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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF INHIBITORS AGAINST *MYCOBACTERIUM TUBERCULOSIS* - METHIONINE S-ADENOSYLTRANSFERASE (MAT)

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ABSTRACT

Methionine S-adenosyltransferase (MAT) is a crucial enzyme for *mycobacterium tuberculosis* in the active as well as chronic phases of bacteria. A small set of tryptamine derivatives was designed and synthesized as MAT inhibitors and evaluated for antitubercular activity using rifampicin as a standard drug. All compounds were screened by the microplate almar blue assay (MABA) which checks cellular growth and viability of the bacteria and the luminescence-based low oxygen recovery assays (LORA) to monitor actively replicating as well as non-replicating persistence (NRP) bacteria. Synthesized compounds were also checked for cytotoxicity on human virulent cell lines. Tryptamine derivative 5,7-dichloro-2-methyltryptamine (IC_{50} = 50.0 µM by MABA and IC_{50} = 95.6 µM by LORA) and 6,7-dichloro-2-methyltryptamine (IC_{50} = 71.8 µM by MABA and IC_{50} = 92.5 µM by LORA) showed moderate antitubercular activity as compare to rifampicin (IC_{50} = 0.10 µM by MABA and IC_{50} = 3.8 µM by LORA) as a standard.

KEYWORDS: Methionine S-adenosyltransferase, MABA, LORA.

INTRODUCTION

Tuberculosis (TB) has become a global health problem due to the morbidity rate as high as nine million cases and mortality rate of almost two million cases per year associated with it.[1, 2] *Mycobacterium tuberculosis* (Mtb), a causative agent for TB has proven itself to be one of most virulent human pathogen. Despite of the revolutionary advances in drug discovery tools and techniques like resolution of whole Mtb genome sequence, boom in identification of novel targets, high throughput screening, and increases in successful applications of *in-silico* drug design methodologies, the Mtb has still remained a challenge.

In addition to this, the well-known DOTS (Directly observed therapy short course), an undoubted successful therapy against TB, which is accessible to 70% of world population has been shown to loose effectiveness for complete eradication of disease and control of spread[3]. There are many reasons for this terrifying long existence and survival of Mtb including its slow growth, dormancy, complex cell envelope, multiple drug resistance TB (MDR-TB), extensive drug resistance TB (XDR-TB), HIV co-infection, intracellular pathogenesis and genetic homogeneity [4]. Hence, the development of novel anti-tubercular agent with the help of unprecedented wealth of information and advances in the field of drug discovery is the need of hour.

The first step for designing new chemotherapeutic agents against TB, in order to overcome the problem of existing drugs resistance, is selection of novel and validated target. One such target is methionine S-adenosyltransferase (MAT). [5] It is responsible for synthesis of S-adenosylmethionine (SAM) using L-methionine and ATP as substrate. The reaction occurs in two steps where the sulfur atom of L-methionine attacks on the C5 atom of ATP cleaving triphosphate group through a bimolecular substitution reaction (SN^2) mechanism and then subsequently it leads to hydrolysis of triphosphate group to pyrophosphate and orthophosphate [6, 7].

SAM in *mycobacterium tuberculosis* plays an important role as a methyl donor catalyzing the a number of reactions [8], while the decarboxylated SAM acts as aminopropyl donor in the synthesis of polyamines such as spermidine and spermine. Consequently Mtb- MAT acts as a striking target for drug design for Mtb in active and chronic phase. In active phase, spermidine and spermine are required for transcriptional regulation and in chronic phase (persistence) methylation and cyclopropylation of mycolipids are necessary for survival [9, 10]. Berger and Knodel have validated MAT as a potential target for design of new anti-TB agents by inhibiting its activity with some non-selective methionine and purine analogs [5].

In our previous work with urge for development of novel anti-TB molecule, a homology model was developed using the crystal structure of MAT from *E. coli* (cMAT) and rat (rMAT) [11].

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In this study through calculations of molecular electrostatic potential (MEP) surfaces of Mtb-MAT and its human counterpart the residues important for Mtb-MAT binding activity and selectivity of the enzyme were identified. Based on these results, *de novo* approach was taken up to design few new and selective inhibitors. The best candidates were selected from designed molecules based on Ludi scores which helped us to predict their dissociation constants (K_i) in the mM to µM range and fulfillment the criteria for drug like properties *i.e.* Lipinski's rule of five. In continuation with this work we have synthesized one of the reported cores *viz*; 2-methyltryptamine and modified this core into newer analogues by achieving similar or good binding interactions with the amino acids of enzymes in the active site pocket through molecular docking. The synthesized molecules were then evaluated for biological activity against active as well as persistent Mtb.

EXPERIMENTAL

Designing studies

All computations and molecular modeling of the MAT enzyme and its inhibitors were carried out on Pentium IV system using the programs Insight II (v 2005; Accelrys Inc., USA) and Sybyl (v7.1, Tripos Inc., USA) and GOLD v3.0.1; Cambridge Crystallographic Data Center, UK) working under the Linux Red Hat Enterprise WS (v 2.1) operating system, .

Protein and Ligand Preparation

The structure of the Mtb-MAT was earlier discovered by homology modeling and de novo Ligand design was reported in literature for this model. Structures of proposed inhibitors of Mtb-MAT (Table 2) were taken from the literature and sketched in Sybyl, the atom and bond types were assigned, and all possible tautomeric states were considered. All ligands were optimized for geometry with energy minimization till a gradient of 0.001kcal/mol/Å was reached, using the MMFF94 force field. By studying synthetic availability of "2-(2,7-dimethyl-1H-tryptamin-3-yl) ethanamine" (tryptamine moiety) was selected for further optimization as Mtb-MAT inhibitor.

Docking Protocol

GOLD (Genetic Optimization for Ligand Docking) is a genetic algorithm (GA) for docking flexible ligands into protein binding sites while considering partial protein flexibility. Active pocket was defined with residues in a 10 Å radius from the Mg^{2+} ion (which is bound through coordinate bonds to the carboxylate groups of Asp179 and Asp259) were defined as the active site for the docking study.

The program was set to terminate when ten solutions within an rmsd of 1.5 Å were identified. The options such as flip ring corners, flip amide bonds, flip pyramidal nitrogen were activated, while unionized carboxylates were allowed to rotate so as to form favorable H–bonds.

Docking was carried out with the default GA settings and the number of GA runs was set to 30. The ten best poses for every ligand were stored for post–docking analysis. The two fitness functions – Gold Score and ChemScore accessible through the GOLD program were selected for scoring the docking poses.

Design of Novel MAT Inhibitors

The binding site of the target protein was used for designing the novel inhibitors through de novo approache. As a de novo design tool, the program Ludi first generates the interaction sites using a set of rules that are intended to cover the complete range of energetically favorable orientations for hydrogen bonds and hydrophobic contacts. Then using the fragment approach, Ludi positions small fragments in the clefts of the protein structure (i.e., active site) such that hydrogen bonds can be formed with acceptor/donor groups, and hydrophobic pockets are filled with hydrophobic groups. A fragment is chosen for its potential to have good hydrophobic and hydrogen bonding interactions with the target receptor.

Synthesis

All the synthesized compounds were characterized by their spectral properties. All solvents and reagents used for synthesis were of laboratory reagent grade. All reactions were monitored by Thin Layer Chromatography using Merck precoated silica plates (GF_{254}) for completion and method development. Melting points were recorded in open capillaries on an electrically heated Thermonik melting point apparatus and are uncorrected; boiling points were recorded in a Thiel's tube. The solvents were recovered using 'BUCHI' rota-evaporator. Infrared spectra were recorded (KBr disc method) on Jasco FT-IR 5300 spectrophotometer for all the intermediates and final compounds. Chemical shift values are reported in 'δ' units (ppm) relative to internal standard tetramethylsilane (TMS).

General method for synthesis of phenylhydrazine hydrochloride

In a 100 ml, three necks round bottomed flask equipped with stirrer, thermometer and dropping funnel, a mixture of 0.0466 mole of substituted aniline and 17.5 ml of water was stirred at room temperature. To this mixture 17.5ml of hydrochloric acid was added dropwise. The reaction flask was placed in an ice-salt bath and cooled rapidly to -15°C. To the cold solution, 0.046 mole of sodium nitrite in 12ml of water was added through a dropping funnel, the tip of which extended nearly to the bottom of the flask. The diazotization reaction required about 10 minutes to complete. The temperature of reaction mixture was allowed to warm to 0°C and poured into a solution of 0.0932 mole of stannous chloride dihydrate in 23.5 ml of hydrochloric acid at 0°C. The resulting mixture was stored at 0°C for overnight. The crystals were filtered and washed with hydrochloric acid and rinsed with ether. The product was recrystallized from distilled water.

General method for synthesis of 5-chloro-2-pentanone

A mixture of 12 ml concentrated hydrochloric acid, 14 ml of water and 0.078 mole of α-acetyl-γ-butyrolactone [3-acetyldihydrofuran-2(3H)-one] was placed in distilling flask fitted with the condenser and receiver. The end of the receiver was immersed in an ice bath. Carbon dioxide gas evolved immediately thereafter. The reaction mixture was heated slowly and the temperature was allowed to raise optimum so that the reaction mixture does not form foam into the condenser. After heating the reaction mixture for about 10 minutes, the color of reaction mixture changed from yellow to orange and then to black.

Once the effervescence subsided, the distillation was continued as rapidly as possible. After 25 ml of distillate was collected, 13 ml of water was added to the distillation flask and another 9.0 ml of distillate was collected. The yellow organic layer in the distillate was separated using separating funnel and the aqueous layer was extracted with three 5 ml portions of diethyl ether. The diethyl ether dried for 1 hour over 2 g of anhydrous calcium chloride. The ether layer was then decanted and evaporated under vaccuo to get product. Yield: 45%

General method for synthesis of Substituted tryptamines

A mixture of substituted phenylhydrazine hydrochloride 0.0126 mole, sodium acetate trihydrate 1.72 g (0.0126 mole), 5-chloro-2-pentanone 1.52 g (0.0126 mole), 4 ml of methyl cellosolve (methoxy ethanol) and 1 ml of water was refluxed on a boiling water bath for 16 h. After completion of the reaction a solution of conc. HCI (1 ml) in water (1 ml) was added to the reaction mixture and the reaction mixture was concentrated on a rotary evaporator under reduced pressure which gave oily mass. The oily mass was cooled and extracted with water in the soxhlet apparatus for 20 h. The extract was neutralized using dilute NaOH solution. The neutral solution was filtered rapidly to discard the impurities. Product separated immediately after leaving the mixture at room temperature for 15 minutes. The crude yellowish brown crystals were filtered and washed with water and recrystallized using 1:1 proportion of alcohol and water. Yield: 55%

2,7-dimethyl tryptamine (Compound 1)
IR (KBr, cm^{-1}): 3418-3327 (N-H), 3275 (N-H secondary amine), 2993 (C-H Ar.), 1H NMR (400 MHz, CDCl3, δ ppm): 1.275 (s, 2H, NH), 6.906-7.374 (m, 3H, Ar-H), 2.41 (s, 3H, CH_3) 2.45 (t, 3H, CH_3) 2.81 (t, 2H, CH_2) 2.94 (s, 2H, CH_2)7.84 (s, 1H, NH).

6,7-dichloro-2-methyl tryptamine (Compound 2)
IR (KBr, cm−1): 3418-3327 (N-H primary amine), 3130 (N-H Secondary amine stretching), 2941 (C-H Ar.), 748 (C-Cl) 1H NMR (400 MHz, CDCl3, δ ppm): 1.330 (s, 2H, NH), 7.108-7.323 (m, 3H, Ar-H), 2.41 (s, 3H, CH3) 2.78 (t, 2H, CH2) 2.92 (s, 2H, CH2) 8.21 (s, 1H, NH).

5-chloro-2-methyl tryptamine hydrochloride (Compound 3)
IR (KBr, cm−1): 3314 (N-H primary amine), 3099 (C-H Ar.), 748 (C-Cl)

5,7-dichloro-2-methyl tryptamine (Compound 4)
IR (KBr, cm−1): 3417 (N-H primary amine), 2958 (C-H Ar.), 748 (C-Cl)

Biological evaluation

Two methods were used for in-vitro evaluation of anti-Mycobacterium tuberculosis activity viz. Micro Plate Alamar Blue assay (MABA) and Luminescence-Based Low Oxygen-recovery assay (LORA).

Microplate Alamar Blue assay (MABA)

Microplate Alamar Blue assay (MABA) is a sensitive, rapid, inexpensive, and non-radiometric method that offers the potential for screening with or without analytical instrumentation, a large number of antimicrobial compounds against slow-growing mycobacteria. The Alamar Blue oxidation-reduction dye is a general indicator of cellular growth and/or viability; the blue, non-fluorescent, oxidized form becomes pink and fluorescent upon reduction by the reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. Growth can therefore be measured with a spectrophotometer or determined by a visual color change thereby generating a quantitative measure of viability and cytotoxicity.

Alamar Blue susceptibility test

Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water, and subsequent two fold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged innocula were initially diluted 1:2 in 7H9GC, and 0.1 ml was added to the wells. Frozen innocula were initially diluted 1:20 in BACTEC 12B medium followed by a 1:50 dilution in 7H9GC. Wells containing drug only were used to detect auto-fluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20 ml of Alamar Blue solution and 12.5 ml of 20% Tween 80 were added to one B well and one M well, and plates were re-incubated at 37°C. Wells were observed at 12 and 24h for a color change from blue to pink and for a reading of 50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorimeter in bottom-reading mode with excitation at 530nm and emission at 590nm. If the B wells became pink by 24 hours, reagent was added to the entire plate. If the well remained blue or 50,000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition. Visual MICs were defined as the lowest concentration of drug

that prevented a color change. For fluorimetric MICs, a background subtraction was performed on all wells with a mean of triplicate micro-wells. Percent inhibition defined as the lowest drug concentration effecting an inhibition of 90% was considered as MIC.

Luminescence-based low oxygen-recovery assay (LORA)

Screening for new antimicrobial agents is routinely conducted only against actively replicating bacteria. However, it is now widely accepted that a physiological state of non-replicating persistence (NRP) is responsible for antimicrobial tolerance in many bacterial infections. In tuberculosis, the key to shortening the 6-month regimen lies in targeting this NRP subpopulation. Therefore, a high-throughput, luminescence-based low oxygen-recovery assay (LORA) was developed to screen antimicrobial agents against NRP *mycobacterium tuberculosis*.

***In vitro* LORA and conventional aerobic culture assay**

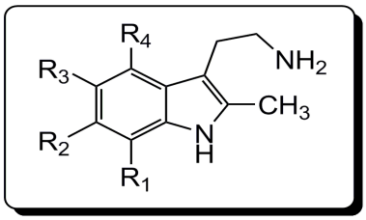
Prior to use, the cultures were thawed, diluted in Middle brook 7H12 broth (Middle brook 7H9 broth containing 1.0 mg/ml casitone, 5.6 g/ml palmitic acid, 5.0 mg/ml bovine serum albumin, and 4.0 g/ml filter-sterilized catalase) and sonicated for 15 seconds. For LORA, the microplate cultures were placed under anaerobic conditions (oxygen concentration less than 0.16%) using an Anoxomat model WS-8080 (MART Microbiology) and three cycles of evacuation and filling with a mixture of 10% H_2, 5% CO_2, and the balance N_2. An anaerobic indicator strip was placed inside the chamber to visually confirm the removal of oxygen. The plates were incubated at 37°C for 10 days and then transferred to an ambient gaseous condition (5% CO_2-enriched air) incubator for a 28 hours "recovery."

The numbers of CFU (determined by subculture onto Middle brook 7H11 agar) during the 10-day incubation did not increase and remained essentially unchanged. On day 11 (after the 28h aerobic recovery), 100 L culture was transferred to white 96-well microtiter plates for determination of luminescence. For the conventional assay, the microplate cultures were placed in an incubator under ambient gaseous conditions (5% CO_2 enriched air) for 7 days and 100 L culture was transferred to white 96-well microtiter plates for determination of luminescence. A 10% solution of *n*-decanal aldehyde (Sigma) in ethanol was freshly diluted 10 fold in PBS, and 100 L was added to each well with an auto-injector. Luminescence was measured in a Victor2 multi label reader (Perkin-Elmer Life Sciences) using a reading time of 1 second. The MIC defined as the lowest drug concentration effecting growth inhibition of 90% relative to the growth for the drug-free controls.

RESULTS AND DISCUSSION

In this paper we have reported design and synthesis of derivatives of tryptamine analogues previously identified through a combined homology modeling and *de novo* lead identification study [11]. Docking study for derivatives of tryptamine analogue (*i.e.* 2-methyl-tryptamine) , substituted at positions 5, 6 or 7- positions with methyl or halo or acetyl or nitro groups molecules revealed maximum ludi scores indicating potential for inhibition of Mtb-MAT enzyme while 6-amino, 5-hydroxy, 7-sulfa amino, 6-nitro, 7-carboxy methyl groups disfavors the anti-TB activity. (Table 1)

Table 1. Docking scores of the designed series of 2-methyltryptamine



Sr. No	R_1	R_2	R_3	R_4	Ludi score
1	-H	-Cl	-F	-H	226
2 (I)*	$-CH_3$	-H	-H	-H	238
3(IV)*	-H	-H	-Cl	-H	240
4(II)*	-Cl	-H	-Cl	-H	246
5	-H	-H	$-NO_2$	-H	261
6(III)*	-Cl	-Cl	-H	-H	269
7	$-COCH_3$	-H	-Br	-H	308

*: molecules selected for synthesis and biological evaluation

These designed molecules showed hydrogen binding interactions with the residues Thr-244, Leu-249, His-16 in the active site pocket. The molecules were selected for synthesis on the basis of their synthetic feasibility, drug like properties and Ludi scores (Table 3) which was calculated by "Energy Estimate 2". The best docking pose of a representative molecule is shown in Fig. 1.

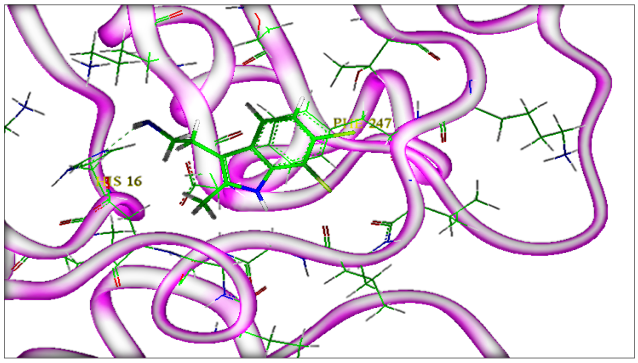
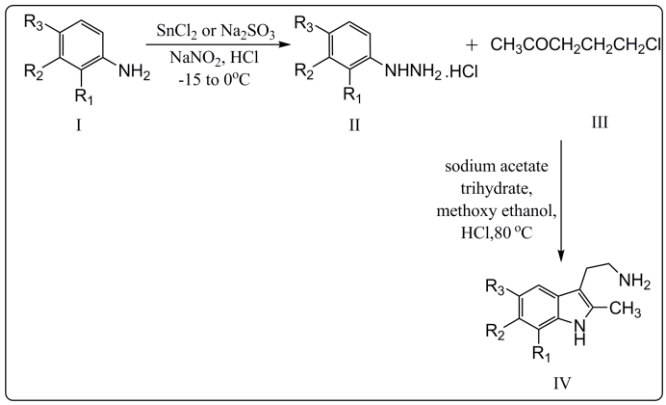


Figure 1. Docking pose for 2-(6,7-dichloro-2-methyl-1H-indol-3-yl)ethanamine. (Compound 2).

Synthesis

From computational study we found halogen substitutions would improve the binding affinity with the receptor Mtb-MAT with Phe-247 with the substituted halogens. Hence we have selected, various halogen containing tryptamine analogs and synthesized using them by using Fischer Indol synthesis [12] as shown in the scheme1.



Scheme1. Synthetic pathway for 2-(5/6/7-substituted-2-methyl-1H-indol-3-yl)ethanamine

Biological evaluation

The compounds were screened against *M. tuberculosis* H37Rv strain using the radiometric BECTEC-12B system (Becton Dickinson Diagnostic Instrument System, Sparks, MD, USA.) until a growth index of 800 to 999 was reached. All compounds were also screened by the Microplate Alamar Blue Assay (MABA) [14] and the Luminescence-based low oxygen recovery assays (LORA) [15, 16]. MABA checks cellular growth and viability of the bacteria while LORA monitors actively replicating as well as non-replicating persistence (NRP) bacteria. Compounds were also checked for cytotoxicity on human virulent cell lines. In both assays, rifampin was the standard drug. The results are summarized in Table 2. Compound 2,7-dimethyl-tryptamine (compound 1) indicated poor inhibitory activity at 128 µM (27%) by LORA assay method and resulted ineffective against mycobacterium species using MABA method. While compound 5,7 dichloro-2-methyl-tryptamine (compound 2) and 6,7-dichloro-2-methy-l-tryptamine (compound 3) are represented the best inhibitors amongst the series of synthesized tryptamine. In which 5,7 dichloro-2-methyl-tryptamine (compound 2) showed inhibition (IC_{50}) on the replicating mycobacterium species in of 50.0 µM concentration using LORA assay method and inhibition of non-replicating mycobacterium species at 95.6 µM using MABA assay method. Compound 6,7-dichloro-2-methyl-tryptamine(compound 3) showed the inhibition of replicating and non-replicating Mtb at 71.8 µM and 92.5 µM using LORA and MABA assay methods respectively. The moderately active compound 5-chloro-2-methyl-tryptamine (compound 4) showed 72% and 56% of inhibition at 128 µM concentration using LORA and MABA methods respectively. The

Table2. The synthetic and activity data of substituted, 2-methyl-5/6/7-substituted tryptamine analogs

Sr. No.	Name of compound	Melting point (°C)	MIC[µM] (% inhibition at 128µM)		Vero cell IC_{50} µM
			LORA	MABA	
1	2,7-dimethyl-tryptamine	149-151°C	> 128 (27%)	> 128 (0%)	>128
2	5,7 dichloro-2-methyl-tryptamine	128-130°C	50.0	95.6	11.6
3	6,7-dichloro-2-methyl-tryptamine	249-252°C	71.8	92.5	11.1
4	5-chloro-2-methyl-tryptamine	128-130°C	> 128 (72%)	> 128 (56%)	86.7

activity of compound 2 and 3 was found to be more than that of compound 1 and 4 may be due to disubstituted halogens.

The tryptamine series was also checked for the cytotoxicity to understand the efficacy of the compounds. Compound 1 found to be non-cytotoxic to the vero cells while compound compound 2 and compound 3 found to be highly cytotoxic to the vero cells and compound IV showed insignificant cytotxicity. Nonetheless the vero cell toxicity was shown much higher for the active compounds 2 and 3 than the inactive compounds 1 and 4. Hence the synthesized compounds could be further modified to enhance the activity and reduce the vero cell toxicity.

CONCLUSION

Methionine S-adenosyltransferase is one of the 256 enzymes deemed necessary for life. The critical role played by this enzyme in both the active and dormant phases of the mycobacterial life cycle makes it an attractive antitubercular target. We have studied few substituted tryptamine analogues having halo substitutions at various positions of the phenyl ring to explore the selective binding profile of Mtb–MAT inhibitors in this paper however detailed study of various substituents on the nucleus are underway. That would help to study the functionally and biochemically important residues at the active site of Mtb–MAT, as there are some differences in the active sites of Mtb and human MAT, which can be exploited to design specific and selective inhibitors of Mtb– MAT.

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