ISSN 0975-6299 Vol 3/Issue 1/Jan – Mar 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOINFORMATICS

ELUCIDATING ISONIAZID RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* USING MOLECULAR DOCKING APPROACH

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ABSTRACT

Isoniazid, an important drug in the anti-tuberculosis therapy, the enzyme catalase-peroxidase (KatG) plays a key role in activating isoniazid (INH). Mutation in *katG* gene is a major mechanism of INH resistance in *M. tuberculosis*. Several derivatives of INH show activity against TB and multi-drug resistant TB. With the aim of finding new compounds, in this study, the derivatives of INH were docked directly with wild type and the mutated models of KatG from *M. tuberculosis* using *in-silico* approaches. Docking results suggests that compounds-10, 12 and 13 were the high scoring derivatives of INH to bind with mutants of KatG. These models represent the first *in-silico* evidence for the binding interaction of KatG and its mutants with INH derivatives. The models may provide useful insights for designing new anti-TB agents in order to overcome the resistance developed with INH.

KEY WORDS

Mycobacterium tuberculosis, KatG, INH resistance, INH derivatives, Mutants, Docking.

INTRODUCTION

Tuberculosis (TB) remains as a major health problem and a leading cause for mortality. The increase in multi-drug resistant (MDR-TB) defined as strains resistant to at least two of the first-line TB drugs - isoniazid [INH] and rifampicin [RIF] has worsened the situation. Further the recent emergence of extensively drug resistant (XDR-TB), defined as MDR-TB that is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs [capreomycin, kanamycin, and amikacin] has compounded the condition still more. Drug resistance in TB is essentially a potential threat to the TB control programmes. However, isolates of *M. tuberculosis* resistant to INH are seen with increasing frequency (1 in 10^6) compared to other drugs [1]. The global proportion of resistance among all incident TB cases was 4.8%. Of the other types of resistance, globally INH resistance was found to be in 10.3% (new cases) and 27.3% (treated cases) [2].

The use of INH (Isonicotinic acid hydrazide-chemical name, Pyridine-4-carbohydrazide-IUPAC name), as an effective antitubercular drug began in 1952, even today, INH remains as one of the most active compounds used to treat and prevent TB worldwide. It is the cornerstone of treatment for drug-susceptible and latent TB infection, acting as a principle component in the current six-month short course chemotherapy. INH is a pro-drug that requires cellular activation by KatG protein to its active form before exerting its toxic effect on the bacillus. This fact was revealed shortly after the introduction of INH in monotherapy when resistance became clearly evident. Such resistance was often accompanied by loss of KatG activity [3, 4]. It has long been recognized that resistance of *M. tuberculosis* to INH correlates with the loss of catalase and peroxidase (CP) activities in resistant strains [5]. There has been considerable interest to know the molecular basis of INH resistance which is not very well characterized, and mutations in several genes have been associated with it. Zhang et al. (1992) demonstrated that mutation in *katG* gene coding for KatG protein is a major mechanism of INH resistance in *M. tuberculosis* [6].

The Ser to Thr mutation (AGC to ACC) in codon 315 of the *katG* gene is considered to be the most prevalent mutation, serving as a reliable marker for the detection of INH resistance [7] and the appearance was most frequent among the MDR strains [8]. However, this mutation was also reported to be associated with intermediate or high levels of resistance to INH (1 to 10 µg/ml) [9]. Based upon the availability of crystal structure of the wild type KatG, certain residues in its active site have been postulated to be involved in enzyme-catalyzed activation of INH [10]. Two other reports depict the structure-function relationships of KatG protein [11, 12]. A number of *in vitro* studies have analyzed the origins of resistance using the S315T mutant as a model [13, 19]. Studies that target mutation other than S315T in KatG has clearly shown that the decrease KatG activity observed in the mutant proteins is well correlated with the structural modifications [20, 21].

More than 25 different KatG mutants were characterized functionally as off now, on varying aspects from all over the world and the number continues to increase each year. In spite of the fact that the above reports has provided extensive kinetic and some structural data related to KatG and its mutants, a

comprehensive structural study is needed, which could explain the cause of INH resistance in the mutants. This still remains as an area to be uncovered. Therefore, in order to understand the rationale behind the point mutants causing INH resistance, in the present study, an attempt was made to explore the interactions of KatG mutants by docking between the KatG mutant models with INH and its derivatives.

Several studies were conducted using different derivatives or analogues of INH to find activity against TB and MDR-TB recently. A study demonstrated that the antimycobacterial pharmacophore moiety of INH has been introduced in 510 moieties to improve their activity against Mycobacteria species, as well as their MDR strains [22]. Many Schiff bases, hydrazones, hydrazides and metal complexes of INH have shown very good activity. Various types of the most active INH derivatives classified based on their structure, their lipophilicity has been calculated and structure-activity relationships were discussed. A series of novel INH derivatives has demonstrated activity against INH^r TB strains. Among the various isonicotinoyl-hydrazinocarbothioamides, 2-isonicotinoyl-*N*-[2-(trifluoromethyl) phenyl] hydrazinecarbothioamide was found to be the most potent compound with (minimum inhibitory concentration) MIC of 0.58 µM against H37Rv and INH^r *M. tuberculosis* [23]. Several nicotinic acid and INH analogues, most of them containing nitro groups were synthesized and evaluated for their *in vitro* antibacterial activity against *M. tuberculosis,* also one of the nitro group containing compound exhibited the best result (1.2 µg/mL) when compared with INH itself could be a good starting point to develop new lead compounds in the treatment of MDR-TB [24]. A study targeting resistant *M. tuberculosis* suggested that N'-(1-alkyl-2,3-dihydro-2-oxo-1H-3-indolyliden)-4-pyridinecarboxylic acid hydrazide derivatives were used to overcome the resistance developed with the therapeutic uses of INH [25].

Although many derivatives or analogues of INH have been synthesized and tested in cell lines for their efficacy against resistant TB, very few of them were turned out be successful. These classical experiments involve whole cell screening and the reasons for the lesser activity of the compound to be tested were multifactorial. On the other hand, in the present study, the derivatives or analogues of INH were docked directly with proteins, wild type (WT) and mutated models of KatG from *M. tuberculosis* rather than the whole cell, using *in-silico* approaches.

MATERIALS AND METHODS

(i) Proteins

The homology models of KatG, WT and the five mutants such as S315T, S315R, S315I, S315N, and N138S (with codon Thr, Arg, Ileu, and Asn instead of Ser, at position 315 and Ser at position 138 instead of codon Asn) were used in this study. The rationale behind the mutant selection was based on our molecular studies both published [26] and accepted [27].

(ii) Ligands

The ligands chosen in this study were the derivatives of INH (Table 1) obtained from NCBI (National Center for Biotechnology Information) Pubchem-database whose characteristics and the chemical structures are provided in supplementary material. The SMILES (Simplified Molecule Input Line Entry System) formulae of INH derivatives obtained from the NCBI-database were converted to chemical structure using Chem sketch software V-10. The structures of the derivatives were then converted into PDB (Protein Data Bank) files using DS. The labeling of the derivatives or compounds is as per the order given in Table 1.

Table 1
Name of Compounds

S.no	IUPAC	SMILES formulae
1	N'-acetylpyridine-4-carbohydrazide	CC(=O)NNC(=O)C1=CC=NC=C1
2	N'-propan-2-ylpyridine-4-carbohydrazide	CC(C)NNC(=O)C1=CC=NC=C1
3	4-amino-2-hydroxybenzoate	C1=CC(=C(C=C1N)O)C(=O)O
4	Sodium 4-amino-2-hydroxybenzoate	C1=CC(=C(C=C1N)O)C(=O)[O-].[Na+]
5	Potassium 4-amino-2-hydroxybenzoate	C1=CC(=C(C=C1N)O)C(=O)[O-].[K+]
6	2-fluoropyridine-4-carbohydrazide	C1=CN=C(C=C1C(=O)NN)F
7	(Pyridine-4-carbonylamino) thiourea	C1=CN=CC=C1C(=O)NNC(=S)N
8	N'-ethanethioylpyridine-4-carbohydrazide	CC(=S)NNC(=O)C1=CC=NC=C1
9	N'-propylpyridine-4-carbohydrazide	CCCNNC(=O)C1=CC=NC=C1
10	N-[(4fluorophenyl)methylideneamino] -1- methoxy-1-pyridin-4-ylmethanimine	COC(=NN=CC1=CC=C(C=C1)F) C2=CC=NC=C2
11	N-(4-oxopentan-2-ylideneamino) pyridine-4-carboxamide	CC(=NNC(=O)C1=CC=NC=C1) CC(=O)C
12	N'-[(E)-(3-methoxy-4-oxo-1-cyclohexa-2,5-dienylidene) methyl]pyridine-4- carbohydrazide	COC1=CC(=CNNC(=O)C2=CC= NC=C2) C=CC1=O
13	Methyl (2-(pyridine-4-carbonyl) hydrazinyl)Methanedithioate	CSC(=S)NNC(=O)C1=CC=NC=C1
14	N-[(4-diethylamino-4-oxobutan-2-ylidene) amino] pyridine-4-carboxamide	CCN(CC)C(=O)CC(=NNC(=O) C1=CC=NC=C1)C
15	N'-(2-oxoindol-3-yl)pyridine-4-carbohydrazide	C1=CC2=C(C(=O)N=C2C=C1) NNC(=O)C3=CC=NC=C3

(iii) Docking software

The GOLD protocol is an implementation of the genetic algorithm. The receptor is held rigid while the ligand is allowed to flex during the refinement. The docking of flexible ligand and a protein with flexible hydroxyl groups makes it a good choice when the binding pocket contains amino acids that form Hydrogen (H) bonds with the ligand. GOLD uses a scoring function that is based on favorable conformations found in Cambridge structural database and on empirical results on weak chemical interactions. The scoring function is force field based, and includes three terms (a H bonding term, a 4–8 intermolecular dispersion potential, and a 6–12 intramolecular potential for the internal energy of the ligand) [28].

(iv) Docking protocol

Docking was performed between 15 derivatives of INH and KatG models, with the help of software GOLD (Version- 4.0.1).

The input atom files for both the proteins and the ligands were created**.** The ligands and the models were added with H atoms using GOLDMINE before docking. The cavity atom file containing the atom number of binding residues was prepared for both heme (Pro77, Leu78, Arg81, Trp84, Tyr206, Asn208, Pro209, Phe229, Leu242, Ile243, Gly245, His247, Phe249, Gly250, Lys251, Thr252, His253 Thr291, Asn292, Trp298, Leu355, Thr357) and INH derivatives (Arg81, Trp84, His85, Tyr206 and Pro209). The binding residues were selected on comparison between binding regions of heme and INH with crystal structures of other molecules (2v2e, 2v2f, 2vcf, 2vcn and 2vcs) similar to KatG. Comparison was done

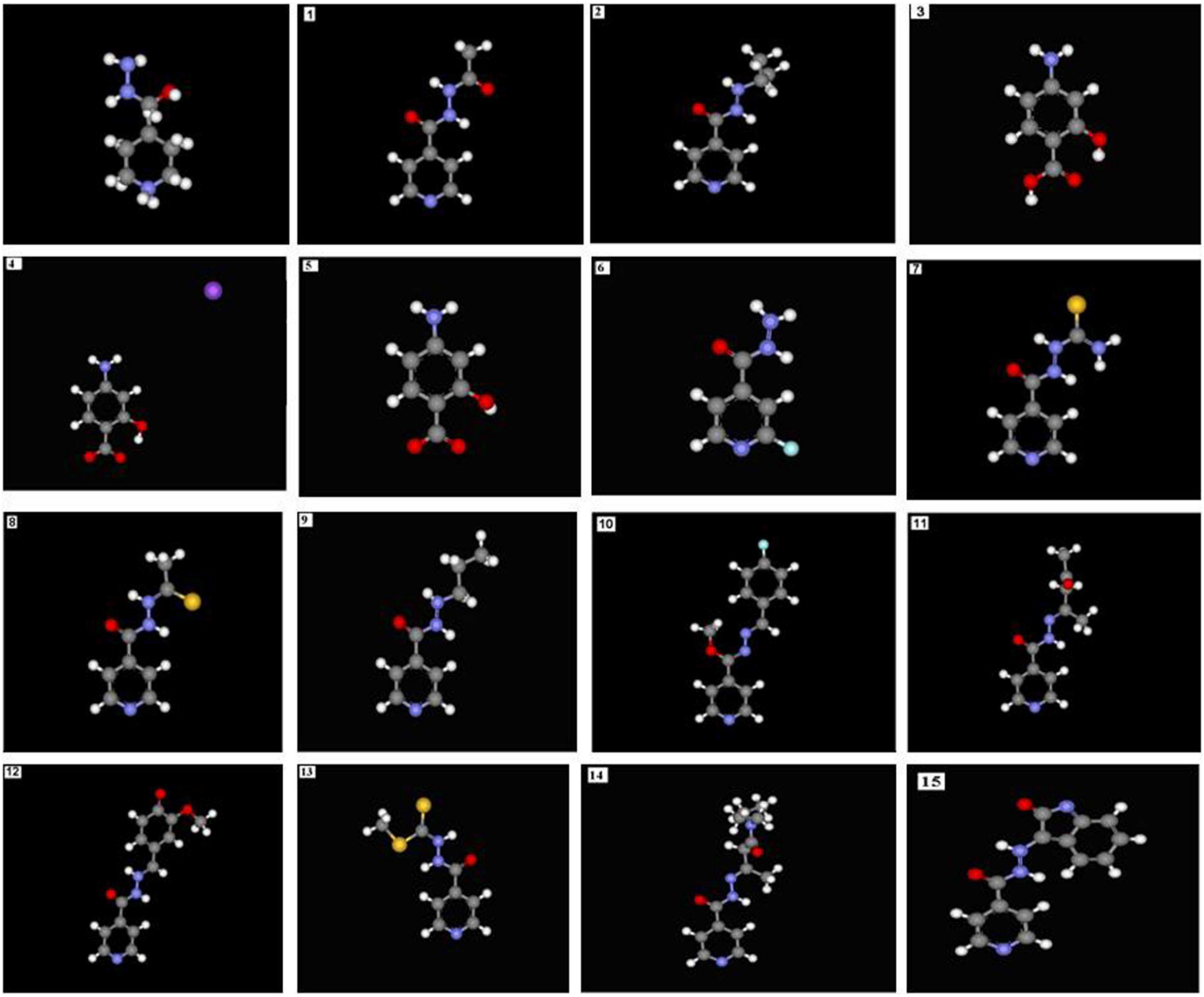

Figure 1

3D structure of INH and its derivatives viewed under Discovery Studio (DS) V-2, (Violet colored-Nitrogen atom, Red colored-Oxygen atom White colored- H atom, Yellow colored-Sulphur atom).

using multiple sequence alignment with ClustalW. Dockings were performed under 'Standard default settings' mode, number of islands was 5, population size of 100, number of operations was 100,000, a niche size of 2, and a selection pressure of 1.1. Ten docking poses were obtained for each ligand. Poses with highest GOLD score were used for further analysis. The docked poses of the ligands were visualized using Hermes software. The scoring function of GOLD provides a way to rank placements of ligands relative to one another. Ideally, the score should correspond directly to the binding affinity of the ligand for the protein, so that the best scoring ligand pose are the best binders.

RESULTS

The models (WT and mutants of KatG protein) were used to evaluate a training set, containing 15 compounds by a process of docking performed using GOLD. The three dimensional (3D) structures of all the derivatives were created as shown in Figure. 1 using DS. All the compounds were selected at random from NCBI-database. Out of the 15 compounds tested for binding activity, six of them are analogues of INH, five (1, 2, 3, 4 and 5) of

Table 2

Docking Score of KatG and its variants with INH and its derivatives

S. no	INH and its derivatives	KatG and its variants					
		S315	**S315T**	**S315I**	**S315R**	**S315N**	**N138S**
	INH	43.82	46.07	45.64	44.87	44.78	43.77
1	N'-acetylpyridine-4-carbohydrazide	45.16	44.66	48.38	47.80	45.10	49.20
2	N'-propan-2-ylpyridine-4-carbohydrazide	**39.51**	41.09	32.21	42.84	**41.16**	**42.91**
3	4-amino-2-hydroxybenzoate	46.72	45.96	46.01	45.63	44.48	45.68
4	Sodium 4-amino-2-hydroxybenzoate	47.08	47.30	44.77	47.51	42.80	45.64
5	Potassium 4-amino-2-hydroxybenzoate	48.57	48.82	44.71	50.31	46.51	47.40
6	2-fluoropyridine-4-carbohydrazide	41.01	41.03	41.48	**40.90**	43.04	40.53
7	(pyridine-4-carbonylamino)thiourea	46.49	46.25	54.41	45.59	49.01	48.84
8	N-ethanethioylpyridine-4-carbohydrazide	56.68	46.50	53.75	52.17	51.44	53.41
9	N'-propylpyridine-4-carbohydrazide	46.30	41.75	37.32	44.62	42.39	46.34
10	N-[(4 fluorophenyl) methylideneamino] -1-methoxy-1-pyridin-4-ylmethanimine	54.35	52.46	**57.07***	**57.67***	56.23	57.27
11	N-(4-oxopentan-2-ylideneamino) pyridine-4-carboxamide	48.95	48.62	47.26	51.08	49.51	48.81
12	N'-[(E)-(3-methoxy-4-oxo-1-cyclohexa -2,5 -dienylidene)methyl]pyridine-4-carbohydrazide	54.11	46.01	49.61	53.19	**57.78***	49.60
13	Methyl(2-(pyridine-4-carbonyl) hydrazinyl)methanedithionate	**57.24***	**54.14***	55.62	57.43	54.26	**57.65***
14	N-[(4-diethylamino-4-oxobutan-2-ylidene)amino]pyridine-4-carboxamide	48.09	**38.13**	**21.14**	44.24	42.48	45.94
15	N'-(2-oxoindol-3-yl)pyridine-4-carbohydrazide	51.93	48.43	45.36	48.11	52.74	51.36

Asterick * indicates highest score and Bold indicates lowest score.

them were compounds with antimycobacterial activity.

(i) Compounds with highest and lowest affinities

In accordance to the general conception, that high score is indicative of high affinity. In the present study, of all the docking scores, top GOLD score was (-57.78 kcal/mol) with the mutant S315N and compound-12 (N'-[(E)-(3-methoxy-4-oxo-1-cyclohexa-2,5 dienylidene) methyl] pyridine-4-carbohydrazide) represents high affinity between the protein and ligand molecule. The scores for the compound-13 (Methyl (2-(pyridine-4-carbonyl) hydrazinyl) methanedithioate) were found to be -57.24, -54.14 and -57.65 kcal/mol for WT and mutants (S315T and N138S) respectively. The score was -57.07 and -57.67 kcal/mol with compound-10 (N-[(4 fluorophenyl) methylideneamino] -1-methoxy-1-pyridin-4-ylmethanimine) for the mutants S315I and S315R respectively (Table 2 and Figure 2A and B). One of the compound-2 (N'-propan-2-ylpyridine-4-carbohydrazide) named iproniazid, known to act against monoamine oxidase was found interestingly to have a remarkable decrease (-39.51 kcal/mol) in degree of binding affinity with WT KatG lower than that of INH itself (-43.82 kcal/mol) and with KatG mutants it imparts some degree of affinity. Also, the other two compounds (6 and 14) displayed lower affinity with WT KatG and mutants compared to INH itself.

(ii) Overall affinity KatG and its mutants with all the compounds

The WT KatG affinity towards compounds -6, 1, 3, 7, 9, 4, 5 and 11 found to have values in range of forty whereas compounds-15, 12, 10 and 8 have values in range of forty whereas compounds-15, 12, 10 and 8 have values in the range of fifty in ascending series. (1) For the mutant S315T, compounds-6, 2 and 9 displayed values in 41 whereas compounds-5, 11 and 15 showed values in 48 series. Other compounds showed varying values ranging from 45-47. However, in spite of its chemically complex nature the compound-14 displayed lower score of -38.13 kcal/mol than INH itself with this mutant. (2) The mutants-S315I and S315R found to discharge higher affinity with compound-10. The compound-14 displayed a lower score than compound-2 and INH itself with S315I mutant of KatG; these results were similar to the mutant S315T. (3) In case of mutant S315R, surprisingly, compound-6 showed the lowest affinity compared to other derivatives along with compound-2 and INH. (4) The mutant S315N affinity towards these compounds varies in a random manner and the striking aspect of this mutant was that several of the compounds namely 4, 6, and 9 in addition to the common compounds 2 and 14 displayed lower affinity than INH itself. (5) The affinity of the mutant N138S with compounds-13, 10, 8 and 15 was higher while the affinity of compounds-2 and 6 presented lower affinity than INH.

(iii) Hydrogen bond profile at the ligand binding sites

The WT (Ser315) shows the presence of 9-H bonds at the ligand binding region with compound-13. Amongst which, residue Ser315 forms 2-H bonds with heme, since INH binds with one edge of heme moiety, therefore any structural changes in the residue 315 would invoke alteration in the heme, which in turn will exert its effect on INH binding ability. This seems to be very important because none of the other mutants (S315R S315I, S315N, S315T and N138S) showed the involvement of

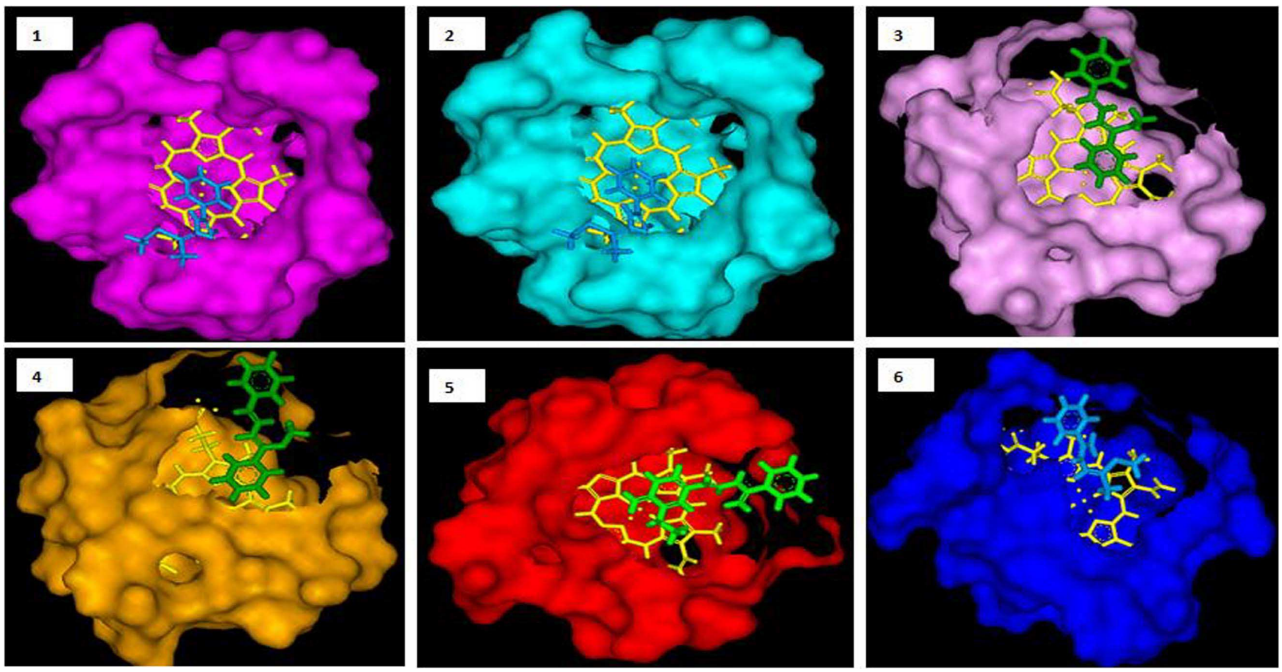

Figure 2A

Surface representation of INH derivatives (Green and Blue colored) docked with Heme (Yellow colored)1-WT and compound 13; 2-S315T and compound 13; 3-S315I and compound 10; 4-S315R and compound 10; 5-S315N and compound 12; 6-N138S and compound 13

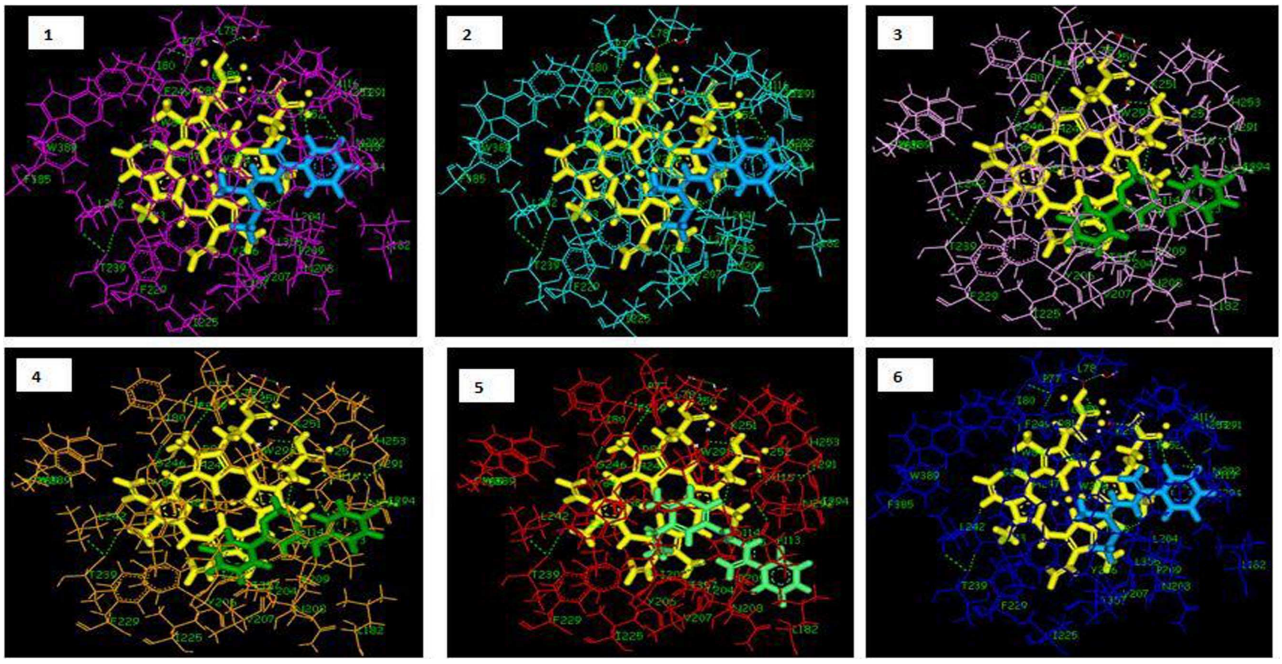

Figure 2B

H bond display in dotted green lines with KatG and its mutant.1-WT and compound 13; 2-S315T and compound 13; 3-S315I and compound 10; 4-S315R and compound 10; 5- S315N and compound 12; 6-N138S and compound 13.

the residue 315 in H bond formation, indicating the residue 315 is not perturb in the WT compared to mutants. Further, the water molecules 7, 11 and 492 forms H bond with heme cofactor in the WT.

The mutated model S315T forms seven H bonds (5 residues and two ligands) with compound- 13 and 10. The models S315N and S315R form five H bonds with the heme group [3 residues and 2 molecules of water] with compounds-12 and 13 respectively showing the highest score. The model with S315I substitution forms two H bonds with compounds 10 and the model N138S forms four bonds (2 residues and 2 molecules of water) with heme group and compound 13. The models *viz.,* S315R, S315I, S315N, S315T commonly contain His247 which forms H bond in connection to heme moiety compared to WT model.

Table 3
H bond formation at the ligand binding site

KatG models	No. of Hb	Hb donor	Hb acceptor	BD
S315 + Cmd13	9	HIS276:N	HEM1500:O1A	2.89
		HIS276:ND1	HEM1500:O2A	2.90
		SER315:N	HEM1500:O1A	2.92
		SER315:OG	HEM1500:O1A	2.55
		WaterHOH7:O	HEM1500:O2A	2.57
		WaterHOH7:O	HEM1500:O1D	2.73
		WaterHOH11:O	HEM1500:O2D	2.67
		WaterHOH492:O	HEM1500:O1D	2.71
		UNK1:H17	HIS108:NE2	1.40
S315T + Cmd13	7	HIS247:HE2	HEM1500:NA	2.33
		LYS251:HN	HEM1500:O2D	1.43
		HIS253:HD1	HEM1500:O2A	2.36
		HEM1500:H	THR252:OG1	2.30
		HEM1500:H	WaterHOH492:OH2	2.41
		UNK1:H16	VAL207:O	2.32
		UNK1:H17	HIS85:NE2	1.47
S315I + Cmd10	2	HIS247:HE2	HEM1500:NA	2.34
		HIS253:HD1	HEM1500:O1A	2.08
S315N + Cmd12	5	HIS247:HE2	HEM1500:NA	2.16
		LYS251:HN	HEM1500:O1D	1.55
		ASN292:HD21	HEM1500:O1A	2.42
		WaterHOH7:OH2	HEM1500:O2A	1.96
		WaterHOH11:OH1	HEM1500:O1D	2.41
S315R + Cmd10	5	HIS247:HE2	HEM1500:NA	2.42
		LYS251:HN	HEM1500:O2D	2.24
		ARG292:HN	HEM1500:O1A	2.03
		WaterHOH7:OH2	HEM1500:O2A	2.77
		WaterHOH7:OH2	HEM1500:O1D	2.44
N138S + Cmd13	4	LYS251:HN	HEM1500:O2D	2.18
		HIS253:HD1	HEM1500:O1A	1.92
		WaterHOH7:OH2	HEM1500:O1A	2.56
		WaterHOH11:H2	HEM1500:O1D	2.44

UNK1 = INH derivative; Hb = Hydrogen bond; Cmd = Compound; BD = Bond distance

More numbers (9) of H bond was found in the WT compared to other mutants. The H bonds present in the KatG-INH complex along with H bond donor and acceptor with their distances are shown in Table 3.

DISCUSSION

An *in-silico* screening based on the structure of chemically versatile analogues/derivatives of INH, with the purpose of contributing to the rational design of tuberculostatic leads, was performed with KatG, in this study. The inter-differences between the binding affinities amongst these structurally diverse molecules were determined for the prediction of better leads which can be used as fundamental raw material for structure based drug designing.

Fifteen molecular descriptors of INH with various types of physicochemical, steric, geometrical, and electronic properties have been tested for its ability to bind with WT and mutants of KatG. The scores indicate that the compound-12 displayed the highest affinity with the mutant S315N; compound-13 showed highest affinity with the WT and also with mutants S315T and N138S, and compound-10 showed the highest affinity with mutants S315I and S315R. Another interesting finding comes from the analysis of the compound-2 followed by compounds-6 and 14 in exhibiting lowest binding affinity (lower than that of parental INH) with WT KatG and its mutant (Figure 3a and 3b).

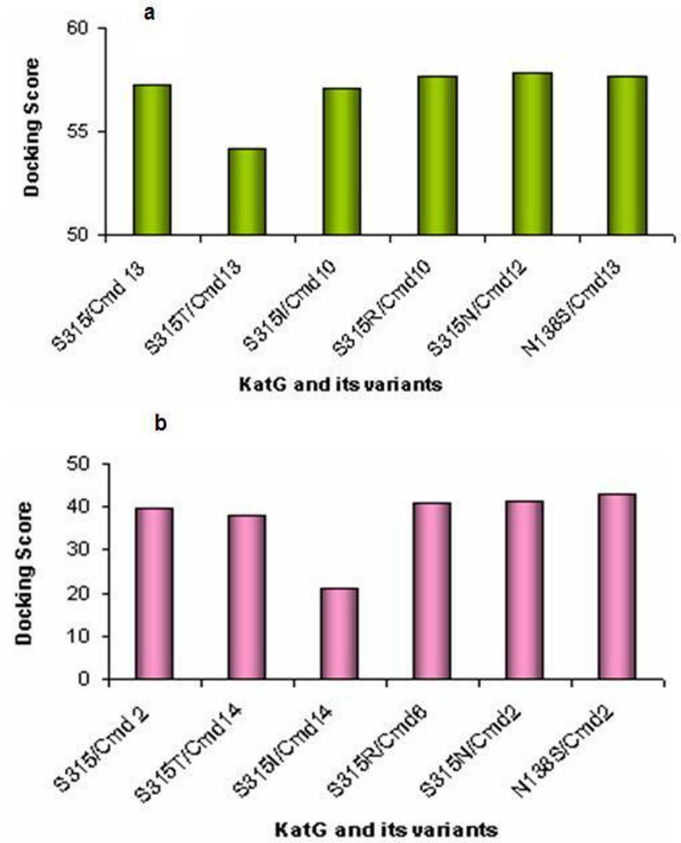

Figure 3a
INH derivatives displaying highest score with KatG and its variants,
3b: INH derivatives displaying lowest score with KatG and its variants. Cmd = Compound

It is well known that H bonds play an important role in the maintenance of stability, structural integrity and function of biological molecules, especially for enzyme catalysis. The presence of more number of H bonds at the ligand binding regions in the WT (Ser315) with compound-13 suggests the stable nature of the molecule compared to others. The changes in H bond interactions between the mutants and heme would eventually be reflected in the binding ability of INH derivatives.

These results indicate that the ligand-12 showed highest affinity with single mutant, whose score was higher than other compounds. Secondly, ligand-10 exhibit high degree of affinity with two mutants and ligand-13 displayed affinity with two mutants and a WT. Hence, these compounds can be used as possible leads in the future for treatment of INH resistant *M. tuberculosis* isolates. On the contrary, compounds such as 2, 6 and 14 owing to their lowest score cannot be considered as leads for the drug development process against INH resistant *M. tuberculosis* isolates. Docking many ligands to the same protein followed by scoring them for their relative strength of interaction has been proposed as a procedure to identify candidates for drug development. However, in the present study, docking results predicts the compounds-10, 12 and 13 as good derivatives of INH which could bind with mutants of KatG.

The compounds-12 and 10 have similar structures and the presence of bulkier side chain (pyridine) in both of them makes ideal candidates for scoring high. In case of compound-13, the presence of elements like sulphur makes it to achieve high score with two mutants and WT. The reason for the lesser score in case of compounds-2 and 6 could be due to absence of functional side chains. This has led to assume that the rationale behind the higher affinity lies in the structural complexity as well as the sterical demands imparted by these ligands. Thus, the findings have provided insights towards understanding the basis for rationalization of INH resistance, in naturally occurring KatG mutant strains of *M. tuberculosis* (Fig. 3a). However, further structural studies are needed to validate the binding aspects of INH and its derivatives in the KatG mutants leading to INH resistance.

In summary, these results suggest that the redesign of INH molecule to improve INH binding may be a viable approach to overcome resistance in KatG. Also, this study presents a basic structural framework for ligand based drug design and the findings could be meaningful for developing QSAR studies.

ACKNOWLEDGEMENTS

This work was supported by Indian Council of Medical Research grant for Senior Research Fellowship.

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