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ARTICLE TYPE

Novel isoniazid-amidoether derivatives: Synthesis, characterization and antimycobacterial activity evaluation

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Abstract: A series of isoniazid-amidoether derivatives was synthesized and screened for their antimycobacterial activity *in vitro* and *in vivo*. Most of the compounds exhibited potent *in vitro* activity against *Mycobacterium tuberculosis* H37Rv strain with MIC_{99} values ranging from 0.39 to 3.125 µM. Five compounds were equally potent to the reference compound isoniazid. The most active compound **3b**, when evaluated for *in vivo* activity exhibited mild reduction in the bacillary load in lungs. However it showed better effect in spleen. All the compounds were also evaluated for their cytotoxicity against THP-1 cell line and no toxicity was observed up to 50 µM concentrations. The calculated ADMET parameters for the compounds validated good pharmacokinetic properties, confirming that these compounds could be used as a template for the development of new anti-tuberculosis agents.

Introduction

Tuberculosis (TB) is an infectious disease mainly caused by *Mycobacterium tuberculosis.* It is a leading cause of death worldwide, infecting about 9.2 million people and kills approximately 2.0 million people annually.[1] After the discovery of many effective anti-TB drugs during the 1950s and 1970s, such as ethambutol, isoniazid, pyrazinamide, rifampicin and streptomycin, there was a drastic decline in the number of TB cases, especially in developed countries. However, since 1980s, the number of TB cases throughout the world has been increasing rapidly due to the emergence of multi-drug resistant tuberculosis (MDR-TB), extensively drug-resistance tuberculosis (XDR-TB) and more recently totally drug resistance tuberculosis (TDR-TB).[2] The MDR, XDR and TDR forms of tuberculosis are more often dreadful and difficult to treat.[3-7] TDR-TB strain has been shown to be resistant to all the first line, second line and third line anti-TB drugs. The situation has become more complicated by the co-infection with human immunodeficiency virus (HIV) as they are more likely to be infected with TB due to their weak immune system.[8,9] However, in recent years several new drug candidates or repurposed drugs namely gatifloxacin, moxifloxacin, rifapentine, TMC207, OPC67683, PA824, linezolid, PNU100480, AZD5847 and SQ109 etc have been developed and some of them are also in the advance stages of clinical trials for the treatment of tuberculosis.[10-18] Only one new drug bedaquiline has been recently approved by FDA for its use in drug resistant tuberculosis.[19-21]

Due to the global impact of this devastating disease, there is an urgent need for the development of new derivatives with promising antimycobacterial activities. Several different approaches such as targeting bacterial virulence, high-throughput screening (HTS), structure-based drug discovery (SBDD), chemical modifications of the known drugs and combinatorial chemistry have been explored to search novel biologically important molecules.[22-24] Among all these strategies, molecular modification approach has been found to be very promising and several drugs available in the market have been developed by using this strategy. Molecular modification is a chemical change in a molecule with the aim to enhance its pharmaceutical, pharmacokinetic or pharmacodynamic properties.

Isoniazid (INH), a first-line anti-TB drug is one of the most effective agents used for the treatment of *Mycobacterium tuberculosis* infection since 1952.[25,26] It is a pro-drug which is activated by the mycobacterial catalase-peroxidase enzyme known as KatG. The activated form reacts with the coenzyme NADH to form isonicotinic acyl-NADH complex,[27,28] that binds with the enoyl-acyl carrier protein (ACP) reductase InhA, which is involved in elongation of fatty acids the mycolic acid synthesis.[29] Thus isoniazid inhibits the synthesis of mycolic acid, required for the mycobacterial cell wall. INH is metabolized in the liver and forms compounds such as hydrogen, which are toxic to the central nervous system and other organs.[30,31] Because INH is an important drug in the therapeutic arsenal for TB treatment, efforts are being made to develop new INH derivatives with greater activity, lower toxicity and fewer side effects than INH.[32-41]

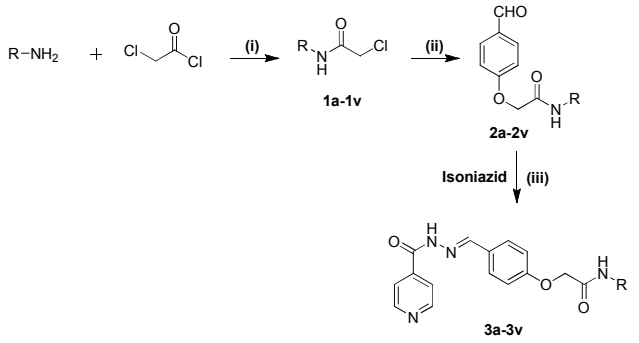
Several recent reports indicate that the incorporation of hydrophobic moieties into the basic structure of INH can enhance penetration of the drug into the highly lipophilic cell wall of the bacterium. Moreover, by functionalizing the hydrazine group of isoniazid and retaining its activity can avoid the toxicity and other severe problems related to the inactivation of isoniazid by the enzyme *N*-acetyltransferase-2 (NAT2).

Several research groups have introduced amido ether functionality in biologically active molecules and the resulting hybrids

exhibited good biological activities.[42-46] Encouraged by the previous studies and in continuation of our efforts towards the synthesis of new anti-tuberculosis agents,[47-51] we proposed to attach amido ether linkage with isoniazid and evaluated for their *in vitro* and *in vivo* anti-TB activity and toxicity against THP-1 cell lines.

Chemistry

For the synthesis of isoniazid-amidoether conjugates (**3a-3v**), firstly different 2-(4-formylphenoxy)-*N*-substituted acetamides (**2a-2v**) were synthesized as shown in scheme 1. The synthesis started with the reaction of 2-chloroacetyl chloride and an appropriate amine in the presence of triethylamine as a base in dichloromethane, that leads to the formation of chloroacetamide derivatives (**1a-1v**) in quantitative yields (scheme 1).[52] These derivatives were then treated with *p*-hydroxy benzaldehyde in the presence of K_2CO_3 as base and potassium iodide as catalyst to give 2-(4-formylphenoxy)-*N*-substituted acetamides (**2a-2v**). These compounds with a free carboxyl group were then condensed with isoniazid in ethanol/H_2O as a solvent to get the desired compounds (**3a-3v**) in good yields. All the synthesized compounds were purified by column chromatography and characterized spectroscopically.



a, R = C_6H_5
b, R = 4-MeC_6H_4
c, R = 4-EtC_6H_4
d, R = 4-*i*-PrC_6H_4
e, R = 2-FC_6H_4
f, R = 3-FC_6H_4
g, R = 4-FC_6H_4
h, R = 2-ClC_6H_4
i, R = 3-ClC_6H_4
j, R = 4-ClC_6H_4
k, R = 2-BrC_6H_4
l, R = 3-BrC_6H_4
m, R = 4-BrC_6H_4
n, R = 4-$OCH_3C_6H_4$
o, R = 4-$NO_2C_6H_4$
p, R = 2-Pyridyl
q, R = 3-Pyridyl
r, R = $CH_2C_6H_4$-4-Me
s, R = $CH_2C_6H_4$-4-OCH_3
t, R = $CH_2C_6H_5$
u, R = $CH_2CH_2C_6H_5$
v, R = 1-Naphthyl

Scheme 1: Reagents and conditions: (i) Et_3N, DCM, 0 ºC to RT, 6-10 h, 70-90%; (ii) 4-hydroxybenzaldehyde, K_2CO_3, KI, RT, 10-15 h, 60-85%; (iii) EtOH/H_2O, RT, 6-10 h, 70-90%

Biological Activity

In vitro anti-tuberculosis activity

A stock culture of *M. tb* H37Rv (ATCC 27294) was grown to Abs_{600nm} of 0.2 in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80, 0.2% glycerol and albumin/NaCl/glucose (ADS) complex. The culture was diluted 1:1000 in 7H9-based medium before aliquoting 50 µL into each well of a 96-well plate. Compounds were dissolved in DMSO (Sigma) to make stock solutions of 50 mM. Compounds (100 µL solution) were added to the first row of the 96-well plate at a final concentration 100 µM. 2-fold serial dilutions were made and 5 dilutions of each compound (50 µM-0.195 µM) were tested for anti-mycobacterial activity. The compounds were diluted 1:1 by the addition of 50 µL of 1:1000 diluted cultures. Row 6 and 12 of the 96-well plates were control with no compound. The plates were incubated at 37 °C and the MIC_{99} values were read macroscopically using an inverted plate reader after 14 days. MIC_{99} is defined as the minimum inhibitory concentration of the compound required for 99% inhibition of bacterial growth. Each measurement was repeated thrice.

In vivo anti-tuberculosis activity

The most active compound **3b** from the series was selected for *in vivo* antituberculosis activity evaluation. For activity evaluation, pathogen-free Balb/c mice of either sex (25-30 g) were procured from the Division of Laboratory Animals, Central Drug Research Institute, Lucknow, India. The animals were maintained in a BSLIII animal facility at University of Delhi South Campus, New Delhi and routinely cared according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. All the experimental protocols included in this study were reviewed and approved by the Institutional Animal Ethics Committee (Ref No. 1/IAEC/AKT/Biochem/UDSC/14.10.2011).

Mice were challenged with *M. tb.* H37Rv bacilli by respiratory route in inhalation chamber (Glascol Inc., USA) pre-calibrated to deliver approximately 1000 bacilli per animal in the lung by using frozen stocks of *M. tb.* H37Rv with their CFU pre-determined. Mice were euthanized on the day after infection to determine the number of CFU implanted in the lungs. Following two weeks of infection, mice were divided into different groups − untreated, DMSO, isoniazid (25 mg/kg), rifampicin (10 mg/kg), compound **3b** (25 mg/kg, 50 mg/kg, 100 mg/kg) and therapy was initiated.

All drugs were administered once daily, five days per week, in a maximum volume of 0.175 mL by oral gavage. Before initiating the chemotherapy, the infection in the animals was verified by euthanizing a group of animals (N = 5) at 2 weeks post infection followed by pathological observation and an enumeration of the bacillary load in the lungs and spleen. Mice (N = 5 each group) were euthanized by CO_2 asphyxiation after three weeks, six weeks and ten weeks time points post therapy and monitored for gross pathological observations and bacillary load. For bacterial enumeration, mice were dissected and lungs and spleens were aseptically removed and homogenized in saline. Appropriate dilutions of the homogenates were plated in duplicates onto MB7H11 agar and the plates were incubated at 37 °C for 3–4 weeks followed by an enumeration of colonies. The results were expressed as log_{10} CFU per organ.

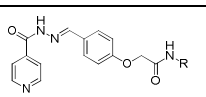
At 3 weeks time point, a bacillary load of 6.38 and 4.19 log_{10} CFU was measured in the lungs and spleens of untreated animals. The disease continued to persist and at 6 weeks post infection also, a bacillary load of 5.91 and 3.68 log_{10} was recorded in the lungs and spleens of untreated animals, respectively. At 10 weeks post infection, the disease further progressed with a bacillary load of 6.04 and 4.02 log_{10} in the lungs and spleens of untreated animals, respectively.

Results and Discussion

The isoniazid amidoether derivatives (**3a-3v**) synthesized under scheme 1 were evaluated for antitubercular activity against *Mycobacterium tuberculosis* H37Rv using isoniazid as reference

compound. The minimum inhibitory concentrations (MIC_{99}) of the compounds are shown in table 1. Five compounds (**3b**, **3n, 3q**, **3r** and **3s**) showed excellent activity with MIC_{99} = 0.39 µM similar to isoniazid. In general, compounds having electron donating groups at *para* position of the benzene ring such as **3n** (C_6H_4-4-OMe), **3r** ($CH_2C_6H_4$-4-Me), **3s** ($CH_2C_6H_4$-4-OMe) and **3b-3d** (alkyl groups) were found to be more active in comparison to other compounds. Electron withdrawing groups lead to slight decrease in activity, as compound **3o** (4-NO_2) and **3v** (1-naphthyl) showed weak activity (MIC_{99} = 1.56-3.125 µM) compared to other compounds.

Table 1: *In vitro* anti-tuberculosis activity of isoniazid-amidoether derivatives

Comp	 R	MIC_{99} (µM)	THP-1 (µM)	ClogP
3a	C_6H_5	0.78	>50	2.30
3b	4-MeC_6H_4	0.39	>50	2.80
3c	4-EtC_6H_4	0.39-0.78	>50	3.33
3d	4-*i*-PrC_6H_4	0.39-0.78	>50	3.73
3e	2-FC_6H_4	0.78	>50	2.10
3f	3-FC_6H_4	0.78	>50	2.70
3g	4-FC_6H_4	0.78	>50	2.70
3h	2-ClC_6H_4	0.78	>50	2.42
3i	3-ClC_6H_4	0.78	>50	3.27
3j	4-ClC_6H_4	0.39-0.78	>50	3.27
3k	2-BrC_6H_4	0.78	>50	2.54
3l	3-BrC_6H_4	0.78	>50	3.42
3m	4-BrC_6H_4	0.78	>50	3.42
3n	4-$OCH_3C_6H_4$	0.39	>50	2.38
3o	4-$NO_2C_6H_4$	1.56-3.125	>50	2.60
3p	2-Pyridyl	0.78	>50	1.63
3q	3-Pyridyl	0.39	>50	1.63
3r	$CH_2C_6H_4$-4-Me	0.39	>50	2.83
3s	$CH_2C_6H_4$-4-OCH_3	0.39	>50	2.25
3t	$CH_2C_6H_5$	0.78	>50	2.33
3u	$CH_2CH_2C_6H_5$	0.78	>50	2.54
3v	1-Naphthyl	1.56-3.125	>50	3.48
Isoniazid		0.39		-0.668

After 3 weeks of treatment with isoniazid, CFU in the lungs of infected animals was reduced by 1.22 log_{10} while after 6 weeks it showed a further CFU reduction in the bacillary load by 2.59 log_{10} and a furthermore reduction of 3.38 log_{10} at 10 weeks when compared with the animals in the untreated group (Fig. 1A). In spleens also, isoniazid treatment resulted in a sharp reduction of bacillary load with a 2.66 log_{10} reduction at 3 weeks when compared with the animals in the untreated group (Fig. 1B). At 6 weeks and 10 weeks time point, no bacilli were recovered from the spleens. Whereas, after 3 weeks of treatment with rifampicin, the CFU in the lungs of infected animals was reduced by 1.93 log_{10} while at 6 weeks it showed a further CFU reduction in the bacillary load by 2.8 log_{10} when compared with the animals in the DMSO group. After 10 weeks of rifampicin administration, no bacilli were recovered from the lungs of the infected animals (Fig. 1A). In spleens also, rifampicin treatment resulted in a marked reduction in the bacillary load with a 1.76 log_{10} and 3.31 log_{10} reduction at 3 weeks and 6 weeks, respectively when compared with the animals in the DMSO group. At 10 weeks time point, no bacilli were recovered from the spleens (Fig. 1B).

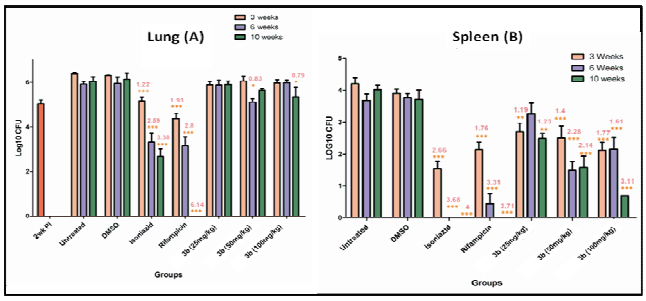


Fig. 1: Bacterial enumeration in lungs (A) and spleens (B) of animals belonging to different groups was carried out at various time points following infection. Bacillary load was measured as described in the materials and methods. Various groups are indicated. PI represents post infection. ***, ** and * mean statistically significant with P value <0.001, <0.01 and <0.05 respectively. The numbers in red represent the log_{10} CFU value by which a reduction in the particular case was observed when compared with the CFU value in the case of control animals.

Compound **3b** was administered at 25 mg/kg, 50 mg/kg and 100 mg/kg concentrations for evaluating the efficacy of this compound. When compound **3b** was given at a low concentration of 25 mg/kg, it exhibited no control towards disease progression even up to 10 weeks as was evident from a comparable bacillary load observed in the case of lungs of animals treated with compound **3b** as compared to DMSO treated animals. When the concentration of compound **3b** was increased to 50 mg/kg or 100 mg/kg still no significant influence of the compound towards pulmonary control of the disease was observed hence, in spite of increasing the concentration up to 100 mg/kg, no significant chemotherapeutic effect was demonstrable under our experimental conditions. At 50 mg/kg concentration of the compound at 6 weeks time point and at 100 mg/kg concentration at 10 weeks time point, there was a slight reduction in the pulmonary bacillary load of compound **3b** treated animals as compared to DMSO treated animals, however, the statistical significance was not of a very high order and the fact that it did not register any significant chemotherapeutic effect even at 100 mg/kg concentration shows that the compound had no significant intrinsic chemotherapeutic value towards the control of pulmonary tuberculosis at least in the murine model as observed in our experiments. Figure 2 depicts the lungs of infected mice either untreated or treated with isoniazid, rifampicin and compound **3b** (100 mg/kg) for 10 weeks.

After the pulmonary infection, the settlement of bacilli in the lung tissues is followed by hematogenous spread which provides another point of control towards protection from the disease hence, the influence of compound **3b** was also measured on the reduction of bacillary load in spleens.

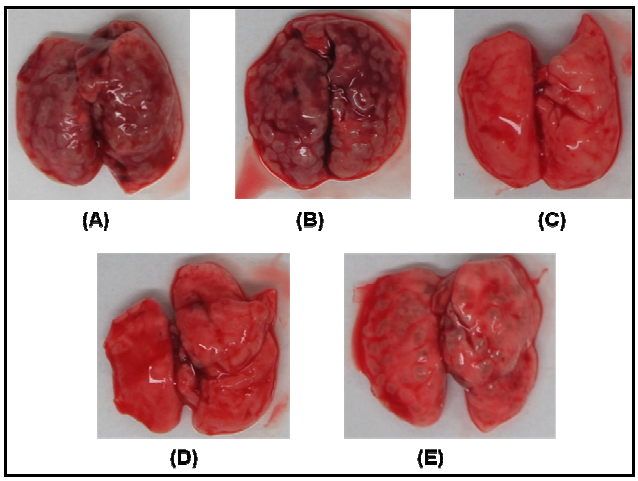


Fig. 2: Lungs of infected mice after treatment for 10 weeks either untreated or treated with different compounds: (A) Untreated, (B) DMSO, (C) Isoniazid, (D) Rifampcin and (E) Compound **3b** (100 mg/Kg).

It was observed that at 25 mg/kg concentration of compound **3b**, there was a reduction in the splenic bacillary load by a value of 1.19 $\log_{10}$ CFU at the end of 3 weeks of chemotherapy when compared with the DMSO treated animals, however, treatment up to 10 weeks further reduced the splenic bacillary load only marginally with 1.23 $\log_{10}$CFU reduction in the bacillary load at this time point (Fig. 1B). With increased concentration of compound **3b** to 50 mg/kg, a more substantial effect was observed on the reduction of splenic bacillary load. We observed that at this concentration, at the end of 3 weeks of chemotherapy the splenic bacillary load exhibited a reduction by 1.4 $\log_{10}$ CFU. Further continuation with the chemotherapy at this dose i.e. up to 6 weeks and 10 weeks resulted in more significant reduction in the splenic bacillary load which was 2.28 $\log_{10}$ CFU and 2.14 $\log_{10}$ CFU less compared to the bacillary load in the spleens of DMSO treated animals. When the concentration of compound **3b** was doubled up to a concentration of 100 mg/kg, it resulted in a more prominent bacillary load reduction in spleens and as compared to 1.4 $\log_{10}$ CFU reduction observed with 50 mg/kg concentration, this concentration resulted in a 1.77 $\log_{10}$ CFU reduction in the splenic bacillary load at the end of 3 weeks of treatment. Further administration of the compound to 10 weeks resulted in a very significant 3.11 $\log_{10}$ CFU reduction in the splenic bacillary load compared to the bacillary load in the spleens of DMSO treated animals (Fig. 1B). All the compounds were further examined for toxicity in THP-1 cell line. The compounds were found to be non-toxic up to a concentration of 50 μM the highest concentration tested (Table 1). It was not possible to test toxicity at higher concentrations due to solubility limitations.

In silico ADMET prediction

We have predicted the ADME properties of test compounds and reference compound isoniazid for the pharmaceutically relevant properties to assess the drug likeness and pharmacokinetic properties. The Qikprop v3.5 (Schrödinger, Inc., New York, NY, 2012) was used for the evaluation of some important absorption, distribution, metabolism and elimination (ADME) parameters and its permissible ranges are listed in the Tables 2 and 3.

Table 2: Prediction of Lipinski's 'Rule of 5' for the active test compounds[a]

Comp	mol_MW (> 500)	Donor HB (<5)	Accpt HB (<10)	QPlogPo /w (<5)	Rule of Five (<4)
3a	374.39	2	7.25	3.139	0
3b	388.42	2	7.25	3.347	0
3c	402.45	2	7.25	3.621	0
3d	416.47	2	7.25	4.272	0
3e	392.38	2	7.25	3.329	0
3f	392.38	2	7.25	3.343	0
3g	392.38	2	7.25	3.577	0
3h	408.84	2	7.25	3.576	0
3i	408.84	2	7.25	3.619	0
3j	408.84	2	7.25	3.904	0
3k	453.29	2	7.25	3.89	0
3l	453.29	2	7.25	3.93	0
3m	453.29	2	7.25	4.078	0
3n	404.42	2	8	3.362	0
3o	419.39	2	8.25	2.814	0
3p	375.38	2	8.25	2.832	0
3q	375.38	2	8.75	2.59	0
3r	402.45	2	7.25	3.206	0
3s	418.45	2	8	3.334	0
3t	388.42	2	7.25	3.106	0
3u	402.45	2	7.25	3.593	0
3v	424.45	2	7.25	4.46	0
INH	137.14	3	4.5	-0.646	0

[a] All values calculated by QikPropv 3.5 and the explanations of the descriptors are given in the text.

Table 3: Calculated ADMET properties

Compd	[a]PercentHuman OralAbsorption (>80% high, <25% poor)	[a]QPPCaco nms^{-1} (<25 poor, >500 great)	[a]QPlogBB (-3.0-1.2)	[a]QPPMDCK (<25 poor, >500 great)	[a]QPlogKhsa (-1.5 to 1.5)	[a]PSA (7.0–200.0)	[a]#rotor (0–15)
3a	91.362	373.559	-1.66	170.656	0.195	107.913	8
3b	92.58	373.506	-1.702	170.63	0.345	107.914	8
3c	94.137	371.023	-1.799	169.404	0.451	107.915	9
3d	100	370.991	-1.819	169.388	0.584	107.917	9
3e	93.157	407.696	-1.523	282.274	0.225	107.448	8
3f	92.52	371.547	-1.559	306.467	0.236	107.923	8
3g	93.934	373.725	-1.557	308.982	0.235	107.912	8
3h	95.15	437.44	-1.428	411.765	0.291	106.704	8
3i	94.178	373.705	-1.516	420.73	0.304	107.912	8
3j	95.849	373.693	-1.515	421.519	0.304	107.913	8
3k	100	454.177	-1.408	463.239	0.308	107.1	8
3l	95.997	373.715	-1.509	452.415	0.326	107.912	8
3m	96.867	373.649	-1.509	453.167	0.327	107.913	8

3n	92.665	373.326	-1.757	170.541	0.201	116.206	9
3o	72.954	44.669	-2.925	17.186	0.146	152.833	9
3p	86.926	265.849	-1.82	118.154	-0.01	119.081	8
3q	83.384	202.307	-1.976	87.949	-0.116	120.825	8
3r	86.5	189.976	-1.803	165.521	0.135	109.005	9
3s	87.243	189.824	-1.858	165.336	-0.011	117.291	10
3t	85.908	189.805	-1.763	165.453	-0.016	108.999	9
3u	89.562	210.386	-1.86	165.226	0.088	109.683	10
3v	100	451.89	-1.601	209.643	0.528	105.996	8
Isoniazid	66.893	277.461	-0.843	123.742	-0.752	81.355	2

[a] Calculated using QikProp v 3.5. Range/recommended values calculated for 95% known drugs

All the compounds were prepared in neutralized form for the calculation of pharmacokinetic properties by Maestro Build module and LigPrep, saved in SD format. In the present study, the test compounds showed good preliminary test of the drug-likeness based on Lipinski's rule of 5 showing zero violation of the rule, proving all the test compounds to be orally active. The descriptor QPPCaco indicating Caco-2 cells permeability, a model used for the gut–blood barrier, showed good values for all the test compounds. Similarly the values of descriptor model such as number of rotatable bond (#rotor) and polar surface area (PSA), used as an indicator of bioavailability for the test compounds lie in expected ranges. Further, the prediction for human serum albumin binding (QPlogKhsa) and QikProp descriptor for brain/blood partition coefficient (QPlogBB) and the blood-brain barrier mimic MDCK cell permeability (QPPMDCK) show satisfactory predictions for all the test compounds (Table 3).

Conclusion

We have synthesized 22 isoniazid-amidoether conjugates and evaluated for their *in vitro* and *in vivo* anti-tuberculosis activity. Most of the compounds exhibited potent activity *in vitro*. When tested for *in vivo* activity the compound exhibited mild activity in case of lungs. However, the influence of the compound on the replication of the pathogen in spleen was far superior as compared to the influence on the lungs. We do not have clear explanation for this observation at present however it might suggest that the bio-availability of these compounds could be the possible reason for the better activity profile in the case of spleen. However, translation of this speculation into real evidence would require further experiments. All the compounds were found to be nontoxic up to 50 μM concentration against THP-1 cell line. Also, the compounds exhibited good pharmacokinetic properties and follow the Lipinski's rule of 5. Thus we believe that these compounds can be considered as a possible lead for the development of new anti-tuberculosis agents.

Acknowledgments

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Notes and references

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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Novel isoniazid-amidoether derivatives: Synthesis, characterization and their antimycobacterial activity evaluation

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Graphical Abstract