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Novel Nicotine Analogues with Potential Anti-Mycobacterial Activity

Paresh T Gandhi[1], Thimmasandra Narayanappa Athmaram[1*] and Gundaiah Ramesh Arunkumar[2]

[1]Advance Research Products, LLC, 608, 21st Avenue, Paterson, New Jersey 07513, USA

[2]Fenny Pharmacy, 129, Newark Avenue, Jersey City, New Jersey 07302, USA

(* For correspondence: athmabiotech@gmail.com)

Abstract

Tuberculosis (TB) is the second leading lethal infectious disease in the world after acquired immuno deficiency (AIDs). We have developed a series of twenty-five novel Nicotine analogues with de-addiction property and tested them for their activity against *Mycobacterium tuberculosis* (MTB). In an effort to increase the specificity of action and directing nicotine analogues to target MTB, four promising compounds were further optimized via molecular docking studies against the Dihydrofolate reductase of MTB. After lead optimization, one nicotine analogue [3-(5-(3fluorophenyl) nicotinoyl)-1-methylpyrrolidin-2-one] exhibited minimum inhibitory concentration of 1 µg/mL (2.86nM) against M. tuberculosis (H37Rv strain), a human pathogenic strain of clinically significant importance. Pharmacokinetic analysis of [3-(5-(3fluorophenyl) nicotinoyl)-1methylpyrrolidin-2-one] with lowest MIC value via oral route in Wistar rats revealed that at a dosage of 5mg/kg body weight gave a maximum serum drug concentration (C_{max}) of 2.86 µg/mL, T_{max} of one hour and a half-life ($T_{1/2}$) of more than 24 hours and Volume of distribution (Vd) of 27.36L. Whereas the parenteral (intra venous) route showed a C_{max} of 3.37 µg/mL, T_{max} of 0.05 hour, $T_{1/2}$ of 24h and Vd equivalent to 23.18L. The acute oral toxicity and repeated oral toxicity studies in female Wistar rats had an LD_{50}>2,000mg/kg body weight. Our data suggests that Nicotine derivatives developed in the present study has good metabolic stability with tunable pharmacokinetics (PK) with therapeutic potential to combat MTB. However, further *in vivo*

studies for anti-tuberculosis activity and elucidation of mode of action could result in more promising novel drug for treating MTB. To the best of our knowledge this is the first report revealing the anti-mycobacterial potential of Nicotine analogue at potential therapeutic concentrations.

Keywords

Nicotine analogues, mycobacterium, pharmacokinetics, anti-TB activity, Binding assay, deaddiction, MABA

1. Introduction

Tuberculosis (TB) caused by intracellular pathogen *M. tuberculosis* (*M.tb*) remains one of the most prevalent and deadly infectious diseases. About one-third of the world's population are infected with *M. tuberculosis*, and 5 to 10% of infected individuals will develop active TB disease in their lifetime, resulting in approximately 9 million new cases of active disease and 1.5 million deaths per year. In 2010, according to the World Health Organization (WHO) 8.8 million new cases and 1.4 million deaths from the disease were reported. [1] In addition, one third of the world population has latent TB, 10% of whom are expected to develop active TB at some point in their lives. TB is caused by acid-fast bacillus M. tuberculosis that mainly infects the lungs (pulmonary TB), and other organs in the body (extra pulmonary TB) including the liver, brain and kidney to some extent. The conventional first-line treatment of drug-sensitive TB infections consists of a four-drug regimen that includes rifampin, isoniazid, pyrazinamide, and ethambutol. [2,3] The treatment is known for its extended time course of treatment that go upto six months because of which many patients stop taking the medication as soon as their symptoms decrease. This leads to the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) forms of TB further extending the treatment duration upto 18–20 months.[4] The existence of MTB in different physiological states during infection, its pathogenesis and complex biology pose specific challenges for drug discovery against TB. It is estimated that without

improvements one billion people will be newly infected, there will be around 125 million people get sick, and 14 million will die in the next ten years. [5-10] Hence there is huge scope for the development of new chemotherapeutic that are more effective, less complex, cheaper and have fewer side effects. The result of inadequate therapy and poor compliance also contribute significantly for the emergence of MDR, resistant to isoniazid and rifampicin, and XDR strains, resistant to a fluoroquinolone [11]. Consequently, there is an urgent need for the development of novel anti-TB drugs that are effective against both drug sensitive and resistant M. tuberculosis[12]. The potential antimicrobial properties of Nicotine have been previously demonstrated [13]. Nicotine devoid of addiction property can be an excellent compound with medicinal value not only for treatment of neurological disorders like Alzheimer`s disease, Alzheimer's or schizophrenia, Parkinson's disease, depression, attention deficit hyperactivity disorder (ADHD) but also microbial infections.

Several Mycobacterium tuberculosis targets Thiamin Phosphate Synthase [14], new drug targets for resistant strains of Mycobacterium tuberculosis have also been discussed [15]. The use of benzo[b]thiophene-1,1-dioxide (BTD) has been recently reported to have activity against M. tuberculosis in a phenotypic assay [16]. One class of compounds that have garnered recent interest as TB antibiotics are the Imidazole [1,2-a] pyridine carboxamides (IPAs) that have been found to be ideally suited to structure activity-relationship (SAR) studies with MIC values ranging from low micromolar to low nanomolar levels that are not affected by serum [17]. Dihydrofolate reductase inhibitor (DHFR inhibitor) a type of antifolate that inhibits the function of dihydrofolate reductase that is required by rapidly dividing cells to make thymine may be used to therapeutic advantage. Bacteria also need DHFR to grow and multiply and hence inhibitors selective for bacterial vs. host DHFR have found application as antibacterial agents [18]. DHFR catalyzes the production of tetrahydrofolate by transferring a hydrogen ion from NADPH (nicotinamide adenine dinucleotide phosphate) to dihydrofolate, thereby releasing tetrahydrofolate and NADP+. Tetrahydrofolate is essential to the bacteria's survival, and is a cofactor that is needed for the

synthesis of the DNA base thymine. Isoniazid is one antibiotic already used to treat tuberculosis by inhibiting an enzyme enoyl-acyl carrier protein (ACP) reductase InhA crucial for the survival of Mycobacterium tuberculosis [19]. A variation of isoniazid could be designed to avoid resistance, and to inhibit DHFR, thereby targeting bacterial DNA synthesis. While designing anti-Tb drugs it is essential to consider the crucial metabolic pathway for survival of persisting organism and identification of safe compounds for prolonged therapy. The most serious side effects of prolonged therapy are drug-drug interactions, since a compound-specific toxicity profile is usually addressed in the safety studies. This aspect can be studied very early in the drug discovery cascade, thus the focus of the unmet challenge shifts to identification of chemical entities with rapid kill kinetics, since this is fundamental for the quick reduction of the bacterial load and, eventually, sterilization. The use of Molecular docking and Insilico pharmacokinetics study will facilitate identification and lead optimization before extensive studies on medicinal chemistry are started. Even though further medicinal chemistry can, indeed, be driven by MIC-based structure activity relationship patterns, knowledge of the target and or mechanism of action would enable studies on possible mechanisms of toxicity.

2. Results

2.1 Insilico Molecular docking analysis

Nicotine along with its analogues were docked with the most predicted crystal structure of Molluscan acetylcholine-binding protein (AChBP) closely related to alpha 7 nicotinic acetylcholine receptor (α7nAChR), an important neurotransmitter of central and peripheral nervous system. From docking study, the active cavities of AChBP were analyzed and the most active docking site was found to be cavity 3 (Figure 1) that was subsequently used further for docking nicotine analogues. The results of docking study for each of the Nicotine analogues are furnished in table 1.

Fig. 1. Determination of the best cavity for Ligand binding on the receptor molluscan acetylcholine-binding protein (AChBP). Five different cavities (in green) are shown in the left panel. Right panel represents the three dimensional structure of the best active cavity no 3 of AChBP identified for docking molecules.

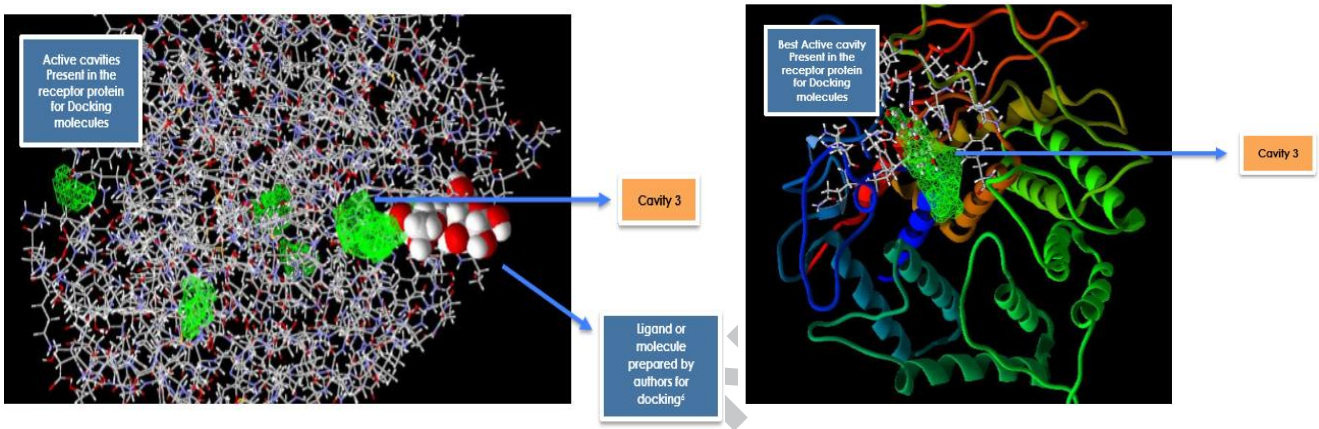


Table 1: Insilico docking study results of Nicotine analogues with molluscan acetylcholine-binding protein (AChBP) with de-addiction property.

Ligand	Docking Score	Mol-Dock Score	Rerank Score	H-Bond energy	No. of H bonds	Interacting residues
Nicotine	-67.0332	-67.7924	-57.5112	-3.97803	2	ARG223(1), IIE217(1)
ARP100101	-124.381	-123.741	-87.6135	-2.5	1	ARG 223(1)
ARP100102	-102.823	-99.1684	-71.7743	-5.13747	3	ARG 223(2), ARG 220(1)
ARP100103	-108.041	-105.487	-78.6417	-14.6255	7	ARG 223(1), ARG 220(4), SER 203(1), CYS218(1)
ARP100104	-108.382	-108.718	-84.2523	-5.0882	3	ARG 223(1), ARG 220(1), SER 66(1)
ARP100105	-107.945	-107.021	-80.637	-7.38651	4	ARG 223(3), SER 66(1)
ARP100106	-108.515	-103.453	-72.5952	-6.82652	4	ARG 223(2), ARG 220(1), SER 215(1)
ARP100107	-102.07	-103.333	-76.3311	-6.52199	3	SER 66(1), LYS 85(1), ASN85(1)
ARP100108	-99.8275	-100.532	-74.1608	-8.5731	6	SER 203(1),GLY262(1), ARG220(2), GLN185(1), LYS 183(1)
ARP100109	-102.633	-95.0548	-71.9546	-4.03186	3	SER 261(1), ARG223(2)
ARP100110	-100.897	-100.8	-77.277	-5.11872	3	ARG 220(1), SER 66(2)

ARP100111	-107.788	-108.897	-81.6242	-7.14768	3	ARG 223(1), ARG 220(1), SER 66(1)
ARP100112	-113.034	-111.802	-89.4765	-9.88698	6	ARG 223(4), ARG 220(1), GLY 262(1)
ARP100113	-106.928	-103.531	-76.5564	-7.87587	5	ARG 223(3), ARG 220(1), SER 66(1)
ARP100114	-103.019	-103.221	-74.0493	-7.16236	4	ASN64 (2), SER 66(2)
ARP100115	-105.921	-106.692	-81.9303	-4.61068	2	ARG 220(1), SER 66(1)
ARP100116	-118.787	-117.107	-89.2821	-6.00452	3	ARG 223(1), SER 66(2)
ARP100117	-107.587	-105.438	-75.838	-10.4887	6	SER203(1), CYS218(1), SER219(2), ARG223(2)
ARP100118	-96.3562	-94.7032	-70.4711	-11.6401	5	GLN 185(1), LYS 183(1), TYR 221(1), SER219(1), ARG 220(1)
ARP100119	-112.126	-110.161	-83.9311	-4.80172	3	ARG 220(1), ARG 223(2)
ARP100120	-124.686	-124.657	-85.7928	-5.80685	3	ARG 223(1), SER 261(2)
ARP100121	-112.709	-112.049	-80.1259	-9.35776	5	ARG 220(1), SER 66(2), ARG 223(1), ASN 64(1)
ARP100122	-98.3354	-92.9773	-67.9322	-4.10609	3	ARG 223(1), SER 261(2)
ARP100123	-109.394	-109.067	-83.5079	-10.1186	5	ARG 220(1), SER 66(2), ARG 223(1), ASN 64(1)
ARP100124	-106.859	-104.113	-77.3977	-4.68828	4	IIE 217(1), ARG 223(3)
ARP100125	-113.416	-106.916	-77.7311	-8.96772	5	ARG220 (1), ARG223(2), SER215(1), LEU 227(1)

2.2 Insilco prediction of Nicotine analogues for Drug likeliness, ADME and toxicity

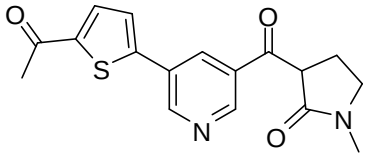
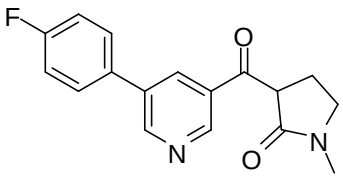
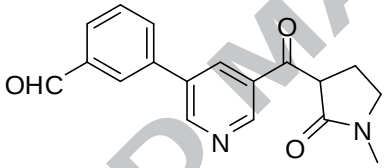
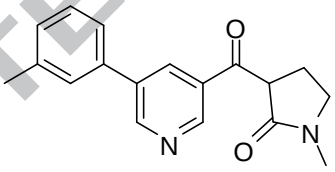
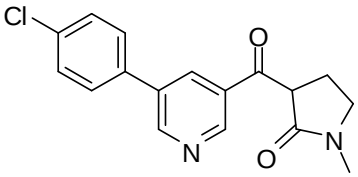
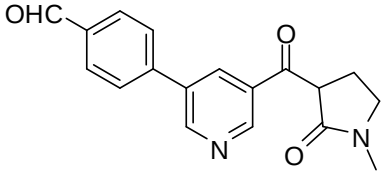
The Insilco analysis of Nicotine analogues designed in the present study qualified all drug likeliness parameters. The molecules were designed for desired Lead-like compounds characteristics with optimized affinity and pharmacokinetic properties by increasing molecular weight and lipophilicity, and are more useful in combinatorial chemistry. In order to Identify and optimize lead compounds as chemical staring points, lead-like rule with low molecular weight (< 350) and ClogP (< 3) and lipophilicity (3-5) were considered. The CMC-like rule was satisfied with a calculated log P values between -0.4 and 5.6 and an average value of 2.52. Molar refractivity was within the qualifying range and was between 40 and 130, with an average value of 97. For the total number of atoms, the qualifying range was between 20 and 70, with an average value of 48 for nicotine analogues in the present study. The World Drug Index (WDI) like rule was also fulfilled by the Nicotine analogues. The reactive functional groups have been selected with no toxicity. In addition, the molecules were predicted to have good human Intestinal

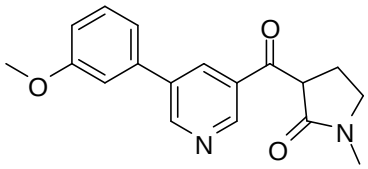
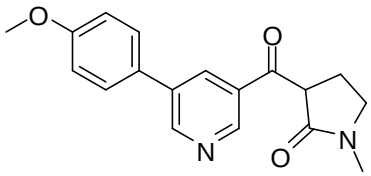
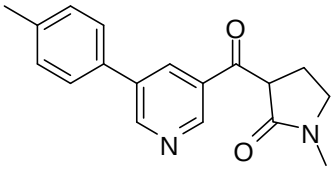
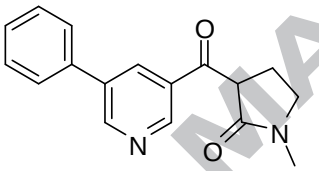
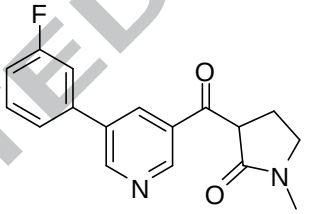
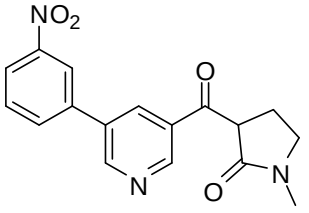
Absorption (HAI %) of 95.6798.33%. The C2C, MDCK & Log kp values predicted show that the molecules are moderately permeable. The predicted plasma protein binding was in the range of 76.09 - 93.83% which indicates better half-life. The blood brain barrier crossing ability is in the range of 0.012 to 0.414 which is poor compared to nicotine (1.15). The molecules appear to be non-carcinogenic in mouse and rat. Considering these Insilco analysis parameters, the Nicotine analogues appeared to be more promising and qualified for further in-vivo evaluation in animal models for their pharmacological action.

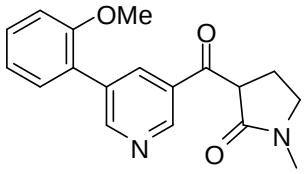
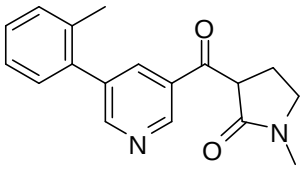
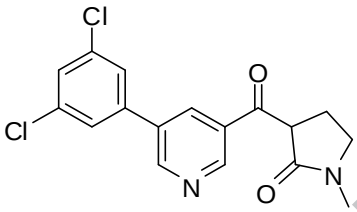
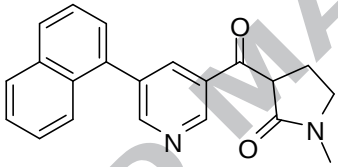
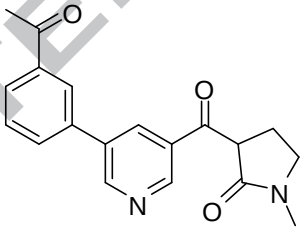
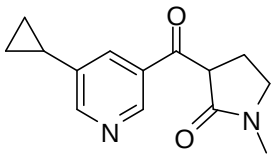
2.3 Synthesis of Nicotine analogues

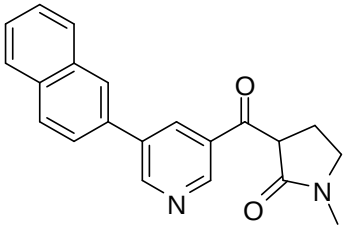
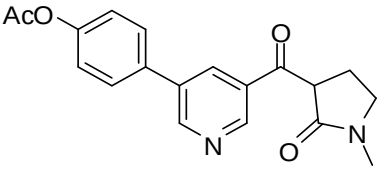
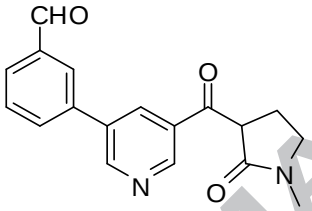
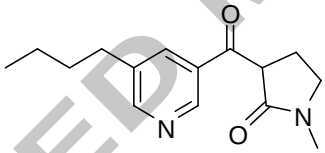
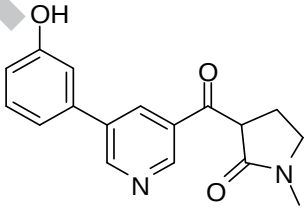
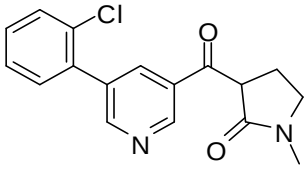
Synthesis of all targeted compounds (Table 2) was confirmed based on spectral and physiochemical analysis of the compounds. The melting points, NMR and IR spectra of all the synthesized compounds were recorded and the physicochemical properties of the nicotine analogues (data available upon request) confirmed the authenticity of the synthesized chemicals. The final synthesized test compounds were analyzed for *in vitro* binding assay before subjecting them for antibacterial screening.

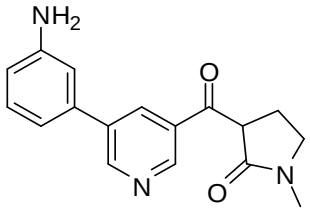
Table 2. Nicotine analogues and their purity levels synthesized in the present study

Compound ID	Compound structure	Purity [Tautomers ratio]
ARP100101		98.94% HPLC (93.43+5.41)
ARP100102		99.47% HPLC (96.42+3.05)
ARP100103		99.74% HPLC (94.07+5.67)
ARP100104		98.21% HPLC (94.14+4.07)
ARP100105		94.68% HPLC (87.72+6.96)
ARP100106		98.93% HPLC (85.05+13.88)

ARP100107		97.30% HPLC
ARP100108		92.50% HPLC (88.30+4.20)
ARP100109		98.39% HPLC (90.37+8.02)
ARP10010		96.50% HPLC (87.17+7.33)
ARP100111		98.25% HPLC (87.89+10.36)
ARP100112		98.13% HPLC (96.70+1.43)

ARP100113		98.13% HPLC (85.74+12.39)
ARP100114		96.36% HPLC (90.09+6.27)
ARP100115		99.18% HPLC (95.93+3.25)
ARP100116		94.58% HPLC (75.54+19.04)
ARP100117		97.12% HPLC [84.20+12.92]
ARP100118		92.72% HPLC (84.83+7.89)

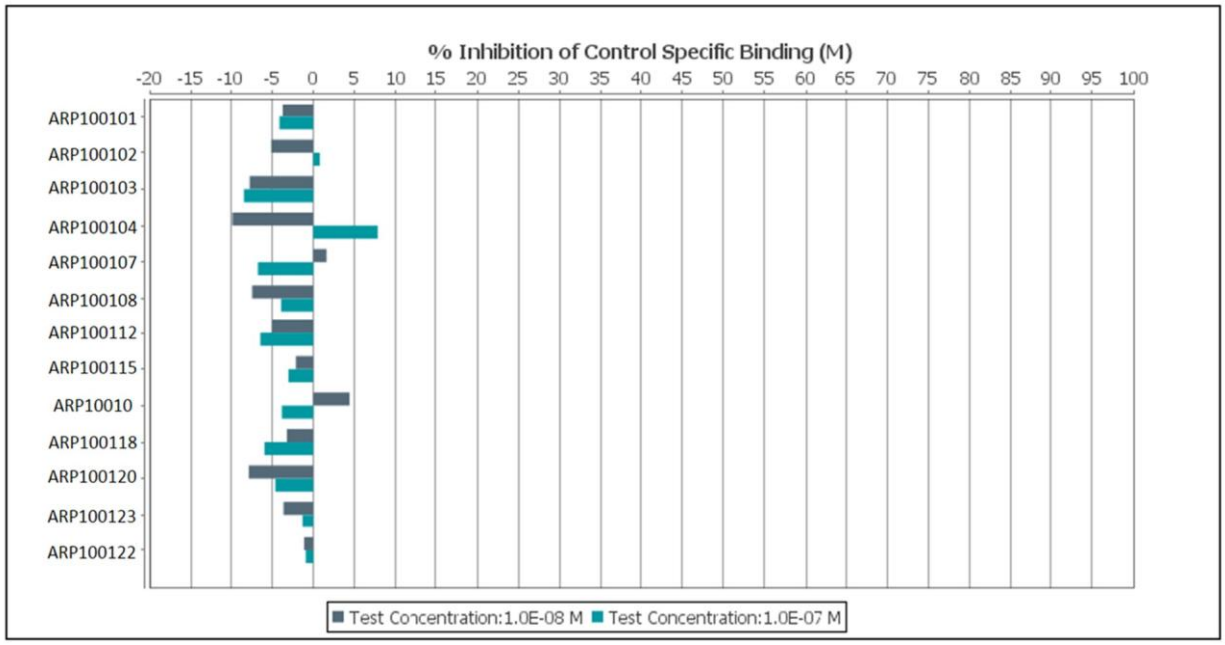
ARP100119		97.40% HPLC (88.06+9.34)
ARP100120		97.08% HPLC (83.55+13.53)
ARP100121		98.97% HPLC (94.35+4.62)
ARP100122		96.64% HPLC (65.73+30.91)
ARP100123		93.59 HPLC (85.95+7.64)
ARP100124		94.97% HPLC (86.72+8.25)

ARP100125		96.51% HPLC [89.80+6.71]

2.4 Invitro pharmacology: Binding assay

As seen in figure.2, all the synthesized Nicotine analogues exhibited very weak to no inhibition to the binding of reference compound (nicotine) to α4β2 radio ligand. Out of thirteen compounds tested, only two compounds ARP 100104 and ARP 100110 exhibited less than 10% inhibition to the reference compound for binding with the α4β2 radio ligand at $1x10^{-8}$M and $1x10^{-7}$M concentration respectively. This data clearly demonstrated that the structural alterations made to the analogues have resulted in the no affinity for α4β2 that significantly contributes towards the deaddiction property of Nicotine.

Figure 2. Binding assay of selected Nicotine analogues. The percent inhibition of nicotine analogues at two different concentrations $1x10^{-7}$ M and $1x10^{-8}$ M towards binding to human recombinant α4β2 agonist radio ligand is represented.



2.5 Invitro Screening of Nicotine analogues against M. tuberculosis H37Rv strain

The bacterial growth in twenty-five compounds treated cultures was observed after 28 days of incubation. The number of colonies on drug-containing slopes and drug free media slopes were counted and as recorded as 3+ for confluent growth (difficult to count the actual number) and 2+ more than 100 colonies. The average number of colonies obtained for the drug containing slopes indicated the number of resistant bacilli. Tests were performed using two different strains of mycobacteria such as; Mycobacterium tuberculosis which is isolated from a clinical sample of sputum and Mycobacterium tuberculosis H37RV (ATCC 25177). The test compounds did not exhibit anti TB activity against Mycobacterium tuberculosis strain isolated from clinical sample, whereas the results of Mycobacterium

tuberculosis H37RV (ATCC 25177) were shown in table 3. In control (drug free media) the growth was recorded as 3+, which indicates confluent growth (difficult to count the number of bacterial colonies). In positive control (Isoniazid and

Rifampicin) no bacterial growth was observed, which indicates that growth was completed inhibited (indicated as 0). As seen in table 3, Nicotine analogues ARP100104, ARP100115 and ARP100120 had less growth (2+) indicating their anti-TB potency at both concentrations i.e. at 50 and 200 µg/mL tested. The other test compounds ARP100103, ARP100106, ARP100108, ARP100112, ARP100117 and ARP100119 and

ARP100124 the growth was scored as 2+, indicating that the compounds have mild anti TB activity at 200 µg/ml concentration. However, no inhibitory activity was found for these compounds at 50 µg/ml concentration. Other compounds screened did not showed activity at the concentrations tested. The positive control drugs exhibited better bacterial inhibition and the negative control had no inhibition.

Table 3: Preliminary screening of Nicotine analogues against Mycobacterium tuberculosis. Two different concentrations of the nicotine analogues were tested against Mycobacterium. The growth observed after the incubation period is interpreted as 3+ for confluent growth or too numerous colonies to count, 2+ to represent >100 colonies and 0 to indicate no colonies)

Name of the test drug	**Concentration**	**Bacterial growth**	
		Replicate 1	Replicate 2
Control (drug free)	0 µg/mL	3+	3+
Isoniazid	0.2 µg/mL	0	0
Rifampicin	40 µg/mL	0	0
ARP100101	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100102	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100103	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100104	50 µg/mL 200 µg/mL	**2+** **2+**	**2+** **2+**
ARP100105	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100106	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100107	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100108	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100109	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100110	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100111	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100112	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100113	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+

ARP100114	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100115	50 µg/mL 200 µg/mL	**2+** **2+**	**2+** **2+**
ARP100116	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100117	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100118	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100119	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100120	50 µg/mL 200 µg/mL	**2+** **2+**	**2+** **2+**
ARP100121	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100122	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100123	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+
ARP100124	50 µg/mL 200 µg/mL	3+ 2+	3+ 2+
ARP100125	50 µg/mL 200 µg/mL	3+ 3+	3+ 3+

2.6 Lead optimization of Nicotine analogues for targeting Mycobacterium tuberculosis

Four nicotine analogues obtained after lead optimization (table 4) based on the most active compound structure ARP100115 selected from preliminary MABA assay had better interaction with the target protein of Mycobacterium tuberculosis Dihydro folate reductase (Fig. 3). All modified structures exhibited negative Mol doc score that ranged from -119 to -136 (table 5).

Table 4. Structures of four lead optimized Nicotine analogues designed to target Mycobacterium tuberculosis Dihydro folate reductase (Black color: Original compound ARP100115, Blue color: Lead optimized compounds of ARP 100115 labelled as B-E)

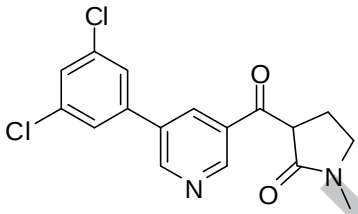
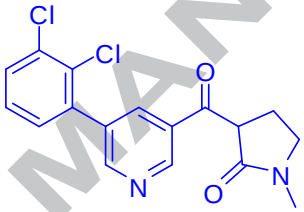
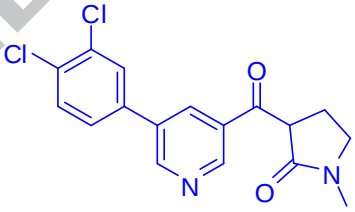
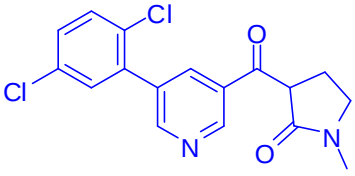
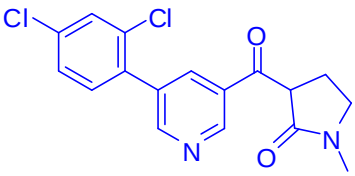
Compound ID	Compound structure
ARP100115	
B	
C	
D	
E	

Figure. 3. Interaction of Mycobacterium tuberculosis Dihydro folate reductase structure with selected ARP100115 (A) and other lead optimized Nicotine analogues (B-E), The site of interactions is shown. F: Nicotine structure alone

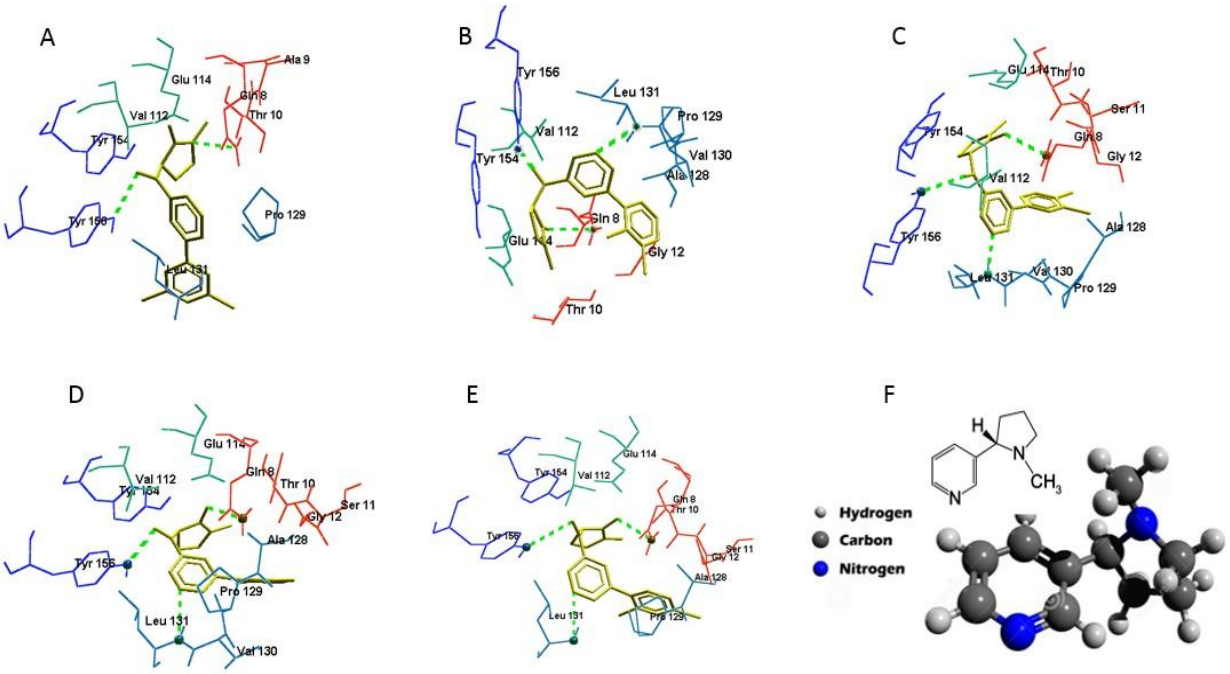


Table 5. Insilico docking study results of Nicotine analogues with Dihydro folate reductase of Mycobacterium tuberculosis

Compound ID	Mol.Doc Score	
	Cavity 1	Cavity 2
ARP100115	-130.87	-120.157
B	-134.057	-119.715
C	-128.88	-121.498
D	-136.303	-120.448
E	-133.209	-120.115

2.7 Lead optimized Nicotine analogues shows enhanced activity against Mycobacterium tuberculosis

All the four compounds after lead optimization were evaluated for their enhanced bactericidal activity using MABA assay. As evident from table 6, one compound (compound no E) had a drastic improvement in its MIC value. This compound was effective at 1µg/mL (2.86nM) and showed anti-bacterial activity comparable to the standard drug isoniazid (MIC 1.45nM). Another compound (compound no B) also exhibited a comparable MIC of 3.25µg/mL (9.3nM).

Table 6. Invitro evaluation of anti-Mycobacterial activity of lead optimized Nicotine analogues (compound 1-5) in comparison to first line anti-Tb drug Isoniazid

Compound	MIC (nM)
Isoniazid	1.45
ARP100115	28.63
Compound B	9.3
Compound C	178.97
Compound D	286.36
Compound E	2.86

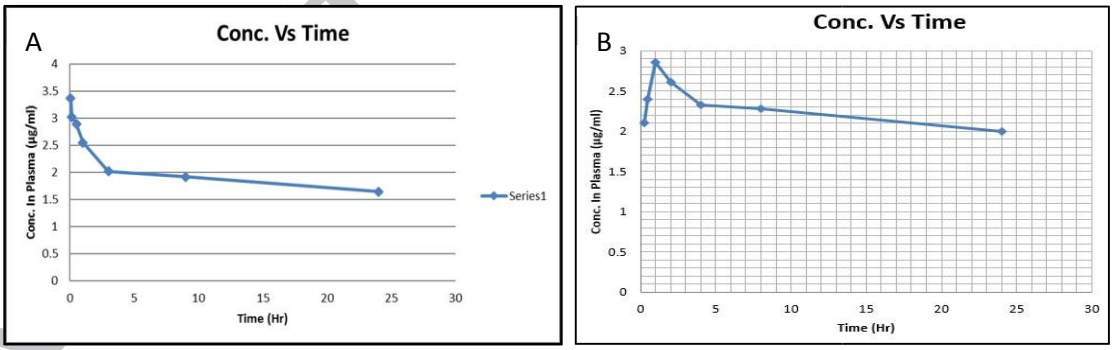
2.8 Pharmacokinetics parameters of Lead optimized Nicotine analogue in Wistar rats

The lead optimized Nicotine analogue that exhibited better activity against Mycobacterium tuberculosis was further studied in wistar rats for Cmax, T max, T1/2 Volume distribution, oral toxicity, repeated oral toxicity. The results are summarized in table 7. The t1/2 of Nicotine analogue was found to be greater than 24 h (Fig. 4) whereas the Nicotine has a biologic half-life of only 2 hours in plasma.

Table 7. Evaluation of Pharmacokinetics parameters in wistar rats

Pharmacokinetic parameter	Oral route (5mg/kg)	Parenteral route/i.v (5mg/kg)
Cmax	2.86 µg/mL	3.37 µg/mL
Tmax	1 hour	0.05h
$T_{1/2}$	More than 24h	24h
Volume distribution (Vd)	27.36L	23.18L
Toxicity (LD50)	>2000mg/Kg	-
Repeated toxicity (LD50)	>2000mg/kg	-

Figure 4. Plasma concentration of Nicotine analogue via Oral (panel A) and Parenteral administration (panel B) in wistar rats



3. Discussion

Nicotine along with its analogues were docked one by one with the most predicted crystal structure of Molluscan acetylcholine-binding protein (AChBP) [20] closely related to alpha 7 nicotinic acetylcholine receptor (α7nAChR) that is considered to be an important human neurotransmitter of central and peripheral nervous system. Ach is produced by enzyme choline acetyl transferase & the action of Ach is mediated by Ach receptors and nicotinic AchRs (nAchRs). We designed structures derived from Nicotine by retaining their beneficial activities but without addiction property so that therapeutic potential of Nicotine can be completely exploited without any undesired properties. The binding assay of the synthesized Nicotine analogues revealed very weak to no inhibition to the binding of reference compound (nicotine) to α4β2 radio ligand. A panel of 25 nicotine analogues used in the present study have previously shown activity against a series of gram positive and gram negative bacteria[21,]. With this background, a preliminary screening of same compounds on standard H37Rv strain of mycobacterium was performed that revealed three promising compounds having marginal activity. To precisely target mycobacterium tuberculosis, a key enzyme Dihydro folate reductase was selected for docking study and the compound ARP100115 was further modified to get four new structures via molecular docking study. Often the presence of hetero atoms or groupings imparts preferential specificities in their biological responses. Several hetero-cycle compounds have been previously reported for application including antimicrobial, anti-inflammatory, anti-HIV, anti-malarial, anti-tubercular, antibacterial and anticonvulsant [22-26] activities. Hence efforts were made in the present study with functional groups at various positions to optimize the nicotine analogues for anti-mycobacterial activity. The low MIC exhibited by one of the lead optimized compound (Compound E) is in par with that of the reference standard drug, Isoniazid known to target InhA of Mycobacterium tuberculosis. The preliminary findings from the present study suggests that DHFR could be an alternate target for anti-mycobacterial action. As the culture method followed in the preliminary screening was labor intensive and required more

time, an alternative robust screening method, MABA was employed for evaluating the lead optimized compounds. MABA being a widely accepted test, the findings were conclusive for assessing the Invitro activity of the compounds. The lead optimized Nicotine analogue that exhibited low MIC value against Mycobacterium tuberculosis had acceptable pharmacokinetics range suggesting that the compound is safe to be administered into animals. It's noteworthy to mention that the t 1/2 of Nicotine analogue was found to be greater than 24 h whereas the Nicotine has a biologic half-life of only 2 hours in plasma. This would significantly support the administration of nicotine analogue as single dosage per day in contrast to the existing multiple doses of standard drug regimen.

Nicotine is extensively metabolized to a number of metabolites by the liver. Six primary metabolites of nicotine have been identified. Quantitatively, the most important metabolite of nicotine in most mammalian species is the lactam derivative, cotinine. In humans, about 70– 80% of nicotine is converted to cotinine. This transformation involves two steps. The first is mediated primarily by CYP2A6 to produce nicotine-Δ1' (5')-iminium ion, which is in equilibrium with 5'-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehyde oxidase. Nicotine iminium ion has received considerable interest since it is an alkylating agent and, as such, could play a role in the pharmacology of nicotine [27]. Although on average about 70–80% of nicotine is metabolized via the cotinine pathway in humans, only 10–15% of nicotine absorbed by smokers appears in the urine as unchanged cotinine [28]. There is a general agreement that the long treatment duration for anti-TB drug regimens contribute to the development of drug-resistant *M. tuberculosis* strains, causing the failure of chemotherapy and continuous *M. tuberculosis* transmission. The Nicotine analogue developed in the present study by itself or in combination with the existing cocktail regimens appears to shorten the treatment length and can potentially reduce *M. tuberculosis* transmission and emergence of drug-resistant strains.

4. Conclusion

Our studies demonstrate that Nicotine analogues designed in the present study have inhibitory effect against *M. tuberculosis* at 1µg/mL (2.86nM) concentration. The results are significant with respect to the reference drug thus suggesting that this class of compounds may be selectively target MTB growth. The pharmacokinetics study of the most promising compound developed in the present study confirms the safety in animals. Future studies pertaining to the use of lead optimized nicotine analogue as first-line or second-line anti-TB drugs to evaluate its activity against various drug-susceptible and drug–resistant *M. tuberculosis* clinical isolates in animal model is in progress. Considering the tolerability and activity, efforts are being made towards clinical evaluation of Nicotine analogue to assess their potential to treat tuberculosis.

5. Materials and Methods

5.1 Insilco docking study of Nicotine derivatives

With an aim to use Nicotine devoid of addiction property in treatment of neurological disorders like Alzheimer`s disease, Parkinson disease etc., 25 molecular structures of Nicotine analogues with altered structures were designed based on alpha 7 nicotinic acetylcholine receptor (α7 nAChR) that plays a key role in the treatment of diseases like Alzheimer's or schizophrenia, Parkinson's disease, depression, attention deficit hyperactivity disorder (ADHD) and nicotine addiction. The X-Ray crystallographic structure of molluscan acetylcholine-binding protein (AChBP) was used as template to model the ligand binding domain of a closely related structure to alpha subunits of the α7nAChR1. The Nicotine analogue structures were converted to 3D structures using Argus lab software and the energies were minimized before docking. Molegro Virtual Docker was used in the present study to dock the molecules. After docking the molecules or ligands with the receptor, the affinity of the molecules or ligands is obtained in

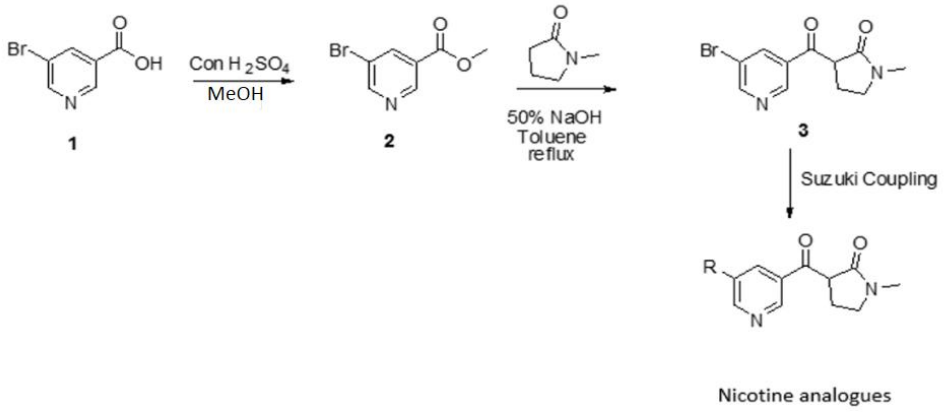
terms of Mol Dock Score or Rerank Score were derived. Mol Dock showed better overall performance in docking simulations when compared with other software.

5.2 Insilco prediction of Nicotine analogues for Drug likeliness, ADME and toxicity

The prediction for drug-likeness, ADME and toxicity was done using PreADMET software, for better understanding the likeliness of the new chemical entities to be selected as lead molecules for further in vivo studies. All the nicotine analogues designed in the present study were analyzed for drug-likeness criteria that includes CMC like rule, Lead like rule, MDDR rule, Rule of 5 and WDI rule.

5.3 Synthesis of Nicotine analogues:

Twenty-five compounds as listed in table 1 were synthesized from Chemveda Life Sciences India Pvt Ltd, Hyderabad, India as described previously [29-30] . The purity and authenticity of the compounds were verified by 1H and NMR analysis using 300MHz Bruker NMR spectrometer. The general steps of chemical synthesis are as shown in the following schematic diagram:



5.4 In vitro pharmacology: Binding Assays

To confirm the de-addiction property of the designed Nicotine analogues, thirteen compounds were randomly selected and subjected to Invitro binding assay as previously described [31] using human recombinant α4β2 agonist radio ligand derived from neuronal SH-SY5Y cells. Nicotine was used as reference compound in the binding assay. For each compound, two different concentrations viz, $1x10^{-8}$M and $1x10^{-7}$M were tested in duplicates. The obtained results were expressed as percent inhibition of control specific binding in the presence of the test compound. The IC50 values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (nH) were determined by nonlinear regression analysis of the competition curves generated with mean replicate values using Hill equation and analyzed using software developed at Cerep (Hill software).

5.5 Anti-TB compounds

Rifampicin (R) and Isoniazid (H) were from Sigma-Aldrich, MO, USA. Rifampicin stock solution (4000µg/mL) and working solutions (500µg/mL) were prepared in absolute methanol. Isoniazid working solution (500µg/mL) was prepared freshly in sterile distilled water.

5.6 Invitro screening of Nicotine analogues against M. tuberculosis H37Rv strain

For preliminary screening of all twenty-four synthetic compounds, standard Mycobacterium tuberculosis culture isolated from a clinical sample of sputum and Mycobacterium tuberculosis H37RV (ATCC 25177) were cultured in Lowenstein-Jensen (LJ) medium (HI-Media [M162] Laboratories, Mumbai, India) prepared as per the manufacturers recommendation as described below.

5.7 Media preparation and inoculation

Lowenstein-Jensen (LJ) medium (HI-Media (M162) Laboratories, Mumbai, India) was prepared following the manufacturer's instructions. Briefly 600 mL of LJ media base was prepared and sterilized by

autoclaving at 121^{o} C (15 psi) for 15 minutes. One liter of whole egg emulsion prepared aseptically was added to sterile LJ media base to obtain the complete LJ media to which the test compounds and standard drugs were added. Appropriate concentrations of the test compounds and standard anti-tuberculosis drugs were added aseptically to L-J fluid before inspissation. A stock solution of the drugs was prepared based on the potency of the drug in sterile distilled water for isoniazid, and in absolute methanol for rifampicin. The solutions of isoniazid and rifampicin were sterilized by membrane filtration. Similarly, all the test compounds were dissolved in DMSO and sterilized by filtration. Suitable working dilutions of the compounds were made. The media containing bottles were placed in a slanted position in an inspissator and the medium was coagulated by heating at 85^{o} C for 50 minutes. After inspissation, the media bottles were incubated at 35 - 37^{o} C for 24 hours as a check for bacterial sterility. The sterile medium was stored in the refrigerator at 2-8^{o} C until used. The pure mycobacterium culture was adjusted to concentration of 1 mg/ml of tubercle bacilli in sterile water in sterile McCartney bottle have standard drugs or the synthetic nicotine compounds in LJ media. A loop-full of culture suspension was inoculated on to media slopes under aseptic condition. The inoculated tubes were incubated at 37^{o}C. Presence or absence of bacterial growth were recorded at 28 days and 42 days post incubation.

5.8 Lead optimization of selected Nicotine Analogues for Mycobacterium tuberculosis Dihydro folate reductase target

In order to enhance the effectiveness of the compounds, one nicotine analogue (ARP100115) showing better activity was selected based on the preliminary screening against MTB and the structure was further optimized by docking against Mycobacterium tuberculosis key enzyme Dihydro folate reductase structure. Four modified structures with highest possible negative Mol doc score and more number of interactions with the target protein were selected for synthesis and were labelled as (B-E).

5.9 In vitro Microplate Alamar Blue assay (MABA) test on optimized Nicotine derivatives

Four lead compounds that showed promising bacterial inhibitory effects on TB culture in LJ medium were selected and further optimized by docking against Mycobacterium tuberculosis Dihydro folate reductase. The anti-mycobacterial activity of compounds was assessed against M. tuberculosis using MABA. The minimum Inhibitory concentrations (MICs) of synthesized compounds were carried out by MABA test [32]. The stock solutions and working concentrations of the Nicotine analogues were made in Dimethyl sulfoxide (DMSO) and were tested on the microbial cultures grown as per standard MABA method. Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. 96 wells plate received 100 µl of the Middlebrooks 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/mL. Plates were covered and sealed with parafilm and incubated at 37^{o}C for five days. After incubation, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. Development of blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. Standard positive controls containing appropriate antibiotics were used in each test.

5.10 Pharmacokinetic study of Nicotine analogue

5.10.1 Animal and Ethic Statement

Female Wistar rats were housed under standard laboratory conditions with temperature 23.4-28.8^{o}C, relative humidity 54-78 %, with a 12 h light and 12 h dark cycle. The animals were housed individually in standard polypropylene cages with stainless steel top grill having facilities for pelleted food and drinking water in bottle; paddy husk was used as bedding material and changed at least twice a week.as per the OECD guideline for the testing of chemicals, Acute oral toxicity study (Acute toxic class method) guideline no.423, adopted on December 17, 2001. This study was carried out in compliance with the

recommendations in the Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the Institutional Animal ethics committee and procedures were used to minimize animal pain and suffering.

5.10.2 Acute oral toxicity studies of selected compounds

Lead optimized Nicotine analogue was tested for its acute oral toxicity in female wistar rats. Limit test at a dose of 2000mg/kg body weight were carried out with six female rats per group. The compound was prepared as suspension of 0.5% carboxy methyl cellulose (200mg/ml concentration and administrated as a single oral dose to overnight fasted (16-18 hours) rats at the dose volume of 10ml/kg body weight.

Mortality, clinical observations, body weights and gross necropsy findings were evaluated.

5.10.3 Repeated dosage toxicity study

To assess the systemic toxic potential of the Compound when administered by gavage to rats, repeated Dose (28 day) Oral Toxicity Study of lead compound was carried out in wistar rats for evaluating the possible health hazards likely to arise from repeated exposures over a limited period of time. This study was conducted in compliance with the OECD Principles of Good Laboratory Practice [ENV/MC/CHEM (98) 17: 1997] and as per OECD Guideline No. 407 for Testing of Chemicals, "Repeated Dose 28-day Oral Toxicity Study in Rodents" adopted on July 27, 1995, in accordance with the Standard Operating Procedures. Based on the acute toxicity data that showed an LD50 > 2000 mg/kg, a limit dose of 1000 mg/kg as the high dose, followed by 500 and 100 mg/kg for mid and low dose, respectively were used for repeated dose study (six animals per each group). The test substance was prepared daily on weight /volume basis using sesame oil. Homogeneity of the test item was maintained during gavage administration by stirring and mixing manually using a glass rod. The test item was administered daily once for 28 and 29 consecutive days in males and females, respectively. Similarly, the vehicle was administered to vehicle control group rats for 28 or 29 consecutive days. The dose volumes were

calculated based on the weekly mean body weights recorded during the first day of each week of the study period. The dose volume of administration was 10 ml/kg body weight. Animals were observed for toxic signs and pre-terminal deaths. Other parameters *viz* neurological/functional examination like movements, respiration, tactile response, response to nociceptive stimuli, tail pinch, locomotor activity, Head shaking, grasping strength, equilibrium test, functional observations for sensory reactivity to visual, auditory and receptive stimuli were assessed.

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Conflict of interest

The authors declare no conflict of interest

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Graphical Abstract