Mechanism of irreversible inhibition of Mycobacterium tuberculosis shikimate kinase by ilimaquinone

Johayra Simithya, Ngolui Rene Fuanta, Judith V. Hobraht, Anna Kochanowska-Karamyan, Mark T. Haman, Douglas C. Goodwin, Angela I. Calderón*

aDepartment of Drug Discovery and Development, Harrison School of Pharmacy, 3306 Walker Building, Auburn University, Auburn, AL 36849, USA.

bDepartment of Chemistry and Biochemistry, 179 Chemistry Building, Auburn University, Auburn, AL 36849, USA.

cDrug Discovery Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom.

dDepartment of Pharmaceutical Sciences, Texas Tech University HSC, 1300 S. Coulter, Amarillo, TX 79106, USA.

eDepartment of Drug Discovery and Biomedical Sciences, College of Pharmacy, Medical University of South Carolina, Charleston, SC 29425, USA.

These authors contributed equally to the work described.

*E-mail: aic0001@auburn.edu
Phone: 334-844-8333
Fax: 334-844-8331

ABSTRACT
Ilimaquinone (IQ), a marine sponge metabolite, has been considered as a potential therapeutic agent for various diseases due to its broad range of biological activities. We show that IQ irreversibly inactivates *Mycobacterium tuberculosis* shikimate kinase (*MtSK*) through covalent modification of the protein. Inactivation occurred with an apparent second-order rate constant of about 60 M⁻¹s⁻¹. Following reaction with IQ, LC-MS analyses of intact *MtSK* revealed covalent modification of *MtSK* by IQ, with the concomitant loss of a methoxy group, suggesting a Michael-addition mechanism. Evaluation of tryptic fragments of IQ-derivatized *MtSK* by MS/MS demonstrated that Ser and Thr residues were most frequently modified with lesser involvement of Lys and Tyr. In or near the *MtSK* active site, three residues of the P-loop (K15, S16, and T17) as well as S77, T111, and S44 showed evidence of IQ-dependent derivatization. Accordingly, inclusion of ATP in IQ reactions with *MtSK* partially protected the enzyme from inactivation and limited IQ-based derivatization of K15 and S16. Additionally, molecular docking models for *MtSK*-IQ were for IQ-derivatized S77 and T111. In the latter, ATP was observed to sterically clash with the IQ moiety. Out of three other enzymes evaluated, lactate dehydrogenase was derivatized and inactivated by IQ, but pyruvate kinase and catalase-peroxidase (*KatG*) were unaffected. Together, these data suggest that IQ is promiscuous (though not entirely indiscriminant) in its reactivity. As such, the potential of IQ as a lead in the development of antitubercular agents directed against *MtSK* or other targets is questionable.

**KEYWORDS:** *Mycobacterium tuberculosis* shikimate kinase, shikimate-3-phosphate, ilimaquinone, irreversible inhibitor, time-dependent inhibition, covalent adduct, liquid chromatography – mass spectrometry, tandem MS, jump dilution, molecular docking.
1. Introduction

Terpenylquinones comprise a class of marine natural products considered attractive scaffolds for drug design due to their ubiquity in nature and their versatile bioactivities [1]. These compounds are characterized as having a bicyclic sesquiterpene skeleton coupled to a quinone
moiety [2]. Among them, ilimaquinone (IQ) (Fig. 1), first isolated in 1979 from the Red Sea sponge *Hippospongia metachromia* [3], has been reported to possess several biological activities of potential therapeutic value, including antiviral [4], anti-inflammatory [4], antimicrobial [5], antimalarial [6] and anti-HIV [7] properties. Most prominently, IQ has been recognized for its ability to degrade Golgi membranes into small vesicular structures (i.e., vesiculated Golgi membranes), a phenomenon which blocks cellular secretion [8–11]. In addition, IQ has shown to induce the transcriptional activation of autophagic target genes in tumor cells, [12,13] and to inhibit the growth of several cancer cell lines, including multiple myeloma [14], prostate cancer (PC-3, DU145 and LNCaP), non-small cell lung cancer (A549), human osteosarcoma (MG63) and hepatocellular carcinoma cells (Hep3B) [15].

![Figure 1. Structure of ilimaquinone (IQ).](image)

Although IQ has proven to exert a myriad of biological activities through multiple pathways, the specific interactions of IQ with its cellular targets have not been clearly elucidated [16–18]. Our interest in this compound arose from an initial observation that IQ inhibited shikimate kinase from *M. tuberculosis* (*MtSK*). The shikimate pathway is responsible for the biosynthesis of aromatic compounds in microorganisms and higher plants, and *MtSK* catalyzes the fifth step of the process. Because this pathway is absent from human metabolism, numerous
investigators have suggested that small molecules inhibitors of shikimate pathway enzymes may produce new leads for the development of antibiotics to treat drug-resistant tuberculosis [19–21].

In this study, we investigated the mechanism of \( MtSK \) inhibition by IQ. Time-dependent inhibition kinetics suggested that IQ was either a slow-reversible or irreversible inhibitor of \( MtSK \). Dilution of IQ-inhibited \( MtSK \) failed to produce an increase in \( MtSK \) activity, suggesting in irreversible mechanism of inhibition by IQ. Accordingly, mass spectra of intact \( MtSK \) showed enzyme derivatized once and twice (+326.3 Da and +652.6 Da, respectively) with IQ lacking the mass of a methoxy moiety, supporting a Michael-addition mechanism. LC-ESI MS/MS analyses of \( MtSK \) trypsinized following its reaction with IQ indicated that threonine and serine residues were the primary targets of IQ-dependent derivatization, including S77, T111, and S44, all of which are in close proximity to the active site. We observed that IQ is also able to derivatize and inactivate lactate dehydrogenase (LDH) but not pyruvate kinase (PK) or \( M. \) tuberculosis KatG (\( MtKatG \)). Thus, IQ shows, at best, modest selectivity for \( MtSK \); however, molecular docking studies show that it may be possible to enhance target specificity by modification of the inhibitor to guide new analog designs.

2. Materials and Methods

2.1 Chemicals.

Dimethyl sulfoxide (DMSO), adenosine-5'-triphosphate (ATP), shikimate, \( H_2O_2 \) (30%), rabbit muscle lactate dehydrogenase (LDH) and rabbit muscle pyruvate kinase (PK) were purchased from Sigma-Aldrich (St. Louis, MO). \( MtSK \) [22] and \( M. \) tuberculosis catalase-
peroxidase (MtKatG) [23] were expressed and purified as previously described. The purity of MtSK and MtKatG were determined by SDS-PAGE and LC-ESI-MS, and aliquots were stored at −80 °C in 50 mM Tris-HCl, pH 7.4; 0.5 M NaCl for MtSK and 5 mM phosphate, pH 7.0, for MtKatG. All organic solvents were HPLC or LC-MS grade and were purchased from Thermo Fisher (Hanover Park, IL). All buffers and media were prepared using water purified by a Milli-Q purification system (Millipore, Billerica, MA).

2.2 Ilimaquinone acquisition and isolation.

Ilimaquinone was obtained with a minimum purity of 90% as analyzed by HPLC or H-NMR. This natural product was obtained from Smenospongia cerebriformis. The sponge was collected from shallow (3-21 m depth) coral reef habitat at Key Largo, Florida, on July 1 and August 7, 2005. Voucher specimens have been deposited in the Natural History Museum, London (BMNH 2007.4.23.5 [University of Mississippi voucher 05FL-020(1)]; BMNH 2007.4.23.6 [University of Mississippi voucher 05FL-061]). The sponge was stored frozen until extracted. Six kilograms (wet weight) of the frozen sponge S. cerebriformis were extracted exhaustively with EtOH in a sonicator. The extracts were then combined, filtered and concentrated in vacuo until dry. The resulting crude extract (260 g) was subjected to vacuum-liquid chromatography with a gradient solvent system starting from hexanes through acetone to methanol, yielding 20 fractions. Nonpolar fractions (mainly 2 and 3) after purification yielded 2.5 g (0.9615% dry weight) of ilimaquinone, which was identified by comparison of 1H NMR and 13C NMR data with a standard.

2.3 LC-MS based time-dependent inhibition assay.
The effect of ilimaquinone on the activity of MtSK was evaluated by an *in vitro* time-dependent inhibition assay. In this assay, MtSK (0.2 µM) was pre-incubated with ilimaquinone (in DMSO) at 0, 5, 10, 25, 50, 100 and 150 µM for 0 - 60 min prior to initiation of the shikimate kinase reaction assay. All assays were performed in 100 mM ammonium acetate, pH 7.6, supplemented with 50 mM KCl and 5 mM MgCl₂. All assays were performed at 25 °C in a final volume of 500 µL. Reactions were initiated by the addition of 5 mM shikimate and 1.2 mM ATP, followed for 30 seconds and quenched by the addition of 2 µL of 98% formic acid and vortexing. To study the effects of substrate competition during inhibition by IQ, time-dependent experiments were performed in the presence of 1.2 mM ATP, added prior to the addition of IQ, and reactions were initiated by the addition of 5mM shikimate. The amount of the product shikimate-3-phosphate (S3P) formed in each reaction was quantified using liquid chromatography-mass spectrometry. LC separation of S3P was carried out as described previously [22].

Data analyses for the time-dependent inactivation of MtSK were performed using least-squares non-linear regression fitting to the appropriate equation. Data were collected in duplicate and initial velocities were calculated by dividing the concentration of S3P formed in each reaction by the reaction quenching time \( V_0 = [S3P]/t_q \). The remaining enzyme activity was determined by comparing the initial velocities in control experiments (in the presence of DMSO) to the initial velocities of the enzyme in the presence of various concentrations of IQ, and plotted against the pre-incubation times. These data were fitted to the equation [1]:

\[ V_t = V_i e^{-k_{obs}t} \]
where \( V_t \) is the measured steady-state velocity after pre-incubation time \( t \), \( V_i \) is the steady-state velocity when pre-incubation time is zero, and \( k_{\text{obs}} \) is the pseudo first-order constant of observed inactivation at a given inhibitor concentration [24]. The \( k_{\text{obs}} \) values obtained were re-plotted as a function of ilimaquinone concentration, and the data were fit using linear regression. The resulting slope corresponded to a second-order rate constant for inactivation \( (k_{\text{inact}}) \)[24]. All data fitting was carried out using GraphPad Prism (version 5.02).

2.4 Inhibition of other enzymes by IQ: Activity assays for MtKatG, PK, and LDH

MtKatG, PK, and LDH (each at 2 \( \mu \)M) were incubated with 100 \( \mu \)M IQ for 1 hour at 25 °C. Reactions with LDH and PK were run in 100 mM Tris, pH 7.6, supplemented with 50 mM KCl and 5 mM MgCl\(_2\), while reactions with MtKatG were run in 5 mM phosphate, pH 7.0. An aliquot was drawn after 1 hr and assayed for its activity. The catalase activity of MtKatG (20 nM final concentration) was monitored by the decrease in absorbance at 240 nm resulting from the consumption of \( \text{H}_2\text{O}_2 \) (\( \varepsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1} \)) [25] in 100 mM phosphate, pH 7.0, as previously described [23]. Activities of IQ-treated LDH and PK (each at 1 \( \mu \)M final concentration) were monitored by decrease in NADH concentration as measured at 340 nm (\( \varepsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1} \)). These reactions all contained 0.18 mM NADH, 1.2 mM ADP, 15 mM PEP. Assays of LDH activity required PK (2 \( \mu \)M) for the generation of pyruvate, and assays of PK activity required LDH (2 \( \mu \)M) for the oxidation of NADH. Interference in the PK assay from the inhibition of LDH by IQ was avoided by the exclusion of LDH from the preincubation cocktail of PK with IQ, resulting in a very short time of exposure of LDH to IQ.

2.5 Dilution experiments.
MtSK (20 µM) was incubated with 250 µM IQ (greater than 50-fold excess over the IC50 for IQ), for 1 hour. The shikimate kinase reaction was initiated by diluting the EI complex (100 fold) into 1.2 mM ATP, 5 mM shikimate in MtSK assay buffer (100 mM ammonium acetate, pH 7.6, supplemented with 50 mM KCl and 5 mM MgCl2). The final concentration of enzyme following dilution was 0.2 µM. The reactions were allowed to run for times ranging from 0 - 60 min prior to quenching with formic acid. The quenched reactions were analyzed by LC-MS as previously described [22] and the concentrations of S3P produced were plotted against reaction time. In reactions where ATP was present during pre-incubation, MtSK-ATP complex was formed prior to the addition of the inhibitor. Subsequent steps were the same as when MtSK was preincubated only with IQ. All data fitting was carried out using GraphPad Prism (version 5.02).

2.6 Intact protein analysis by mass spectrometry

MtKatG, LDH and PK (each at 1 µM) were incubated with 100 µM IQ at 4 °C for 30 hr; MtSK (1 µM) was incubated with 100, 10 and 1 µM. With the exception of MtKatG, all reactions were carried out in 100 mM ammonium acetate, pH 7.6, supplemented 50 mM KCl and 5 mM MgCl2. Reactions with MtKatG were performed in phosphate, pH 7.0. chromatograms and mass spectra were collected each hour and analyzed using Agilent MassHunter BioConfirm software version B.06.00.

2.7 Nano liquid chromatography – tandem mass spectrometry (nLC-MS/MS) analysis of MtSK tryptic peptides.

Post pre-incubation of MtSK (1 µM) and Ilimaquinone (100 µM) at 25°C for 1, excess, unbound ilimaquinone was removed by filtration using Macrosep® Advance centrifugal device prior to enzymatic digestion. Desalting was carried out following manufacturer’s instructions.
using MtSK assay buffer as the wash buffer. Modified and unmodified MtSK were digested with trypsin at a ratio of 1:20 (w/w) trypsin/substrate overnight at 37 °C in an assay buffer consisting of 100 mM ammonium bicarbonate pH = 8. Tryptic peptides were separated using a 75 μm i.d × 17 cm Reprosil-Pur C18-AQ (3 μm; Dr. Maisch GmbH, Germany) nano-column (packed in-house) using an EASY-nLC nano HPLC (Thermo Scientific, Bremen, Germany). The mobile phases consisted of water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B). Peptides were eluted using a gradient of 2 - 50% B in 50 min followed by 50 - 98% B in 5 min and maintained over 10 minutes at 300 nL/min. The nano-HPLC was coupled to an Orbitrap Fusion™ Tribrid mass spectrometer (Thermo Scientific, San Jose, California). Spray voltage was set at 2.3 kV and capillary temperature was set at 275 °C. The mass spectrometer was set to perform a full MS scan (350 – 1200 m/z) at 120,000 FWHM resolving power (at 200 m/z), followed by sequential HCD (higher-energy collisional dissociation) MS/MS fragmentation at normalized collision energy of 27% and 7,500 FWHM resolution. The monoisotopic precursor selector (MIPS) filter for peptides was activated. All MS/MS scans were performed in the ion trap mass analyzer (rapid scan rate) using an isolation window of 2.0 m/z. Maximum injection times of 50 ms and 80 ms were defined for MS and MS/MS scans, respectively. AGC values were set to 5 x 10^5 for MS and 5 x 10^4 for MS/MS. MS data were collected in profile mode and MS/MS data were collected in centroid mode. All acquisitions were performed under positive mode polarity. Thermo Proteome Discoverer (PD, version 2.2.0.388) was used to perform database searching against UniProt shikimate kinase Mycobacterium tuberculosis FASTA database. Search engine Sequest-HT implemented in Thermo Proteome Discoverer was applied for all MS raw files. The search parameters were set to 10 ppm for precursor tolerance and 0.5
Da for MS/MS fragment ions and a maximum of three missed cleavages. Custom modifiers were created for the mass of ilimaquinone minus 32 Da. \((C_{21}H_{26}O_3)\) with preference for the target amino acids (S, T, Y, R and K) and each sample was searched against each modifier separately as fix custom modifications.

**2.8 Prediction of covalently bound ilimaquinone binding poses.**

All computations were performed using the Small Molecule Drug Discovery Suite of the Schrödinger software package, 2016-1 release (Schrödinger, LLC). Two MtSK X-ray structures (PDB entry codes 2IYT and 2IYY) were prepared using the Protein Preparation Wizard suite of tools. Hydrogens were added, protonation states assigned, and any ligands present removed. The structures were relaxed through restrained minimization within 0.3 root-mean-square-deviation (RMSD) of heavy atoms from the original crystal structures using the OPLS3 force field, further referred to as prepared crystal structures. Following preparation with LigPrep, IQ was docked into both structures in three binding site regions, in proximity of S44, S77 or T111. In order to include protein flexibility and increase sampling of ligand binding poses the Induced Fit docking method was applied with extended sampling, without constraints. Default parameters were used except for increasing the residue refinement region around the ligand to 8 Å. Out of docked poses that placed the 5-methoxy substituent of the benzoquinone ring in close proximity of the S/T modification sites two favorable docked poses were selected: one with the methoxy group near S77 and one near T111. No suitable pose could be obtained for IQ in the region of S44. Both selected poses originated from docking based on the same X-ray structure (PDB 2IYY). Side chain conformations of the following residues close to the covalent modification sites were adjusted: S77 and T111 side chains conformers were selected from the side chain rotamer
library. In the complex structure with the IQ pose suitable for linking with T111, side chain conformations of R117 and T115 were also adjusted. IQ was then covalently linked to T111/S77 using modeling tools, followed by restrained energy minimization of the two complexes using Protein Preparation Wizard suite of tools with default parameters. The protein in the obtained structures was discarded and the ligand merged with the original prepared crystal structure (PDB code 2IYY), followed by the same side chain conformational adjustments described above. The ligand pose was covalently linked to either S77 or T111 and the two structures relaxed through restrained energy minimization. This cycle of extracting the ligand poses, merging with the MtSK structure prior to energy minimization, and restrained energy minimization of the complexes was repeated until the energy minimized IQ poses were readily accommodated through covalent linking to S77/T111. Structural refinement of the obtained two complexes was achieved through Prime Protein-Ligand Complex Refinement of the region within 8 Å of the covalently linked IQ using default parameters. The root-mean-square deviation of the final structures with covalently bound IQ compared to the MtSK crystal structure used (PDB 2IYY) is 0.39 in case of IQ linked to S77 and 0.45 in case of IQ linked to T111.

3. Results and Discussion

3.1 Time-dependent and irreversible inhibition kinetics of MtSK by IQ

Preincubation of IQ with MtSK revealed an exponential decrease in MtSK activity with time, and the pseudo-first order rate of inactivation ($k_{obs}$) increased linearly with IQ concentration (Fig. 2). From the slope of $k_{obs}$ dependence on IQ concentration, an apparent second-order rate constant for inactivation was estimated to be $80 \pm 10 \text{ M}^{-1}\text{s}^{-1}$. In an alternative approach, we observed that inclusion of IQ in assays of MtSK activity produced an exponential
decrease in the rates of S3P formation following the initiation of reactions with MtSK (Fig. 3). There was a linear increase in $k_{obs}$ with IQ concentration. From the slope of the line, an apparent second-order rate constant was estimated as $45 \pm 10 \text{ M}^{-1}\text{s}^{-1}$ which was in reasonable agreement with preincubation studies.

**Figure 2. Effect of IQ preincubation on MtSK activity.** The loss of MtSK activity as a function of incubation time with IQ (A) was evaluated for 0 (●), 5 (×), 10 (▲), 25 (■), 50 (●), 100 (▲), and 150 (■) μM IQ. All reactions contained 0.2 μM MtSK. Shikimate phosphorylation was initiated at the indicated time by the addition of 5 mM shikimate and 1.2 mM ATP. The data were fit to a single-exponential function as described in Materials and methods, and the observed pseudo-first order rate constants ($k_{obs}$) are shown as a function of IQ concentration (B). The slope ($k_{obs}$ vs [IQ]) represents the second-order rate of inactivation $k_{inact}$. The data shown are the average of two injections per sample. The graphs presented are representative of duplicate experiments. The error bars represent the range.
Figure 3. Time-dependent loss of MtSK activity in the presence of IQ. Production of shikimate-3-phosphate (S3P) was initiated by adding MtSK (0.01 µM) to solutions containing 5.0 mM shikimate, 1.2 mM ATP, as well as 0 (●), 10 (×), 25 (▲), 50 (○), 100 (▼), and 150 (✗) µM IQ (inset). Reactions were quenched at the indicated times, samples were withdrawn and S3P concentration determined by MS. Except for the control (fit by linear regression), the rest of the data were fit to single exponential function. The observed rate of inactivation ($k_{obs}$) is plotted against IQ concentration. The data shown are the average of two injections per sample, and error bars indicate the range (inset). The graphs presented are representative of duplicate experiments.

The time-dependent component of MtSK inhibition by IQ is consistent with either a slowly reversible or irreversible inhibition mechanism. To differentiate between these two possibilities, we preincubated 20 µM MtSK with 250 µM IQ and then monitored S3P production following dilution of MtSK-IQ into an enzyme assay cocktail. A low level of activity (10%) was observed following dilution of IQ-treated MtSK compared to the untreated MtSK control (Fig. 4). This was a far greater extent of inhibition than could be accounted for by the post-dilution concentration of IQ present in the assay (2.5 µM). In addition, little if any acceleration of S3P production was observed following dilution (Fig. 4), indicating that the inhibition achieved by IQ during preincubation was irreversible. Taken together, these data suggest a slow, one-step
irreversible inactivation of MtSK by IQ governed by a second-order rate constant ($k_{inact}$) of about 60 M$^{-1}$s$^{-1}$ as depicted in the following scheme:

\[
E + I \xrightarrow{k_{inact}} E-I
\]

**Figure 4. Recovery of activity following dilution of IQ-inhibited MtSK.** MtSK (20 µM) was preincubated for 1 hour in the presence (●) and absence (■) of 250 µM IQ. Following incubation, each mixture was diluted 100-fold into a reaction cocktail containing 1.2 mM ATP and 5.0 mM shikimate. Reaction time ranged from 3 – 3600s. With two injections per sample, samples were withdrawn and S3P production evaluated. Data were fit to single-exponential function. The most rapid reaction rates were observed at earliest time points, as seen in the first 300s of product generation (inset). The data shown are the average of two injections per sample, and the graphs presented are representative of duplicate experiments. The error bars represent the range.

### 3.2 Analyses of ilimaquinone-dependent covalent modification of MtSK

One of the most common mechanisms by which irreversible inhibition is achieved is through covalent enzyme modification by the inhibitor. We explored IQ-dependent covalent alteration of MtSK by LC-MS. LC-MS total ion chromatograms showed an elution time of 5.3 min for intact MtSK, and the deconvoluted ESI-MS spectra for our MtSK preparations revealed...
two species with average molecular masses of 19648.74 and 19517.57 Da. As described previously, the difference, 131.2 Da, is consistent with the post-translational removal of the N-terminal methionine [22]. Incubation of MtsK with IQ produced a time-dependent decrease in the intensity of the chromatographic peak at 5.3 min concomitant with the appearance of a shoulder at 5.5 min and a new peak eluting later in the chromatogram at 6.0 minutes (Fig. 5). For MtsK incubated with IQ for 30 h, deconvoluted mass spectra collected across the chromatographic range (5.164 – 6.615 min.) showed three pairs of peaks (Fig. 6A). In each pair, the lighter and heavier masses were separated by 131 Da, and the intensity of the peak corresponding to the heavier mass was ~60% of the intensity of the peak corresponding to the lighter mass. Further, the lighter of each pair was separated from one another by increments of 326 Da, starting at 19517.73, the mass of MtsK lacking its N-terminal methionine. The same pattern was observed for the heavier mass of each pair, starting at 19648.97, the mass of intact, full-length MtsK. These data suggested that MtsK (with or without its N-terminal Met) was covalently modified by IQ, and this could occur at more than one site on the MtsK intact protein.

Figure 5. Effect of IQ incubation time on MtsK LC retention. MtsK (1.0 µM) was incubated with 100 µM IQ at 4°C for 30 hours. At the times indicated, a sample was withdrawn.
and evaluated by LC-MS. MS signals corresponding to unmodified MtSK (*, retention time = 5.3 min) diminish over time. MS signals corresponding to IQ-derivatized MtSK (#, retention times = 5.5 and 6.0 min) result from incubation of MtSK with IQ.

No IQ-MtSK adducts were observed at 5.25 min (5.164 – 5.438 min range) (Fig. 6B). Deconvolution of the MS spectrum of the peak shoulder observed at 5.5 min (5.438 – 5.841 min.) (Fig. 6C) showed nearly equal contributions of underivatized MtSK and MtSK derivatized once by IQ. Finally, the deconvoluted MS spectrum of the peak eluting at 6.0 min (5.841 – 6.615 min.) was dominated by MtSK derivatized twice by IQ (Fig. 6D). The mass shifts, in each case 326.4 Da, precisely match the molecular mass of one IQ with an average loss of 32.2 Da, which corresponds to the loss of H-OCH₃ from IQ upon each reaction with MtSK.

Intensities of the deconvoluted MS spectra of MtSK preincubated with IQ for 30 hrs, showed a gradual loss of the mass of free enzyme - with or without its N-terminal Met (Supplementary Material, Fig. S1A). Concomitantly, there was a transient increase in the intensity of the mass of singly modified MtSK derivative (MtSK-IQ), reaching a maximum at around 15 hours. Its intensity was constant over a five hour range, after which it slowly declined (Supplementary Material, Fig. S1B). As shown in the supplementary material (Fig. S1C), the appearance of the doubly modified IQ-MtSK adduct (MtSK-IQ₂) was relatively slow, marked by a slow increase in intensities for both forms of MtSK. It reached a maximum at around 20 hrs, after which there was slow decline in peak intensities.

Reaction of MtSK with 10-fold lower concentrations of IQ (i.e., 10 µM), resulted in the loss of unmodified MtSK (5.25 min retention peak) and an increase in derivatized species (retention time > 5.4 min). However, in both cases these were observed to a lesser extent than in
reactions using 100 μM IQ. In addition, only once-derivatized MtSK-IQ species were observed in deconvoluted mass spectra (Supplementary material, Fig. S2). As would be expected from IQ-dependent inactivation kinetics (see Fig. 2), reaction of MtSK with equimolar IQ (i.e., 1 μM) produced minimal though detectable modification of MtSK (data not shown).

3.3 Reactivity of IQ with other enzymes

To evaluate the specificity of IQ for covalent alteration of MtSK, we investigated three other enzymes, MtKatG, PK, and LDH. Incubation of MtKatG and PK with 100 μM IQ for 1 hour produced no discernable loss of enzyme activity relative to controls lacking IQ, but LDH lost nearly 90% of its activity under the same conditions (Supplementary material, Fig. S3). We evaluated each intact protein by LC-MS in a manner similar to MtSK. No change in the total ion current LC retention profile was observed for MtKatG and PK. Likewise, mass spectra collected across the LC range showed no evidence of covalent derivatization of these enzymes by IQ (e.g,
Figure 6. Deconvoluted ESI-MS spectra for MtSK incubated with IQ. MtSK (1.0 µM) was incubated with 100 µM IQ for 30 hours at 4 °C prior to LC separation and MS analyses of the intact protein. The deconvoluted mass spectrum corresponding to the entire elution envelop (5.16 – 6.62 min) (A) is compared to that of the first (5.16 – 5.44 min) (B), second (5.44 – 5.84 min) (C), and third (5.84 – 6.62 min) (D) elution features of the chromatogram. Masses of 19648.97 and 19517.73 in panel A represent intact MtSK with and without its N-terminal methionine, respectively. Each mass shift of 326 Da in panels C and D corresponds to addition of an IQ derivative which lacks a methoxy group plus hydrogen (32 Da.).
PK in Supplementary material Fig. S4). However, in a manner very similar to MtSK, the total ion current LC retention profile for LDH showed a transition to species with longer retention times that became more pronounced with longer times of LDH preincubation with IQ. As shown in Supplementary Data (Fig. S5), mass spectral analyses revealed the appearance of LDH-IQ adducted proteins, with a mass difference of 326 Da as observed with MtSK. As an aside, issues such as these reveal the problems inherent in coupled assays for evaluation of inhibition kinetics and mechanism. The coupling enzymes themselves (LDH is commonly used for this purpose) can be subject to the action of candidate inhibitors.

3.4 Identification of IQ-reactive sites in MtSK.

MS/MS spectra of tryptic peptides from MtSK reacted with IQ were searched with Sequest-HT for automated detection of modified residues. Peptides that were identified through Sequest-HT with a XCorr values above 2.0 were considered positive hits for covalent adduction. Examples of MS/MS spectra used to confirm the modification of these residues are shown in Supplementary Material (Fig. S6).

Tandem MS/MS analyses showed evidence for the modification of several amino acid residues (Fig. 7A). Serines and threonines, and to a lesser extent, lysines and tyrosines were the most susceptible to modification by IQ. Several IQ-modified sites were remote from the enzyme’s substrate binding pockets, active site, and conformationally dynamic lid domain (highlighted in green in Fig. 7A). It is possible that derivitization of one or more of these sites
may account in part for IQ-dependent inactivation of MtSK, but one would anticipate that such would arise from a generic impact on protein structural stability.

Notably, several prominent sites of modification (Lys 15, Ser 16, Thr 17, Ser 77, Thr 111, and Ser 44) were in close proximity to the active site (highlighted in red Fig. 7A). Indeed, all three residues of the Lys 15 - Thr 17 sequence comprise the C-terminal end of MtSK’s P-loop (i.e., phosphate-binding loop or Walker A motif), a widely distributed structure in ATP- and GTP-utilizing enzymes. In addition, Thr 111 is part of MtSK’s conformationally dynamic lid domain, a structure which closes over the active site upon substrate binding [26]; Ser 77 is adjacent to Ser 16, and Ser 44 sits on the edge of the shikimate binding pocket (Fig. 7B – D).

A

```
MAPKAVLVGL PGSKSTIGR RLAKALGVGL LTDVAIEQR TGRSIAIDIFA
TDEQEFRRRI EEDVVLAAAL DHDGVLSLGG GAVTSPGVRA ALAGHTVYVL
EISAAEGVRR TGGNTVRPLL AGPDRAEKYR ALMAKRAPLY RRVATMRVDT
NRRNPAGAVVR HILSRLQVPS PSEAAT
```
Figure 7. Sites of MtSK adduction by ilimaquinone. MtSK residues for which LC-ESI-MS/MS evidence for IQ-based modification was observed are highlighted in green or red, with those in close proximity to the active site highlighted in red (A). The conformational positions of IQ-derivatized active site residues K15, S16, and T17 (Walker A motif), T111 (lid domain), S77 (Walker B motif), and S44 (extended substrate binding domain) are shown in substrate-free MtSK (PDB 2IYT) (B), the MtSK-ATP complex (PDB 2IYW) (C), and the MtSK-ADP-shikimate ternary complex (PDB 2IYQ (D) (26). Images were produced using PyMol (version 1.3).

Modification of any of these sites could easily account for the loss of MtSK activity caused by IQ. In order to explore this possibility further, we evaluated the effect of the ATP substrate on IQ-dependent inactivation of MtSK. Inclusion of a saturating concentration of ATP limited both the rate and extent of enzyme inactivation. Inclusion of ATP in time-dependent inhibition and inhibitor dilution experiments (Fig. 8A and 8B, respectively) confirmed that the presence of ATP prevented (or at least delayed) a substantial proportion of MtSK inactivation; however, it was also clear that, as before, what IQ-inactivated MtSK was formed during the incubation period was irreversibly inactivated. Interestingly, trypsinization and MS/MS analyses of MtSK reacted with IQ in the presence of ATP indicated that ATP partially limited Lys 15 and Ser 16 derivatization, particularly when IQ concentrations were low (i.e., 1 and 10 µM). Conversely, clear protective effect of ATP could not be discerned with respect to derivatization of Ser 44, Ser 77 or Thr 111. These data suggest that modification of P-loop amino acids accounts for some but not all IQ-dependent inactivation of MtSK, and inactivation due to modification of these residues is prevented to some extent when ATP is also present.

Based on the nature of the residues modified by IQ and the consistent neutral loss of 32 Da, we propose a mechanism of IQ-dependent inactivation of MtSK which proceeds by Michael addition (Fig. 9). Here, MtSK Ser and Thr (and to a lesser extent, Lys and Tyr) side chains act as
nucleophiles and attack the benzoquinone C5 position, generating a methoxy leaving group. Together with the intact protein analyses and time-dependent inhibition kinetics, our results suggest that IQ inhibits the *MtSK* activity irreversibly as a protein modification reagent.

**Figure 8. Effect of ATP on *MtSK* inactivation by IQ.** The *MtSK* activity remaining following preincubation with 50 μM IQ in the presence (■) and absence (○) of 1.2 mM ATP is shown in panel A. Recovery of *MtSK* activity following dilution of IQ-inactivated enzyme is shown in panel B. In these experiments, *MtSK* (20 μM) was preincubated for 1 hour with no additives (■), with 250 μM IQ (○), or with 250 μM IQ and 1.2 μM ATP (●). Following incubation, each mixture was diluted 100-fold into a reaction cocktail containing 1.2 mM ATP, 5.0 mM shikimate. Aliquots were withdrawn from the assay mixture and quenched as described in Materials and Methods spanning a time range from 3 – 3600 s. The inset shows reaction at early time points. These data represent duplicate experiments. The error bars represent the range.

**Figure 9. Proposed mechanism for *MtSK* modification by ilimaquinone.** Ser, Lys and Thr-based nucleophiles attack the quinoid ring (C5) of ilimaquinone, resulting in the formation of an enolate ion. Due to charge stabilization, the methoxy group is eliminated. The result of this is
ilimaquinone bound to protein via Ser/Thr residue, with a corresponding loss of HOCH₃ from the inhibitor, as observed by MS.

For residues where it was not as clear that ATP might interfere with derivatization by IQ (i.e., Ser 44, Ser 77, and Thr 111), we explored possible binding modes of IQ covalently linked to MtSK at these residues. These were predicted through docking, restrained energy minimization and complex structural refinement, as described under Materials and Methods. For generating binding mode hypotheses we utilized two MtSK crystal structures: an apo structure with open lid domain (PDB 2IYT) and a closed lid structure, co-crystallized with shikimate phosphate (PDB 2IYY) [26]. The latter structure resulted in more favorable non-polar contacts with IQ in the shikimate binding site region compared to the apo structure. Therefore, our best binding models are based on the closed lid MtSK structure (PDB 2IYY), describing IQ covalently bound to S77 and to T111. No favourable fit could be obtained for the IQ structure in proximity of S44. The two IQ bound complexes developed based on the structure (PDB 2IYY) were superimposed onto MtSK with co-crystallized ATP and Mg ion (PDB 2IYW) to compare the position of ATP to that of the predicted IQ poses (Fig. 10).
IQ covalently bound to S77 binds to a site adjacent to the ATP binding pocket. This pose would displace two crystal waters coordinating Mg$^{2+}$ (marked with red arrows in Fig. 10). However, in this pose IQ may contribute to metal coordination through a hydroxyl group that closely overlaps with one of the crystal waters. In this pose IQ does not penetrate into the ATP binding site and shows no interference with bound ATP. In the IQ pose covalently linked to T111, the ligand partially penetrates the ATP binding pocket resulting in severe steric clashes with an ATP phosphate group restrained by Mg$^{2+}$ coordination. As discussed below, the benzoquinone ring of IQ is sandwiched between R117 and P11 while covalently bound to T111, which interactions would prevent re-orientation of the benzoquinone ring away from the phosphate group. Therefore, this IQ binding model is not compatible with ATP binding. A close-up view of the binding site of covalently bound IQ in the two models is illustrated in Fig. 10.
Figure 11. IQ binding models based on MtSK (PDB 2IYY) with IQ covalently linked to A. S77 and B. T111. Residues shown form the binding site of IQ. Carbon atoms of MtSK are colored dark green, while ligand carbons are colored pink or light brown. All other atoms are colored by atom type (as in Fig. 10). Polar interactions discussed in text are indicated by dashed lines.

In both IQ binding models the hydrophobic part of the ligand is accommodated in a mainly non-polar region, partially overlapping with the shikimate binding site, where lid domain residues form part of the IQ binding pocket. In the IQ model covalently bound to S77 the hydrophobic part of the ligand forms favourable non-polar contacts with P11, I45, F49, F57, P118, the aliphatic chain of R117 and CB atom of the D34 side chain. Its benzoquinone forms a hydrogen bond with R20. The side chain conformation of R20 is restrained through hydrogen bonding/salt bridging interactions, as shown in Fig. 11A. In the IQ model covalently linked to T111 the following residues participate in non-polar contacts with the ligand: P11, I45, F49, F57, P118, L119, L120 and the aliphatic part of the R117 side chain. In this pose the benzoquinone moiety of IQ is sandwiched between P11 and R117, forming aromatic – cation interactions with the guanidinium group of R117, and a weak hydrogen bond with G12 (Fig. 11B). Both IQ binding modes show excellent shape complementarity with their binding sites, as illustrated by the electrostatic potential surface of MtSK displayed in Fig. 12.
Although our data suggest that IQ inactivates MtSK by derivatizing residues in or near the active site, IQ has a clear capacity toward collateral modification of MtSK amino acids well removed from the enzyme’s active site. Similarly, we observed IQ cross reaction with other proteins/enzymes. Out of three other enzymes evaluated, LDH activity showed a similar sensitivity to IQ, and it was derivatized by IQ. Notably, IQ neither inhibited nor derivatized two other enzymes, KatG and pyruvate kinase. This indicates that though IQ may be promiscuous in its reactivity, it is not indiscriminant as a protein modification reagent; across the four enzymes evaluated there are numerous solvent-exposed Ser, Thr, Lys and/or Tyr residues. A substantial proportion of these do not appear to be reactive with IQ. All things considered, in its present form, IQ does not exhibit stellar specificity for MtSK, and there is a clear capacity to cross react with unintended targets. As such, the potential of IQ as a lead in the development of antitubercular agents directed against MtSK or other targets is questionable.

4. Conclusion
IQ-based irreversible inactivation of *Mt*SK is via covalent adduct formation. Covalent inhibitors have proven to be more potent than typical reversible inhibitors and highly profitable [27]. Acetylsalicylic acid [aspirin], esomeprazole [Nexium], and clopidogrel [Plavix] are a few examples among many. Covalent inhibitors possess several advantages over non-covalent inhibitors including increased biochemical efficiency of target disruption, longer duration of action that outlasts the pharmacokinetics of the compound, and the potential to prevent the emergence of acquired resistance conferred by mutations [28,29]. However, off-target modification is a major concern for drugs acting through covalent mechanisms. By virtue of its tendency to covalently derivatize Ser and Thr as well as Lys and Tyr residues and its ability to inactivate two targets across a limited set of four enzymes, IQ has all the appearances of a non-specific or PAINS-type inhibitor. Certainly, caution is warranted in the use of IQ in drug-discovery applications. On the positive side, IQ is not indiscriminant. Across the four enzymes evaluated, a large proportion of available residues (i.e., solvent exposed Ser, Thr, Lys, and Tyr) were not modified, and two enzymes, KatG and PK, were entirely unaffected by IQ. Molecular docking studies indicate that there may be potential paths forward for modification of the IQ scaffold to increase its specificity and suitability as an *Mt*SK active site-directed agent.

ACKNOWLEDGMENTS

We thank Dr. Benjamin A. Garcia and his graduate student Mariel Coradin for facilitating the instrumentation and performing a part of the mass spectrometry analysis at the University of Pennsylvania. JS is grateful to the Secretaría Nacional de Ciencia y Tecnología” (SENACYT) in
collaboration with the “Instituto para la Formación de Recursos Humanos” (IFARHU) of the Panamanian government for Ph.D. scholarship.

**FUNDING**

This work was supported by Auburn University Intramural Grants Program (AU-IGP), through the Office of the Vice President for Research (OVPR) to A.I.C and D.C.G. M.T.H. Thanks NCCIH, CSC, The Charles and Carol Cooper Family and The Abney Foundation for Financial Support.

**REFERENCES**


