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Development of gallic acid formazans as novel enoyl acyl carrier protein reductase inhibitors for the treatment of tuberculosis Vanita D. Saharan^{*}, Supriya S. Mahajan

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ABSTRACT

The enoyl acyl carrier protein reductase (InhA) of *Mycobacterium tuberculosis* (MTB) is an attractive target for developing novel antitubercular agents. A series of gallic acid formazans, were computationally designed and docked into the active site of InhA to understand their binding mode and potential to inhibit InhA. Nine compounds from the designed series were identified as potential InhA inhibitors, on the basis of good Glide score. These compounds were synthesized in the laboratory and evaluated for in vitro antitubercular activity against drug-sensitive and multi-drug resistant strains of MTB. Out of nine compounds, three compounds exhibited the most promising MIC of $< 2 \mu$ M against the sensitive strain of MTB, H37Rv. The compounds were evaluated against five resistant strains of MTB. Most of the compounds exhibited activity superior to the standard, linezolid, against all these resistant strains. The mechanism of action of these compounds was concluded to be InhA inhibition, through InhA enzyme inhibition study. Insignificant cytotoxicity of these compounds was observed on RAW 264.7 cell line. Inactivity of all these compounds against gram positive and gram negative bacteria indicated their specificity against MTB. The compounds were further analyzed for ADME properties and showed potential as good oral drug candidates. The results clearly identified some novel, selective and specific InhA inhibitors against sensitive and resistant strains of MTB.

Keywords: Tuberculosis, Formazans, Enoyl acyl carrier protein reductase, Glide, Cytotoxicity.

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (MTB), leading to serious levels of morbidity and mortality, particularly in developing countries.¹ The TB epidemic is further complicated by emergence of multi-drug resistant² (MDR-TB) and extremely-drug resistant-TB (XDR-TB) strains.³ According to WHO report 2015, there has been 14 % increase in MDR-TB patients in comparison to 2014. It is estimated that as many as 50 million people worldwide have been infected with MDR-TB strains, which has adversely affected patient care and public health. Also on average, an estimated 9.7 % of people with MDR-TB have XDR-TB.⁴

The development of new antitubercular agents with superior activity has been slow. There is an urgent need to develop antitubercular agents against unique drug targets expressed by MTB organisms and indispensable for their growth and survival. Moreover, development of the antitubercular agents effective against the drug resistant strains is the need of the hour.

Mycolic acids are the hallmark of these species, essential for the integrity of the mycobacterial cell wall.⁵ The 2-trans-enoyl-acyl carrier protein reductase or MTB InhA, is the last key enzyme involved in elongation cycle of fatty acids in MTB.⁶ Inhibition of InhA disrupts the biosynthesis of mycolic acids, unique to the species, and induces accumulation of saturated fatty acids, leading to cell wall alterations, lysis and consequently, to death of the organism.⁷ The enzyme InhA has been identified as the primary target of isoniazid (INH), one of the most effective first-line anti-TB drugs.⁸ The INH requires activation within the mycobacterial cell by catalase-peroxidase (KatG). The major mechanism of INH resistance arises from mutations in KatG.⁹ To overcome the INH resistance associated with mutations in KatG, called direct InhA inhibitors, are developed as new promising agents against the ever increasing threat from drug resistant MTB strains.^{10,11} Hence, many research groups have been attempting to develop direct InhA inhibitors, such as triclosan,¹² diphenyl ethers,¹³ pyrrolidine carboxamide,¹⁴ arylamide,¹⁵ piperazine,¹⁶ thiadiazole¹⁷, and oxoquinazolin acetamide¹⁸ derivatives.

In continuation with our earlier work¹⁹ and in search of developing novel direct InhA inhibitors, the design and synthesis of gallic acid formazans were undertaken. Gallic acid is a naturally existing antioxidant,²⁰ possesses various biological activities such as analgesic,²¹ antimicrobial, anticancer,²² antityrosinase, antimycobacterial²³, etc. Formazans are known for their spectrum of biological activities such as antimycobacterial, antibacterial, anti-

inflammatory, anticonvulsant and antifungal.^{23, 24} Further careful literature survey for functional groups, which could be considered as pharmacophores for antitubercular activities, revealed that the hydrazone moiety (R¹R²C=N-NH) is common among most of the antitubercular agents. Considering these therapeutic values, hydrazones were selected to synthesize newer derivatives of Schiff's bases as antimycobacterial agents.²⁵ Our idea behind this project was to combine gallic acid and azohydrazones in one single molecule by using hybridization technique, to get novel formazans and explore their potential as antitubercular agents.

In silico studies: The computational chemistry approach of docking serves as a tool in screening a large number of compounds against the target and helps to speed up the drug discovery process from hit to lead. Molecular docking study was carried out to develop gallic acid formazans as novel enoyl acyl carrier protein reductase (lnhA) inhibitors for the treatment of tuberculosis. A series of gallic acid formazans were designed *in silico* using the software *Maestro* 10.3 from Schrodinger. The crystal structure of MTB lnhA, complexed with the reference inhibitor, N-(4-methylbenzoyl)-4-benzylpiperidine (PDB ID: 2NSD with a resolution of 1.9 0 A), was downloaded from the protein data bank. (Available online: <u>http://www.rcsb.org/pdb/home/home.do</u>).

Initially, widely cited X-ray crystallographic structures with PDB IDs 2H7M, 2H7P, 2H7I, 2H7L and 2NSD was selected for docking. This protein structures, had co-crystallized reference inhibitors, good resolution, complete protein structure available, were not wild type or mutant. But it was decided to continue the docking studies with the PDB ID, 2NSD, since the reference inhibitor in 2NSD gave higher G-score (G = -10.346) better than the other structures like, 2H7M (G = -8.067), 2H7I (G = -9.538), 2H7L (G = -8.278) and 2H7P (G = -8.453). G-score is one of the important parameters to evaluate the docking score. The higher the negative G-score, higher is the energy released by the system (protein-ligand complex) and stronger is the binding of the ligand with the protein.

Further, the downloaded protein was pre-processed by using the program '*Protein Preparation Wizard*', from *Maestro* 10.3, available in the molecular modeling suite of Schrodinger. Various parameters like assign bond orders, add hydrogens, treat metals, find overlapping of amino acid chains, delete water molecules and orientation of amino acids, were selected for pre-processing of the protein. Finally, energy minimization was done using

OPLS-2005 force field to get the refined, energetically balanced and optimized structure of the protein.



Figure 1. Structures of (A) Grid generation around the inhibitor of InhA (B) Superimposition of the re-docked pose of the reference inhibitor and the original pose of the reference inhibitor

Grid generation program *Glide*, was used to define docking space and to generate the grid box. The protein InhA, with PDB ID 2NSD, has a co-crystallized reference inhibitor placed in the active pocket of the protein. Hence, the grid box was generated around the co-crystallized inhibitor, as shown in **Figure 1A**.

In the present study, the docking protocol was validated by extracting the native ligand from the crystal structure and docking it back into the binding site of InhA. The root mean square deviation (RMSD) between the original conformation of the reference inhibitor and the conformation obtained from its re-docking into the crystal structure was found to be 0.8 ⁰A, which was less than the acceptable limit of 1 ⁰A, thus validating the reliability and reproducibility of the docking procedure. **Figure 1B** shows the super-imposition of the re-docked pose of the reference inhibitor and the original pose of the reference inhibitor.

A library of more than hundred gallic acid formazans was designed *in silico* and docked into the active site of InhA. Chemical diversification was introduced on two ends a) aryl and heteroaryl substituted aldehydes and b) substituted amines, so as to develop a strong structure-activity relationship (SAR) profile and also to understand the ideal site for

introducing chemical diversity. The choice of substituents on parent compound was based on *in silico* studies (Glide Score, H-bonds, bad bonds, ugly bonds and van der Waal interactions).

Based on the input from protein-ligand interactions, it was observed that unsubstituted benzaldehyde displayed better enzyme inhibition than substituted benzaldehyde, with substituents like chloro, nitro, methoxy, methyl, hydroxyl etc. Even heteroaryl substituted aldehyde like furfuraldehyde, did not display promising results. Hence unsubstitued benzaldehyde was taken up for further study.

Similarly, substituted amines showed better enzyme inhibition than that of unsubstituted amine. Hence, various substituted amines were introduced and their activity was mapped for contributing towards the antimycobacterial activity. From the docking study, it was revealed that chloro, methoxy, nitro and fluoro substituted amines display enhanced antimycobacterial activity. The presence of electron withdrawing groups increased the hydrophobicity due to hydrophobic interactions with the active site, which was observed crucial for inhibition. However, further introduction of bulky substituent like 4-*tert*-butyl on the amine, abolished activity, indicating that for optimal activity there is a size limit for substituents on the primary amine. Such bulky substituents may interfere with binding in the active site.

Out of the series of compounds docked, nine promising compounds showed G-score in the range of -7.732 to -9.781 kcal/mol (**Table 1**). The G-score of the reference ligand (N-(4-methylbenzoyl)-4-benzylpiperidine) and isoniazid was found to be -10.346 and -6.257 kcal/mol, respectively. All the compounds, except for the compounds **C1** and **C6**, showed G-score more than that of isoniazid and close to the reference ligand. **Figure 2** represents compounds **7** and **9** docked into the active site of InhA.



Figure 2. (A) Compound 7 and (B) Compound 9 docked into the active site of InhA (PDB ID: 2NSD)



Figure 3. Interacting amino acids of InhA with compounds (A) C1 and (B) C7

Visual inspection of the docked complexes of compounds **C2-C9** revealed that all the ligands could snugly fit into the active site of InhA, occupying positions very close to that of the native ligand in the crystal structure. The compounds **C2-C9** were found to be buried into the hydrophobic pocket of InhA and were found to make contacts mainly with Tyr158, Ile215, Met155, Leu218, Pro156, Ala157, Met199 and Met161 (**Figure 3**).

The hydrogen bonding network with Tyr158 and NAD⁺ cofactor, seemed to be a conserved feature among all the lnhA-inhibitor complexes, identified so far.^{10, 11} This hydrogen bonding network probably served as the key feature governing the orientation of the compound within the active site. The ligand-protein interaction was further studied in detail to understand the interaction of the compounds at molecular level. The protein-ligand interaction (Figure 3) revealed that all the compounds except compound C1, formed hydrogen interactions with Tyr158 and NAD⁺. The enzyme bound conformation of the compounds C2-C9 showed that the carbonyl oxygen atom of the compounds was involved in forming a hydrogen bond with Tyr158. The compounds C2-C9 displayed higher G-scores as compared to the G-score of the compound C1 (Table 1). Figure 3 represents the orientation and interaction pattern of compounds C1 and C7 in the active site of InhA. The compound C1 is oriented in a different manner as compared to compounds C2-C9, hence, it displayed lower G-score. The hydrogen bonding helped in good fitting of the compounds C2-C9 with the active site of the enzyme, making them more potent. Hence, the compounds C2-C9 displayed good InhA enzyme inhibition, in silico. The phenolic hydroxyl groups also forms H-bonds with other amino acids which reflects the stability of a protein-ligand complex.

Hence, compounds **C1-C9** were found to be favourable in improving the interaction with the active site residues, and hence were attempted in synthesis and further biological evaluation.

Synthesis: The compounds C1-C9 were synthesized by using simple reactions depicted in Scheme 1. The intermediate galloyl hydrazide was prepared in good yields by refluxing propyl gallate with hydrazine hydrate. Further, galloyl hydrazide was condensed with benzaldehyde, to yield Schiff's base. This Schiff's base was further reacted with various diazotised primary amines to obtain compounds C1 to C9, the gallic acid formazans. These compounds were obtained in good yields and purity, using inexpensive and commonly available reagents. The compounds were fully characterized by spectroscopic analysis, IR, ¹H-NMR, ¹³C-NMR and mass spectral data and were in agreement with the proposed structures as reported in the literature.²⁶

The infrared spectra exhibited characteristic stretching vibrations of C=N (1650-1630 cm⁻¹), N-H (3460-3400 cm⁻¹) and N=N (1590-1550 cm⁻¹). Additionally, intense bands, corresponding to C=O stretching was observed in the range 1756-1733 cm⁻¹. Further, the characteristic N-H signal of formazan, was observed as a singlet of integration intensity

equivalent to one hydrogen between 14-16 ppm in the ¹H NMR spectra. Similarly, the ¹³C NMR spectrum of the compounds showed the characteristic peaks values of C=O (162-163 ppm) and C=N (150-152 ppm). The molecular ion peaks of the compounds **C1-C9** in their mass spectra were in full agreement with their molecular weights.

Antitubercular activity screening: The antitubercular activity of all the compounds C1-C9 was determined by measuring inhibition of growth against MTB H37Rv sensitive strain (ATCC27294) using agar dilution method.²⁷ The concentrations in the range of 50 μ g/ml to 0.78 μ g/ml, in duplicates, were used for the study. The minimum inhibitory concentration (MIC) was determined for each compound as the minimum concentration of a compound required to inhibit the complete growth of bacteria. Isoniazid, ethambutol and ofloxacin were used as the reference compounds for comparison. The antitubercular activity of gallic acid formazans along with the standard drugs are presented in **Table 1**.

Relevant mycobacterial activity as defined by the Clinical and Laboratory Standards Institute (CLSI) relates to MIC values below 25 μ M (<25 μ M) for pure compounds. The synthesized compounds **C1-C9** showed activity against MTB with MIC ranging from 1.752 to 38.109 μ M. The MIC of the compound **C1** against MTB was 38.109 μ M, highest among all the compounds, whereas for the remaining compounds, MIC was below 25 μ M. Thus, as per the CLSI norms, all the compounds, except compound **C1**, can be said to display good antitubercular activity. All the eight compounds, **C2-C9**, inhibited MTB with the MIC of < 16 μ M. The compounds **C3**, **C5** and **C9** exhibited the most promising MIC of < 2 μ M. The MIC of the compounds **C4** and **C8** was <10 μ M, whereas for the compounds **C2**, **C6** and **C7**, it was approximately 15 μ M.

For antitubercular activity of compounds against the sensitive strain, isoniazid, ethambutol and ofloxacin were used as standards. The MICs of compounds C3 (1.752 μ M), C4 (4.00 μ M), C5 (1.921 μ M), C8 (7.422 μ M) and C9 (1.979 μ M) were found to be lower than that of ethambutol (7.64 μ M). Thus, these compounds are more potent than ethambutol. The compounds C3, C5 and C9 were found to be more active than ofloxacin (2.16 μ M).

Encouraged by the promising antitubercular activity, the compounds **C1-C9** were further evaluated for their activity on multi-drug resistant (MDR) strains. ²⁸ **Table 2** shows MIC values obtained against MDR strains of MTB, including HR (Isoniazid, Rifampicin-resistant strain), HRKS (Isoniazid, Rifampicin, Kanamycin, Streptomycin-resistant strain), HRKCpm

(Isoniazid, Rifampicin, Kanamycin, Capreomycin-resistant strain), HRKSXMfxLev (Isoniazid, Rifampicin, Kanamycin, Streptomycin, Ofloxacin, Moxifloxacin, Levofloxacinresistant strain), and HRS (Isoniazid, Rifampicin, Streptomycin-resistant strain). Linezolid was used as the reference compound for the comparison.

The compounds displayed promising results when tested against various resistant strains of MTB. In HR resistant strain, the MIC of the compounds C2 (2.9 μ M), C3 (0.33 μ M), C5 (1.90 μ M) C8 (2.85 μ M) and C9 (0.49 μ M) were found to be lower than that of the standard linezolid (3.70 μ M). The activity of compounds C6 (5.89 μ M) and C7 (5.46 μ M) were also found to be active at MICs little higher than linezoild. In HRKS resistant strain, compound C9 (0.73 μ M) was found to be more active as compared to the standard linezolid (2.78 μ M) and activity of compound C5 (2.92 μ M) was found to be close to linezolid. The activity of compound C3 (5.16 μ M) was slightly less than that of linezolid. The compounds C6 (16.02 μ M) and C8 (22.32 μ M) were found to be moderately active against HRKS resistant strain.

In HRKCpm resistant strain, the MIC of compound C9 (1.92 μ M) was found to be very close to that of linezolid (1.86 μ M). The compounds C3 (7.43 μ M) and C5 (7.62 μ M) were also found to be active, whereas the compound C8 (14.0 μ M) was moderately active. The MIC of compounds C3 (11.44 μ M) and C5 (10.56 μ M) was higher than that of linezolid (1.86 μ M) when tested against HRKSXMfxLev resistant strain, but they did show moderate activity against this strain.

In HRS resistant strain, compounds C2 (2.95 μ M), C3 (1.34 μ M), C5 (1.90 μ M) and C9 (0.96 μ M) were found to be more active than linezolid (3.70 μ M). The compounds C6 (5.89 μ M) and C8 (5.46 μ M) were also found to be active, whereas compound C7 (14.84 μ M) displayed a moderate activity.

Thus, most of the compounds showed good activity against various multi-drug resistant strains tested, when compared with linezolid. These results are promising for the development of new effective compounds against the growing number of multi-drug-resistant strains of MTB.

Cytotoxicity: Cytotoxicity studies are usually conducted to eliminate toxic compounds that can limit the progression of a novel chemical molecule and to evaluate, if the compounds are

toxic only to the mycobacterial cells (selective toxicity). The compounds were tested for cytotoxicity in RAW 264.7 cell line (mouse leukemic monocyte macrophage) at concentrations of 100 and 50 μ M. Macrophage cell line was selected to test toxicity since MTB resides inside the macrophages and drug molecules should not possess any toxicity against these macrophages. After 72 h of exposure of the cell line to the test compounds, the viability of the cells was assessed on the basis of cellular conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a formazan product using the Promega Cell titre 96 non-radioactive cell proliferation assay.²⁹ Per cent inhibitions are reported in **Table 1**. Negligible toxicity, at concentrations of 50 and 100 μ M, was observed for the most active compounds **C3** (12.08 and 21.54 % inhibition), **C5** (4.51 and 15.02 % inhibition) and **C9** (11.85 and 23.68 % inhibition). Thus, they can be considered safe for administration.

Tuberculosis infected macrophage assay: In tuberculosis infection, macrophages act as reservoirs for replicating mycobacteria. In this study, *in vitro* macrophage model was used to investigate the ability of the compounds to kill intracellular mycobacteria. The compounds **C2-C9** were profiled for their activity against MTB residing inside the mouse macrophages, in the concentrations of 0.1, 1, and 10 µg/ml, using the standard procedure.³⁰ In the initial screening of compounds in the tuberculosis infected macrophage assay, compounds **C3**, **C5** and **C9** effectively inhibited the intracellular growth of MTB at concentration lower than 1 µg/ml. Further, to determine IC₅₀ values for these compounds, six different doses ranging between 0.1-1.0 µg/ml were used. The compounds **C3**, **C5** and **C9** were found to be effective against intracellular mycobacteria with IC₅₀ of 0.35 \pm 0.02, 0.56 \pm 0.04 and 0.62 \pm 0.02, respectively.

Antibacterial activity: Antibacterial activity of all the nine compounds was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* at the concentrations of 50 and 100 μ g/ml using Cup-plate agar diffusion method.³¹ All the compounds showed very poor or no activity against gram positive and gram negative bacteria, undertaken in the study. Inactivity of all these compounds against these tested bacteria indicated their specificity toward MTB. Hence, all these compounds were found to possess selectivity and specificity in their action.

InhA enzyme inhibition study: Recombinant MTB InhA was expressed in *E. coli* and subsequently purified.³² The reduction reaction of 2-trans-dodeceenoyl decenoyl-CoA (DD-CoA) catalysed by MTB InhA was spectrophotometrically measured, monitoring NADH oxidation to NAD⁺. The enzyme inhibition assay was carried out in the presence of the synthesized compounds using 1% DMSO as the solvent (this concentration did not interfere with the assay conditions). As a control, the maximal rate of the enzymatic reaction (100 % Mt InhA activity) was determined in the absence of inhibitors and in the presence of fixed non-saturating concentrations of NADH and DD-CoA in the presence of 1% DMSO as the solvent. The inhibitory activity of each compound tested was expressed as the per cent inhibition of MTB InhA activity, at 10 μ M concentration, with respect to the control.³³ The IC₅₀ values for the compounds (**C2-C9**), showing more than 50 % inhibition of InhA at 10 μ M concentration, were also determined. The IC₅₀ value is defined as the concentration of the inhibitor that reduces the enzyme velocity by half.

The InhA enzyme inhibition assay was carried out to explore the mechanism of action of the compounds. As shown in **Table 1**, all the compounds showed % inhibition of MTB InhA in the range of 42.23 ± 0.37 to 87.12 ± 1.64 at 10 µM concentration. Out of 9 compounds tested, 8 compounds exhibited InhA enzyme inhibition with IC₅₀ less than 10 µM. A good correlation was observed between *in silico* study, *in vitro* antitubercular activity, and InhA enzyme inhibition study. The compounds **C1** displayed the lowest G-score of -7.732, the lowest antitubercular activity with the MIC equal to 38.109 µM and also the lowest InhA enzyme inhibition of 42.23 ± 0.37 % at 10 µM. The compound **C9** emerged as the most potent inhibitor of InhA with IC₅₀ value of 3.42 ± 0.48 µM and MTB MIC of 1.979 µM. It also displayed very good interaction with InhA enzyme, *in silico*, with G- score of -9.481. The highest % inhibition of InhA at 10 µM was shown by the compound **C9**. Thus, IC₅₀ value for this compound was the lowest of all. Since the enzyme inhibition was less than 50 % at 10 µM for the compound **C1**, it was not considered for determining IC₅₀ value against MTB InhA.

The compounds C3 and C5, which showed IC_{50} of 5.22 ± 0.36 and $5.79 \pm 0.22 \mu$ M, respectively, in the MTB InhA enzyme inhibition assay, exhibited MTB MIC of 1.752 and 1.921 μ M, respectively. The compounds C3 and C5 also showed good G-score of -8.731 and -8.828. Similarly, compound C4 displayed good G-score of -8.736, inhibited MTB with MIC of 4.00 μ M and IC_{50} of 6.66 \pm 0.59 in the MTB InhA enzyme inhibition assay. The

compounds **C2**, **C7** and **C8** inhibited MTB with higher MIC values of 15.225, 14.845 and 7.422 μ M, respectively, and also showed higher IC₅₀ values of 9.16 ± 0.58, 8.46 ± 0.32 and 8.16 ± 0.34, respectively, in InhA enzyme inhibition assay. However, compound **C6**, with MIC equal to 15.394 μ M failed to inhibit MTB despite good potency in the InhA enzyme inhibition assay (IC₅₀ = 3.32 ± 0.34 μ M) and good docking score (G = -8.020). It is also possible that this compound is actively extruded from the bacterial cell by efflux pumps, and this could be a major challenge in TB drug discovery. It was observed that, the IC₅₀ values of the compounds in the InhA inhibition study were 2-4 fold higher than MTB cellular activity. The difference may be due to intracellular accumulation, differential sensitivity of InhA between *in vitro* and *in vivo* conditions and also potential direct or indirect effects including secondary molecular targets of the compounds inside the cells.

Biophysical characterization: One of the active compound C3 from the series was further investigated using a biophysical technique, differential scanning fluorimetry (DSF). The ability of the compounds to stabilize the catalytic domain of the InhA protein and of the protein bound with the ligand was measured.³⁴ Complex with compound C3 was heated stepwise from 25 °C to 95 °C in step of 0.1 °C rise in the presence of a fluorescent dye (sypro orange), whose fluorescence increased as it interacted with hydrophobic residues of the InhA protein. As the protein was denatured, the amino acid residues became exposed to the dye. A right side positive shift of *Tm* in comparison to native protein meant higher stabilization of the protein-ligand complex, which was a consequence of the inhibitor binding. In our study, compound C3 showed significant positive *Tm* shift of 2.8 °C confirming the stability of the protein-ligand complex as shown in the Figure 4, which depicts the curves obtained in the DSF experiment for the MTB InhA protein (pink) and protein-compound C3 complex (green).



Figure 4. DSF experiment for the compound C3 (protein-ligand complex, green) showing an increase in the thermal shift of 2.8 °C compared with the native InhA protein (pink). Protein Tm 39.90 °C and Protein with compound C3 Tm 42.70 °C.

In silico ADME predictions: A promising lead is often defined as a compound which combines potency with an admirable absorption, distribution, metabolism, excretion and toxicity (ADMET) profile. As such, compounds with unfavourably predicted pharmacokinetic profiles are either completely dismissed from the list of potential drug candidates (even if they prove to be highly potent) or the drug metabolism and pharmacokinetics (DMPK) properties are 'fine tuned' in order to improve their chances of making it to clinical trials. Approximately 40 % of drug candidates fail in clinical trials, owing to poor pharmacokinetics and toxicity properties. This late-stage failures contribute significantly to the cost of new drug discovery endeavours. Therefore, ability to detect problematic candidates in the early stage of drug discovery, significantly reduces the amount of time and resources being wasted on molecules that are doomed to fail in the later stage.

The virtual predictions are not always reliable, but it can be often used as initial tools to eliminate compounds likely to present uninteresting pharmacokinetic profiles and unacceptable levels of toxicity from the list of potential drug candidates, still a major bottleneck in drug discovery process.

With this objective, *in silico* ADME prediction was undertaken for the active molecules to gauge their drug-likeliness using *QikProp* tool (Schrodinger, LLC., New York) incorporated in Schrodinger molecular modeling suite. It provides ranges for comparing the properties of molecules with those of 95 % of known drugs.³⁵ The descriptors calculated were molecular weight (MW), #stars, logarithm of partition coefficient (Log P), Lipinski's rule of five, % human oral absorption (% HOA), CNS activity (blood-brain barrier partition coefficient) and Caco-2 cell permeability (gut-blood barrier permeability; absorption of orally administered drugs).³⁵ The *in silico* ADME prediction data of the compounds are summarized in **Table 3**.

In silico ADME prediction was further a guiding tool in identifying the promising compounds that can be taken up for further study. Lipophilicity is one of the most important physico-chemical properties determining the biological activity of molecules, affecting the non-specific diffusion through biological membranes. It is well known that antimycobacterial activity is often enhanced by increased lipophilicity, which facilitates the penetration of compounds through highly lipophilic mycobacterial cell wall. The acceptable range predicted for this parameter is **-2.0 to 6.5**. The lipophilicity of all the synthesized compounds, as obtained from the software *QikProp*, was found to be in the range of **1.864 to 4.49**. Due to good lipophilicity, compounds exhibited good antitubercular activity against drug-sensitive and multi-drug resistant strains of MTB.

Drugs targeting the central nervous system (CNS) are expected to cross the blood brain barrier in order to reach their destination while drugs with peripheral site of actions are expected to have no brain penetration to avoid related side effects. Lowest values of -2 (**Table 3**), predicted from blood-brain barrier partition coefficient, signify that these molecules have very low propensity to cross the blood-brain barrier, thereby eliminating the chance of CNS related toxicity.

The value of absorption >80% is considered good and <25% is considered poor, according to the *QikProp* prediction. Except compounds **C7** and **C8**, all the compounds displayed fairly good oral absorption with very low susceptibility to acid hydrolysis in the stomach as reflected from the % human oral absorption data. The predicted values of apparent Caco-2 cell permeability, which predicts absorption of orally administered drugs, further supports these findings. The parameter *metab* computed for the compounds was in the range of 4-5

(range for 95% of drugs is 0 to 15). Hence the compounds are proposed to be metabolically stable.

In conclusion, we have designed a series of gallic acid formazans and docked in the active site of the enzyme InhA. Nine compounds exhibiting good G-score were identified as inhibitors of InhA. Thus, docking helped to filter out those candidates with lower G-score as compared to the reference ligand and isoniazid and hence, lower antitubercular activity, in *silico*. The compounds C3, C5 and C9 exhibited the most promising MIC of $< 2 \mu$ M against MTB H37Rv. These compounds also displayed good G-score in docking study. The compounds C2 and C3 were found to be active against all the five resistant strains tested. The InhA enzyme inhibition assay established the mode of action of these compounds as InhA inhibition. The compounds C2-C9 showed good enzyme inhibition in the range of 57.12 \pm 1.12 to 87.12 \pm 1.64 % at 10 μ M. The compounds C3, C5 and C9 emerged as the most promising inhibitors of InhA, with IC_{50} values of 5.22 \pm 0.36, 5.79 \pm 0.22 and 3.42 \pm 0.48 μ M, respectively. The compounds C3, C5 and C9 were also effective against intracellular mycobacteria, with IC₅₀ values of 0.35 ± 0.02 , 0.56 ± 0.04 and 0.62 ± 0.02 , respectively. These compounds also displayed insignificant activity against gram positive and gram negative bacteria and negligible cytotoxicity against RAW 264.7 cell line. Thus, these compounds were selective and specific in their action against the mycobacteria. The molecular modeling tool was also used as a guiding tool for the prediction of ADME of nine synthesized compounds. The ADME analysis of all the compounds showed that, compounds C3, C5 and C9 possess potential as good oral candidates. The results clearly indicated that the formazans C3, C5 and C9 were good InhA inhibitors and specifically effective against sensitive and resistant strains of MTB.

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Scheme 1. Chemical reactions for the synthesis of gallic acid formazans

		% Inhibition of InhA at 10 µM ^a	IC-c in uM		Cytotoxicity ^c		
Compound code	Glide			MIC (µM)	(% inhibition)		
	Score			MTB ^b	At 100 μM	At 50 μM	
C1	-7.732	42.23 ± 0.37	NT	38.109	41.38	13.62	
C2	-8.842	57.12 ± 1.12	9.16 ± 0.58	15.225	32.65	24.46	
C3	-8.731	73.18 ± 1.65	5.22 ± 0.36	1.752	21.54	12.08	
C4	-8.736	71.66 ± 0.78	6.66 ± 0.59	4.00	52.67	46.51	
C5	-8.828	69.34 ± 2.14	5.79 ± 0.22	1.921	15.02	4.51	
C6	-8.020	81.44 ± 2.77	3.32 ± 0.34	15.394	41.50	32.69	
C7	-9.147	62.32 ± 1.72	8.46 ± 0.32	14.845	5.43	2.11	
C8	-9.197	57.12 ± 1.67	8.16 ± 0.34	7.422	34.63	11.12	
C9	-9.481	87.12 ± 1.64	3.42 ± 0.48	1.979	23.68	11.85	
Reference Ligand	-10.346	NT	NT	NT	NT	NT	
Isoniazid	-6.257	NT	NT	0.72	NT	NT	
Ethambutol	-	-	-	7.64	-	-	
Ofloxacin	-	-	-	2.16	-	-	

Table 1. Docking and biological activity results of gallic acid formazans

IC₅₀, 50% inhibitory concentration; MTB, *Mycobacterium tuberculosis*; MIC, minimum inhibitory concentration; NT, not tested.

^a MTB InhA enzyme inhibition activity.

^b In vitro activity against MTB H37Rv strain.

^c Against RAW 264.7 cells.

Compound		ΜΙC (μΜ)						
code	HR ^a	HRKS ^b	HRKCpm ^c	IRKCpm ^c HRKSXMfxLev ^d				
C1	NA	NA	NA	NA	NA			
C2	2.9	NA	NA	NA	2.95			
C3	0.33	5.16	7.43	11.44	1.34			
C4	23.85	NA	NA	NA	NA			
C5	1.90	2.92	7.62	10.56	1.90			
C6	5.89	16.02	NA	NA	5.89			
C7	5.46	NA	NA	NA	14.84			
C8	2.85	22.32	14.0	NA	5.46			
C9	0.49	0.73	1.92	NA	0.96			
Linezolid	3.70	2.78	1.86	1.86	3.70			

Table 2. MIC of gallic	acid formazans against	resistant strains	of	M	T
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NA, Not Active

^aHR: Isoniazid, Rifampicin-resistant strain

^bHRKS: Isoniazid, Rifampicin, Kanamycin, Streptomycin-resistant strain

^cHRKCpm: Isoniazid, Rifampicin, Kanamycin, Capreomycin-resistant strain

^dHRKSXMfxLev: Isoniazid, Rifampicin, Kanamycin, Streptomycin, Ofloxacin,

Moxifloxacin, Levofloxacin-resistant strain

^eHRS: Isoniazid, Rifampicin, Streptomycin-resistant strain

Compound code	#Stars ^a	CNS ^b	QP log Po/w ^c	QPP Caco ^d	%HOA ^e	Rule of Five ^f	#metab ^g
C1	0	-2	4.085	221.17	89.992	0	4
C2	0	-2	3.6	221.38	92.83	0	5
C3	0	-2	3.791	226.87	95.40	0	5
C4	0	-2	2.798	68.64	76.20	0	4
C5	0	-2	2.648	64.86	74.83	0	4
C6	0	-2	4.49	250.26	92.06	0	4
C7	0	-2	1.951	12.32	44.93	1	4
C8	0	-2	1.864	7.73	40.79	1	4
С9	0	-2	2.769	64.73	75.57	0	4

Table 3. QikProp analysis of gallic acid formazans

^a#stars - this property indicates the number of property or descriptor values that fall outside the 95% range of similar values for known drugs. The range predicted for this parameter is 0-5; where 0 indicates no violation or the best candidate.

^bCNS - this exhibits the predicted central nervous system activity, acceptable range predicted for this parameter using *QikProp* being -2 to 0 for inactive compounds, and 0 to 1 for active compounds.

^cQP log Po/w - this gives the predicted octanol/water partition coefficient. The acceptable range predicted for this parameter using *QikProp* is -2.0 to 6.5.

^dQPPCaco - this gives the predicted Caco-2 cell permeability in nm/s. Caco-2 cells are a model for the gut-blood barrier. The *QikProp* predictions are for non-active transport, where <25 is considered poor and >500 is considered excellent.

^ePer cent human oral absorption - this gives the predicted human oral absorption on 0-100% scale. The prediction is based on a quantitative multiple linear regression model. Value of absorption >80% is considered good and <25% is considered poor.

^tRule of five - this property denotes the number of violations of Lipinski's rule of five. The rule includes MW<500, QPlogPo/w<5, donor HB \leq 5 and acceptor HB \leq 10. Compounds that satisfy these rules are considered to possess drug-like action.

^gmetab - range for 95% of drugs is 0 to 15.

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In silico design of gallic acid formazans as InhA inhibitors for treatment of

tuberculosis

THE



Gallic acid formazans docked in active site of InhA



active site of InhA



Compound C7 docked into the Interacting amino acids of InhA with compound C7

Comp	MIC (µM)	MIC (µM) in Resistant Strains				<i>In -Vitro</i> InhA Enzyme inhibition study		
	H37Rv	HR	HRKS	HRKCpm	HRS	% Inhibition	IC ₅₀	
C3	1.752	0.33	5.16	7.43	1.34	73.18 ± 1.65	5.22 ± 0.36	
C5	1.921	1.90	2.92	7.62	1.90	69.34 ± 2.14	5.79 ± 0.22	
С9	1.979	0.49	0.73	1.92	0.96	87.12 ± 1.64	3.42 ± 0.48	

- > Novel InhA inhibitors against sensitive and resistant strains of MTB
- > C3, C5 and C9 exhibited promising MIC of $< 2 \mu$ M against MTB H37Rv
- > C2 and C3 were found to be active against all the five resistant strains
- > Compounds also displayed selectivity and specificity against the mycobacteria
- . fr > Compounds C3, C5 and C9 possess potential as good oral candidates, from ADME