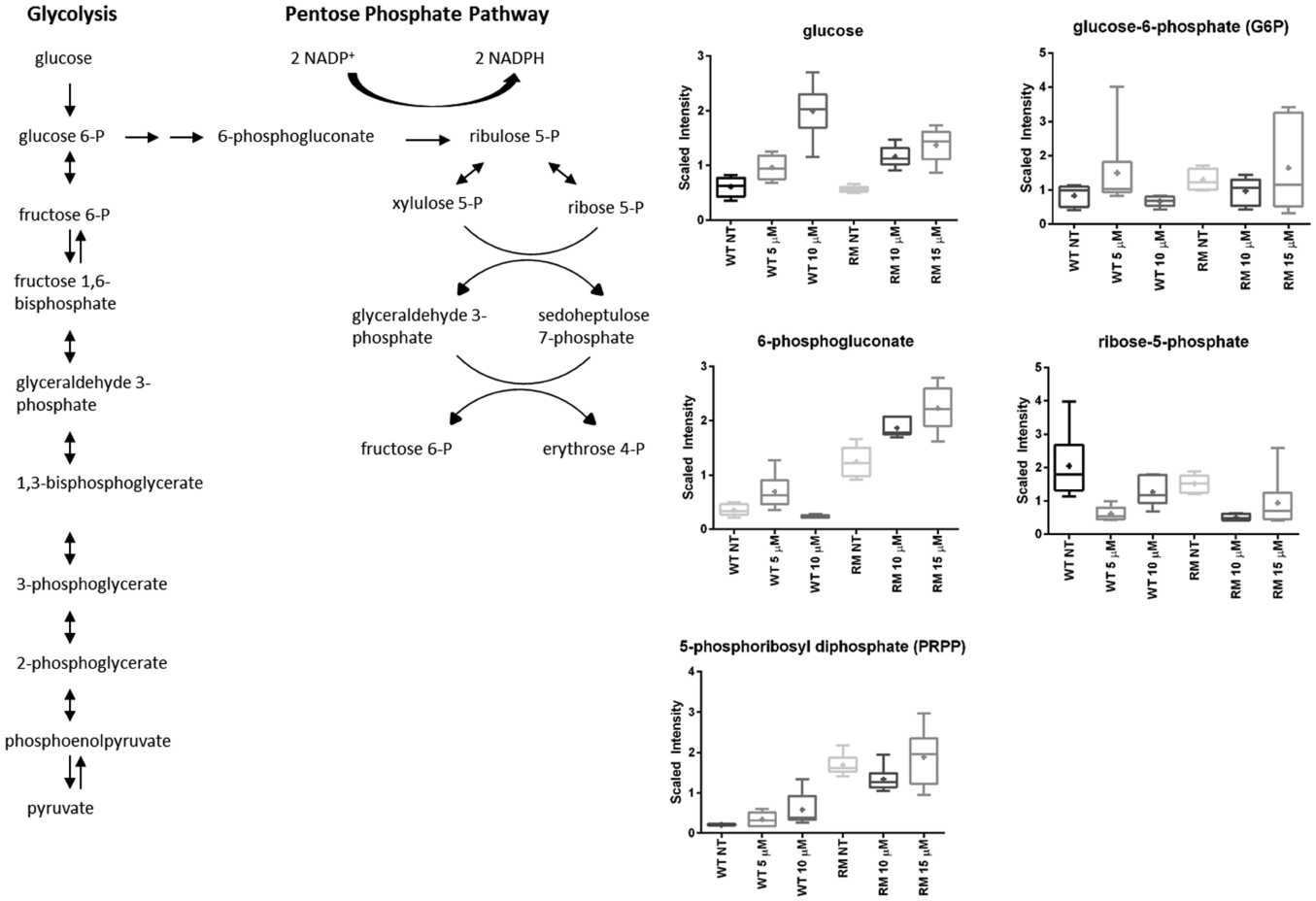

Figure 6. Metabolomic signatures in the wild-type and resistant mutant strains after exposure to econazole: glucose metabolites. Box plots show the levels of metabolites present in H37Rv (LP) and LP-0105935-RM5 ± econazole. Data are the average of six replicates. Metabolites highlighted here are important for glycolysis and the PPPs.

The imidazole concentration required to induce the production of ROS within the cells was equivalent for all strains tested, as demonstrated by similar EC_{50} values, again showing no correlation with imidazole sensitivity to growth inhibition.

To further understand the relationship between ROS stress and imidazole killing of *M. tuberculosis*, we determined the MIC of strains expressing altered ROS-detoxifying genes. The deletion of *katG* or *ahpCF* has previously been shown to confer hyperlethality to diverse antibiotics.[36] Two *M. tuberculosis* strains with different versions of nonfunctional *katG* genes were found to have imidazole MICs identical to the wild-type. The overexpression of these genes has been shown to mitigate ROS-mediated damage.[37,38] We found that the overexpression of *sodC* also had little to no effect on imidazole MICs relative to the wild-type. We evaluated whether the addition of various antioxidants would be protective to *M. tuberculosis* upon incubation with imidazoles. Thiourea (a redox-active thiol), NALC (a thiol-containing antioxidant), and Tempol (a redox-shuttling nitroxide capable of detoxifying a wide range of ROS and reactive nitrogen intermediates) all had no effect on imidazole MICs of *M. tuberculosis*.

A previous work showed a role for *icl1* in antioxidant defense against antibiotics with diverse modes of action.[30] The presence, absence, or increased level of expression of Icl1 had no discernible effect on the MIC for imidazoles. Interestingly, we detected much higher levels of ROS produced in response to imidazole incubation with the Δ*icl1/2* strain compared with the parental Erdman strain. The combination of all of these data suggests that while ROS generation is increased in response to imidazole exposure within *M. tuberculosis* bacteria, the bacteria have sufficient detoxifying mechanisms to prevent an irreparable amount of damage from occurring within the cells. The lack of cell death directly attributable to ROS production or activity suggests that imidazoles act by an alternative mechanism yet to be elucidated.

To better understand the effect of imidazoles on *M. tuberculosis*, we conducted metabolomic analysis of wild-type and econazole-resistant bacteria exposed to two sublethal concentrations of econazole for 24 h. A comparison of the global biochemical profiles revealed many differences in the metabolite levels in response to econazole treatment in both the wild-type and resistant strains. In several instances, the levels of metabolites in the untreated resistant samples were similar to the levels in the treated wild-type strain. This suggests that the changes we saw upon exposure to econazole were an adaptive response to drug treatment, rather than a downstream effect of target inhibition.

Both wild-type and econazole-resistant strains had numerous alterations in pathways contributing to the synthesis of cell wall components. The wild-type strain had decreased in levels of metabolites involved in peptidoglycan synthesis as well as components of PIMs, lipomannan, and lipoarabinomannan; however, the precursors of these metabolites were elevated in the treated wild-type samples. These observations may reflect

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ACS Omega 2017, 2, 41−51

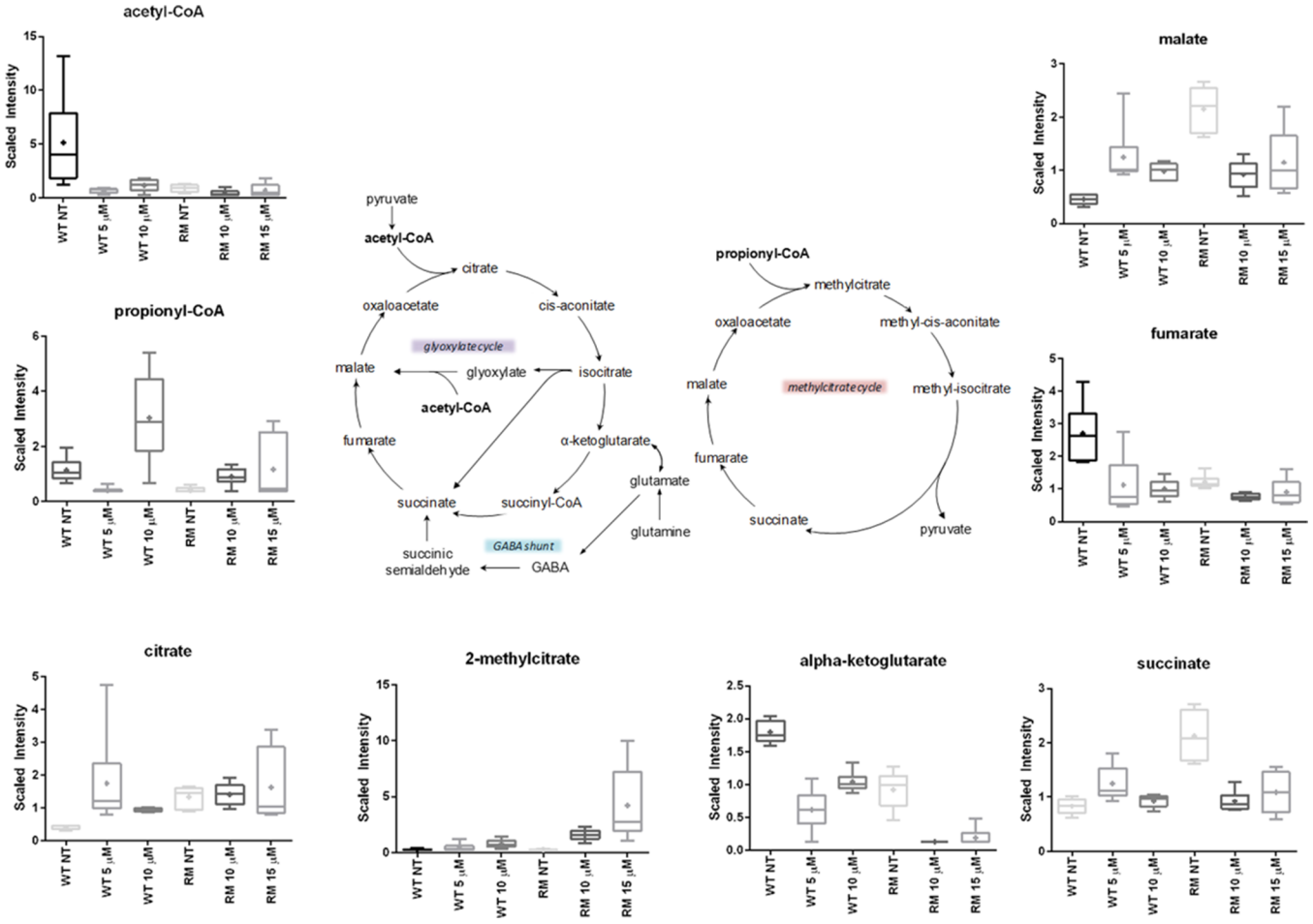

Figure 7. Metabolomic signatures in the wild-type and resistant mutant strains after exposure to econazole—changes in TCA cycle metabolites. Box plots show the levels of metabolites present in H37Rv (LP) and LP-0105935-RM5 ± econazole. Data are the average of six replicates. Metabolites highlighted here are important for energy metabolism.

either an increased demand for cell wall components in the presence of econazole or a block in their synthesis that results in accumulation of these precursors. The untreated econazole-resistant strain had similar changes to the treated wild-type strain in the levels of these metabolites. The untreated econazole-resistant strain contained higher levels of cell wall biosynthesis components than the treated wild-type strain. Drug treatment led to a significant decrease in these metabolites, in contrast to the increase observed in the treated wild-type cells. Taken together, these observations raise the possibility that the econazole-resistant strain has adapted to alterations in cell wall synthesis in the absence of the drug that allows for greater resistance to perturbations induced by econazole treatment.

Econazole treatment resulted in alterations in metabolism in both the wild-type and econazole-resistant strains. The decreased levels of glucose-6-phosphate observed in the treated econazole-resistant strain relative to the wild-type strain, coupled with the increased levels of PPP intermediates may indicate increased PPP activity in the econazole-resistant strain that could influence the availability of NADPH. Increased levels of NADPH in the resistant strain could contribute to the econazole resistance phenotype of this strain owing to its reducing power by augmenting the ability of these bacteria to detoxify higher levels of ROS stress compared with the wild-type strain. The untreated econazole-resistant strain appears to rely more on the glyoxylate pathway for energy production than the untreated wild-type strain. Drug treatment induced similar changes in the wild-type strain, suggesting that the glyoxylate cycle is increased as a result of drug treatment. Econazole treatment also appeared to induce increased utilization of the methylcitrate cycle in both the wild-type and resistant strains.

The changes related to glyoxylate and methylcitrate cycles may reflect the increased catabolism of odd- and even-chain fatty acids to propionyl-CoA and acetyl-CoA. Although acetyl-CoA levels decreased with drug treatment, this hypothesis is supported by increased levels of medium-chain fatty acids and the ketone body 3-hydroxybutyrate (BHBA) along with decreased levels of free coenzyme A (CoA) in both the wild-type and econazole-resistant strains in response to drug. The increase in the medium-chain fatty acids and BHBA was greater in the resistant mutant strain than in the wild-type strain, suggesting a differential capacity for the catabolism of fatty acids for energy.

In conclusion, we have demonstrated that imidazoles induce ROS in a transient fashion, which is not associated with the bacterial viability, but that econazole treatment leads to metabolic remodeling, which may represent adaptations to the drug treatment. Further work to elucidate whether there is an intracellular target for imidazoles or whether another general mechanism of death is induced is warranted.

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MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Chemicals. *M. tuberculosis* strains are summarized in Table 1. Strain H37Rv (LP) is ATCC 25618; strain H37Rv (MA) is ATCC 27294; strains were sequenced and several point mutations[27] were identified. *M. tuberculosis* was grown in Middlebrook 7H9 medium containing 10% v/v OADC (oleic acid, albumin, dextrose, and catalase) supplement (Becton Dickinson) and 0.05% w/v Tween-80 (7H9-Tw-OADC) or on Middlebrook 7H10 agar containing 10% v/v OADC. Gentamicin (Sigma) was added to 10 μg/mL and hygromycin B (Roche) to 50 μg/mL where required.

Determination of Minimum Inhibitory Concentrations. MICs were determined in a liquid medium as described previously.[39] Briefly, the compounds were solubilized in dimethyl sulfoxide (DMSO) (Fisher Scientific) and assayed as a 10-point 2-fold serial dilution series. Bacterial growth was measured by OD_{590} after incubation at 37 °C for 5 days. MIC was defined as the minimum concentration required for the complete inhibition of growth. MIC_{99} was determined on a solid medium using the serial proportion method.[40] MIC_{99} was defined as the minimum concentration required for the prevention of 99% growth.

Kill Kinetics. For kill kinetics during replicating conditions, a late log phase culture of *M. tuberculosis* was adjusted to OD_{590} = 0.1 in 7H9-Tw-OADC; 50 μL was used to inoculate 5 mL of 7H9-Tw-OADC containing compounds (final DMSO concentration of 2%). The cultures were incubated standing at 37 °C, and the serial dilutions were plated to determine CFUs after 4 weeks incubation at 37 °C.

Construction of *sodC* and *icl1* Overexpression Strains. Gateway compatible entry plasmids bearing the *sodC* and *icl1* genes were obtained from the Pathogen Functional Genomics Resource Center and cloned into the pDTCF expression vector[41] via an LR Clonase reaction (Life Technologies). This expression vector contains an anhydrotetracycline-inducible promoter and appends a C-terminal FLAG tag on the protein. The plasmids were electroporated into *M. tuberculosis*,[42] and the transformants were selected on plates containing hygromycin B.

ROS Measurement. A mid-log-phase culture of *M. tuberculosis* was adjusted to OD_{590} = 0.5, harvested and washed with 7H9-Tw (no OADC), and resuspended in 7H9-Tw containing 40 μM DCFDA. Bacteria were incubated at 37 °C for 30 min, harvested, washed, and resuspended in 7H9-Tw. DCFDA-loaded cells were added to black-walled clear-bottom 96-well plates (Greiner) containing a 10-point 2-fold dilution series of compounds. DMSO was used as a vehicle control. Fluorescence (Ex485/Em535) was measured using a Biotek Synergy H4 microplate reader; background fluorescence measured in the untreated DCFDA-loaded *M. tuberculosis* was subtracted. Each experiment was performed three times. To measure ROS over extended time periods, *M. tuberculosis* was grown in the presence of econazole, the cells were loaded with DCFDA and fluorescence measured.

Metabolomics. *M. tuberculosis* and an econazole-resistant mutant (LP-0105935-RM5)[13] were grown under aerobic conditions in roller bottles at 100 rpm to an OD_{590} of 0.4 before the addition of econazole or DMSO. Bacteria were harvested and delipidated with chloroform−methanol after 24 h of incubation. Metabolomic and statistical analyses of samples was conducted at Metabolon, Inc. as previously described.[43,44] Briefly, the delipidated *M. tuberculosis* samples (N = 6/group) were subjected to methanol extraction. The extracts were split into aliquots and processed for analysis using ultrahigh performance liquid chromatography/mass spectrometry (LC/MS) in the positive- or negative-ion mode and using gas chromatography/MS (GC/MS). For LC/MS, the platform was based on a Waters Acquity ultraperformance liquid chromatography and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization source and a linear ion-trap mass analyzer. The sample extract was split into two aliquots, dried, and then reconstituted in acidic or basic LC-compatible solvents; each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive-ion optimized conditions and the other using basic negative-ion optimized conditions in two independent injections using separate-dedicated columns. The extracts reconstituted under acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, whereas the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. For GC/MS, the samples were redried under vacuum desiccation for a minimum of 24 h before being derivatized under dried nitrogen using BSTFA. The GC column was 5% phenyl, and the temperature ramp is from 40 to 300 °C in a 16 min period. The samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization.

Proprietary software was used to match ions to an in-house library of standards for metabolite identification and for metabolite quantitation by peak area.[45] For statistical analysis and data visualization, any missing values were assumed to be below the limits of detection. To determine the statistical significance, Welch's two sample *t*-tests and two-way analysis of variance were used to compare the means of two populations. P values $\leq$0.05 were considered highly significant, and P values between 0.05 and 0.1 were considered less significant. An estimate of the false discovery rate (Q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies, with Q < 0.05 used as an indication of high confidence in a result.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.6b00212.

Validation of DCFDA fluorescent probe for detection of ROS. Growth curves in the presence of econazole in aerobic roller culture. Membrane potential of *M. tuberculosis* is unaffected by exposure to econazole (PDF)

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Notes

The authors declare no competing financial interest.

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