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Revisiting Activation of and Mechanism of Resistance to Compound IQG-607 in *Mycobacterium tuberculosis*

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ABSTRACT IQG-607 is a metal complex previously reported as a promising anti-tuberculosis (TB) drug against isoniazid (INH)-resistant strains of *Mycobacterium tuberculosis*. Unexpectedly, we found that INH-resistant clinical isolates were resistant to IQG-607. Spontaneous mutants resistant to IQG-607 were subjected to whole-genome sequencing, and all sequenced colonies carried alterations in the *katG* gene. The *katG*(S315T) mutation was sufficient to confer resistance to IQG-607 in both MIC assays and inside macrophages. Moreover, overexpression of the InhA(S94A) protein caused IQG-607's resistance.

The pentacyano(isoniazid)ferrate(II) complex ($Na_3[Fe^{II}(CN)_5(INH)] \cdot 3H_2O$), also known as IQG-607, is an isoniazid (INH) analog that contains a cyanoferrate moiety bound to the pyridinic nitrogen of the INH molecule (Fig. 1) (1). So far, two alternative hypotheses were proposed to explain its mechanism of action. Enzymatic inhibition studies demonstrated that IQG-607 inhibits the wild-type NADH-dependent enoyl-[acyl carrier protein] reductase (InhA) enzyme (Enzyme Commission no. EC 1.3.1.9) and its structural mutants (I21V, S94A, and I47T) in a time-dependent manner (2, 3). Interestingly, this inhibition was observed in the absence of NAD^+ or NADH and without requiring activation by the catalase-peroxidase KatG (EC 1.11.1.21), suggesting that IQG-607 could be used against INH-resistant *Mycobacterium tuberculosis* strains whose mechanism of resistance involves mutations in the *katG* (Rv1908c) gene (2, 3). Another possible explanation involves an intramolecular electron transference mediated by the metal center that would self-activate the INH moiety from IQG-607 inside the host macrophages, thus forming a hypothetical adduct with NADH (4). Despite the extensive characterization of IQG-607 interaction with the InhA enzyme, its activity in mice (5), and its favorable toxicological profile in rats (6) and mini pigs (7), it is still unknown if the compound is active against *M. tuberculosis* strains carrying mutations in the *katG* gene, hence our work focused on elucidating the mechanism of resistance of the *M. tuberculosis* to the compound IQG-607 by employing a genetic approach with a set of different INH-resistant strains.

We first tested the compound activity against several multidrug-resistant tuberculosis (MDR-TB) clinical isolates by determining MICs on Middlebrook 7H9 liquid medium as described previously (8). Eight MDR-TB clinical isolates carried the most common mutation found in the *katG* gene (Ser315Thr) (9), while one INH-susceptible clinical

FIG 1 Chemical structure of IQG-607 (Na$_3$[Fe^{II}(CN)$_5$(INH)] · 3H$_2$O). This inorganic complex contains a pentacyanoferrate(II) moiety bound to the nitrogen atom of the heterocyclic ring of isoniazid. This moiety is thought to activate the isoniazid molecule.

isolate carried the wild-type gene. As shown in Table 1, all strains were resistant to both INH (MIC > 25 mg/liter) and IQG-607 (MIC > 100 mg/liter), except for the CDCT-28 isolate. This result was unexpected, since it suggests an association between the presence of the *katG*(S315T) mutation and an increase of at least 64× in the MIC values of IQG-607.

Next, spontaneous mutants resistant to IQG-607 were isolated on 7H10 plates using compound concentrations above the MIC (≥2 mg/liter). We confirmed the resistance phenotype by determining MICs of six colonies (named IQG1 to 6) selected from the highest compound concentration tested (16 mg/liter). The six selected mutants displayed cross-resistance only to INH (Table 2). We retested the resistance of one representative mutant (IQG1) by monitoring its growth rate, as previously described (10), in the presence of IQG-607 or INH above their MIC values, and indeed neither of the compounds prevented the growth of this mutant (see Fig. S1 in the supplemental material).

We performed the whole-genome sequencing (by MiSeq platform) of three colonies (IQG1 to 3) to uncover the mutations associated with the resistance phenotype, and we found that all colonies carried mutations in the *katG* gene. This result was confirmed by target DNA sequencing of the six mutants (IQG1 to 6) using several primers described in Table S1 in the supplemental material. Indeed, all six mutants carried alterations in the *katG* gene (Table 2). IQG1, 2, and 5 had a single deletion of cytosine 120 (120del1),

TABLE 1 Activity of IQG-607 and several anti-TB compounds against nine clinical isolates[a]

Isolate[c]	*katG* mutation	*inhA* promoter genotype	*rpoB* mutation	MIC (mg/liter) for[b]			
				INH	IQG-607	RIF	MFX
H37Rv	WT	WT	WT	0.39	1.56	<0.2	<0.2
CDCT-1	S315T	WT	H526D	>100	>100	>100	<0.2
CDCT-2	S315T	C(−15)T	H526D	100	>100	>100	<0.2
CDCT-3	S315T	WT	D516V	25	>100	6.25	<0.2
CDCT-4	S315T	WT	S531L	>100	>100	>100	<0.2
CDCT-5	S315T	WT	D516V	50	>100	>100	<0.2
CDCT-10	S315T	WT	H526D	100	>100	>100	<0.2
CDCT-16	S315T	C(−15)T	D516V	100	>100	>100	<0.2
CDCT-27	S315T	WT	WT	25	>100	<0.2	<0.2
CDCT-28	WT	WT	D516A	0.39	1.56	<0.2	<0.2

[a]TB, tuberculosis; WT, wild type.
[b]MIC values reported here were observed in two independent experiments or were the highest value found among three independent tests. INH, isoniazid; IQG-607, pentacyano(isoniazid)ferrate(II); RIF, rifampin; MFX, moxifloxacin.
[c]Clinical isolate identification.

TABLE 2 Activity of IQG-607, INH, ETH, and MFX against several laboratory strains of *M. tuberculosis*

Strain	*katG* genotype	*inhA* promoter genotype	*inhA* genotype	MIC (mg/liter) for[a]			
				INH	ETH	MFX	IQG-607
H37Rv	WT	WT	WT	0.39	3.13	0.08	1.56
IQG1[b]	120del1	WT	WT	>100	3.13	0.16	>100
IQG2	120del1	WT	WT	>100	1.56	0.08	>100
IQG3	T(271)C	WT	WT	>100	3.13	0.16	>100
IQG4	1298del1	WT	WT	>100	1.56	0.08	>100
IQG5	120del1	WT	WT	>100	1.56	0.02	>100
IQG6	C(1249)G	WT	WT	>100	3.13	0.08	>100
pNIP::*furA*+*katG*(WT)	WT	WT	WT	<0.2	3.13	<0.2	0.78
pNIP::*furA*+*katG*(S315T)	G(944)C[c]	WT	WT	>100	0.78	<0.2	>100
pNIP::Ø	WT	WT	WT	0.39	6.25	<0.2	1.56
pNIP::InhA(WT)	WT	WT	WT	0.39	6.25	<0.2	3.13
pNIP::InhA(S94A)	WT	WT	T(280)G[d]	25	>100	<0.2	>100

[a]MIC values reported here were observed in two independent experiments or were the highest value found among three independent tests. ETH, ethionamide.
[b]IQG1-6 refers to the IQG-607 spontaneous mutant strains.
[c]This mutation changes the serine-315 residue by a threonine (S315T).
[d]This strain still expresses the underlying level of the wild-type *inhA* gene but carries the mutant *inhA* gene [T(280)G], cloned into the integrative plasmid pNIP40/b. This mutation changes the serine-94 residue by an alanine (S94A).

which changed the reading frame of *katG* and introduced a premature stop codon at position 45. The resultant defective protein lacks almost all the residues from the N-terminal domain, including the key active site residues (11) and the entire C-terminal domain. The deletion of adenine 1298 (1298del1) in the mutant IQG4 introduced a premature stop codon at position 467 and led to a truncated protein lacking only the C-terminal domain. These findings are in agreement with previous work using INH to select spontaneous mutants (12, 13). The W91R mutation found in IQG3 was already described in clinical isolates at a very low frequency (14–17), and it is expected to confer resistance to INH by a still unclear mechanism, possibly involving a disruption of an important H-bonding network in the active site of KatG (18). To the best of our knowledge, the H417D mutation found with IQG6 has never been described in either clinical isolates or in laboratory strains. Although the role of this particular histidine residue is not known, some studies have already uncovered the importance of the neighbor residue Arg-418 for catalysis and its involvement in INH resistance (18–20).

To establish a causal relationship between the clinically relevant mutation *katG*(S315T) and resistance to IQG-607, we knocked out the wild-type *katG* gene from *M. tuberculosis* H37Rv strain by using an allelic exchange approach (21) and complemented it with the wild-type, *furA*+*katG*(WT), or the mutated allele, *furA*+*katG*(S315T) (see Fig. S2 in the supplemental material). MIC for IQG-607 increased by more than 64-fold in the mutated strain (Table 2), clearly suggesting that the compound depends on KatG to be active. However, MIC assays lack the oxidizing environment of the macrophage, and therefore would not allow a route for the redox-mediated self-activation of IQG-607 previously proposed (4). To test this premise, we infected murine macrophages as described previously (22) with Δ*furA*::*katG* laboratory strains carrying the wild-type gene or the S315T mutant and treated them with IQG-607. The mutant strain preserved its resistance phenotype toward IQG-607 inside macrophages (Fig. 2), indicating that the host intracellular environment is not sufficient to trigger the self-activation mechanism proposed for IQG-607, which reinforces the necessity of the KatG enzyme for the activity of IQG-607.

Finally, we evaluated the antimicrobial activity of IQG-607 in mycobacterial cells constitutively overexpressing the S94A mutant InhA enzyme, a mutation that is known to cause INH resistance (23). The mutant *inhA* gene (Rv1484) was cloned into an integrative plasmid (pNIP40/b) under the control of the *hsp60* promoter. In addition to the expected resistance to INH and also to ethionamide (ETH), the expression of the InhA(S94A) mutant enzyme (see Fig. S3 in the supplemental material) resulted in mycobacteria also resistant to IQG-607 (Table 2). This result genetically validated the InhA protein as the target of IQG-607. However, it also showed that the Ser-94 residue
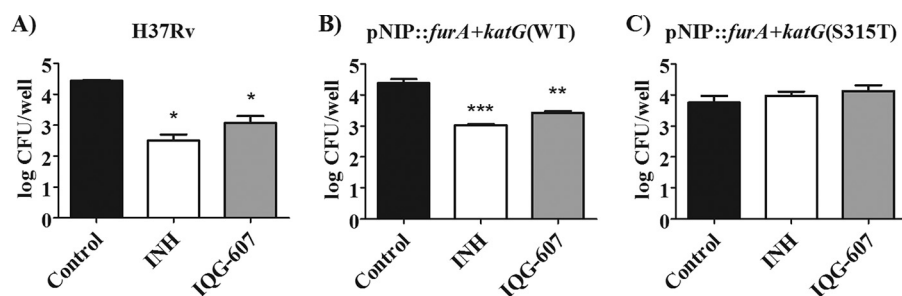
FIG 2 Treatment of macrophages infected with (A) H37Rv, (B) pNIP::*furA*+*katG*(WT), or (C) pNIP:: *furA*+*katG*(S315T) laboratory strains of *M. tuberculosis* with 50 μM (6.85 mg/liter) of INH (white bar) or 50 μM (23.2 mg/liter) of IQG-607 (gray bar). Data are expressed as mean numbers of the logarithms of CFU per well from triplicates. Two-way ANOVA with Bonferroni's *post hoc* test was employed to compare the treatments. Asterisks (*/**/***) represent P values below 0.05, 0.01, and 0.001, respectively, compared with the untreated control group (black bar).

is essential for the compound's interaction with the enzyme, somehow contradicting the inhibition observed against the purified InhA(S94A) enzyme (3).

In conclusion, we demonstrated that IQG-607 does not activate itself inside macrophages or mycobacteria. As with INH, IQG-607 also requires the activity of KatG in order to be active against *M. tuberculosis* strains. These findings challenge its planned usage against INH-resistant strains. Nonetheless, the compound has already been reported to be less toxic than INH in a preclinical study using mice (5, 24). This is a remarkable feature, since INH was previously associated with hepatotoxicity in animal and humans due to its toxic metabolites, such as hydrazine (25). We performed a checkerboard assay as previously described (26) and found that IQG-607 has an indifferent effect when combined with other anti-TB drugs (see Table S2 in the supplemental material), showing it could be used in association. Further experiments will be required to understand its metabolism inside mycobacteria and to establish its utility as an INH surrogate with low toxicity.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .02222-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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