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Computer assisted drug repurposing: Anti TB activity in non antibiotics

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TB drug development is a formidable challenge at all times. As a part of our anti TB drug development program, we recently identified sertraline as a potential anti TB agent with an MIC-1.6µg/mL against MtbH37Rv. Sertraline is a popular marketed anti-depressant drug, hence we have planned to use the pharmacophoric signature of sertraline for virtual screening of drug database.We have performed shape based virtual screening of Drug Bank 5.0 database using vROCS software. Twenty chemically diverse drug molecules have been selected using Tanimoto COMBO score (TC>1.0). This gives chlormidazole, an obsolete anti-fungal drug as a Hit molecule with TC=1.67 (ranked 6th) and shows excellent anti TB activity (MIC 1.6µg/mL). It has been subjected to lead optimization studies and various chlormidazole analogues have been synthesized (**1b-9b**). They have been further screened for *in vitro* anti TB activity studies. Our results have successfully identified chlormidazole (**5b**) with MIC=1.6 µg/mL which can be repurposed as anti TB drug. Compounds **1b** and **4b** show selective potent anti TB activity. The compound **8b** shows selective antibacterial activity against *Staphylococcus aureus* (gram+ve) bacteria. Compounds **2b**, **3b**, **5b** and **6b** show potent anti TB activity along with antifungal activity against *Aspergillus niger*. In conclusion, our study has successfully identified potent anti-TB activity in Chlormidazole and its analogues.

Keywords: Shape based virtual screening, Tanimoto combo score, MABA assay, MTT assay, Axenic assay

TB is the oldest and dreadful diseases existing on earth and considered as one of the major global health problems. The persistence of this disease has its roots in the resilience of mycobacterium to antibiotics/host-immunity, patient non-compliance and public ignorance. Steady raise in multidrug resistant infections and paltry presence of novel anti TB drugs in the pipeline is posing a severe threat to humanity. Anti TB drug discovery and development has several bottlenecks. For generations, TB remained as a "poor people's" disease. Globally, TB patient care is largely managed by their respective governments, hence no assured financial gains. Secondly, clinical trials for TB drugs are hampered by ethical issues. Lastly, an antibiotic drug, when listed for TB therapy, loses its ground for treating other diseases. Financial and time constraints made TB drug research a non-productive task for many pharmaceutical companies. Bedaquiline and Delamanid, are the only two new drugs entered into market recently due to persistent concerted efforts of TB Alliance (a consortium formed in the year 2000 by industry, academia and NGOs).

Repurposing is using old or obsolete drugs for alternate clinical applications. As the drugs were already FDA approved, if they found beneficial in the treatment of other diseases, these can be brought to clinics with fairly less efforts. In drug repurposing, Phase I and dosing trials, which take years to complete and needs millions of dollars in funding can be bypassed. Secondly, a lot of information on the chemistry, SAR and toxicity profile of old drugs is available, which immensely helpful in the structure optimization studies. Repurposing old/obsolete drugs for 'neglected' diseases is recently gaining much attention. Thioridazine was reported to possess clinically useful antitubercular activity against multi-drug/extremely drug resistant tuberculosis[1]. Recently we found potential anti TB activity in several non-antibiotic molecules[2], in which sertraline (antidepressant) had shown Minimum inhibitory concentration (MIC)-1.6µg/mL against *M. tuberculosis* (Mtb)H37Rv. Antimicrobial activity was earlier reported in antidepressant and psychotropic drugs[3-6]. A few earlier studies on SSRIs ascertained their synergism with existing antibiotics and inhibition of efflux pump mediated drug resistance[7-8].This inspired us to further search for anti TB activity in non-antibiotic drugs. As it is virtually impossible to screen all available drugs for anti TB activity, we chose to employ computational tools for virtual

screening of the existing drugs database. Sertraline, though showed potential anti TB activity, it is widely used as an antidepressant with a global market worth 86 million dollars[9]. Hence we planned to use the molecular pharmacophoric signature of sertraline for screening the drug database.

Shape based virtual screening has been successful in predicting bioactivity in structurally diverse chemical molecules using pharmacophoric features of a drug molecule. 3D Shape based screening method-Rapid Overlay of Chemical Structures (ROCS) is a very effective technique in identification of bioactivity in a chemically diverse set of compounds. This technique uses descriptors, which compare molecules based on their molecular shapes, by assessing atom-centered overlapping Gaussians and calculating the maximal intersection of the volume between molecules[10]. In this method, shape derived from the low energy 3D conformer of a compound is used for virtual screening. During the screening process, this query shape is used to check fitment of 3D conformers present in test compound library. Molecules fitting the query shape are expected to interact with similar molecular targets. The similarity of the two molecules is assessed by Gaussian parameter and the volume of overlap on the available heavy atoms. Concurrently, complementary properties of chemical functionalities are also calculated. vROCS Combo Score (CS) is evaluated by measuring extent of overlap in both shape (Tanimoto score) and chemical functionality (Scaled Colour Tanimoto Score). The CS ranges from 0 to 2, and 2 represents identity. Molecules with combo score (CS>1.0) are considered as hit molecules in the vROCS screening process.

Results and Discussion

Computational studies

The *in silico* screening library for this study was generated from Drug Bank online repository. The database used in this study contained a set of 5783 molecules (after excluding proteins and biologicals). Structures of individual drug molecules were checked for correctness and all salt forms were converted to unionized form to facilitate proper ionization during conformer generation.

Sertraline, which was found to have potent anti TB activity in one of our earlier studies was selected as a model for this study. For thorough probing of conformational space, OMEGA 2.4.6 software was used to generate conformations for sertraline. A total of 12 conformers (Figure 1) were obtained and are used for building shape based model. Conformers were also generated for the compounds in drug bank database.

vROCS 3.1.2 module (Open Eye Scientific Software: Santa Fe, NM, 2014. www.eyesopen.com.) was used for the preparation of query (Figure 2) and performing virtual screening. The query for our search, sertraline was prepared by the default protocol and all important structural features including hydrophobic, hydrogen bond donor/acceptor and aromatic rings are considered. The fitment was evaluated by using COMBO Score (CS).vROCS Combo Score (CS) is evaluated by measuring the extent of overlap in both shape (Tanimoto score) and chemical functionality (Scaled Colour Tanimoto Score). The CS ranges from 0 to 2, and 2 represents identity. Molecules with combo score (CS>1.0) are considered as hit molecules in the vROCS screening process. Hits (Table I) were selected based on the diversity in structural/ biological activity and Combo score (CS>1.0). Among the hit molecules, Chlormidazole (ranked 6th in 5783 molecules (Figure 3) was selected as it is one of the oldest and obsolete drug.

Synthesis of compounds 1b-9b

The compounds 1b-9b were synthesized (Scheme I) by benzylation of benzimidazoles/2-methyl benzimidazoles/benzotriazole with appropriate benzyl chlorides. The products were purified by either crystallization or column chromatography and further identified by using spectral techniques.

Biological screening

All the compounds (1b-9b) were screened for Anti microbial and Anti tubercular activity.

Antibacterial activity

The synthesized compounds (1b-9b) were tested for antibacterial activity against *Staphylococcus aureus* bacteria (Gram +ve) and *Escherichia coli* (Gram –ve) by broth dilution method and agar well diffusion method (Table II). Among the synthesized compounds, compound **8b** showed activity against *Staphylococcus aureus* with an MIC of 10µg/mL. Compounds **2b** and **3b** possessed activity against *Staphylococcus aureus* and *Escherichia coli* with an MIC of 15µg/mL. Minimum bactericidal concentrations (MBC) were also determined for potent compounds and they were found to be at 15µg/mL (**8b**) and 30µg/mL (**2b**and **3b**) respectively.

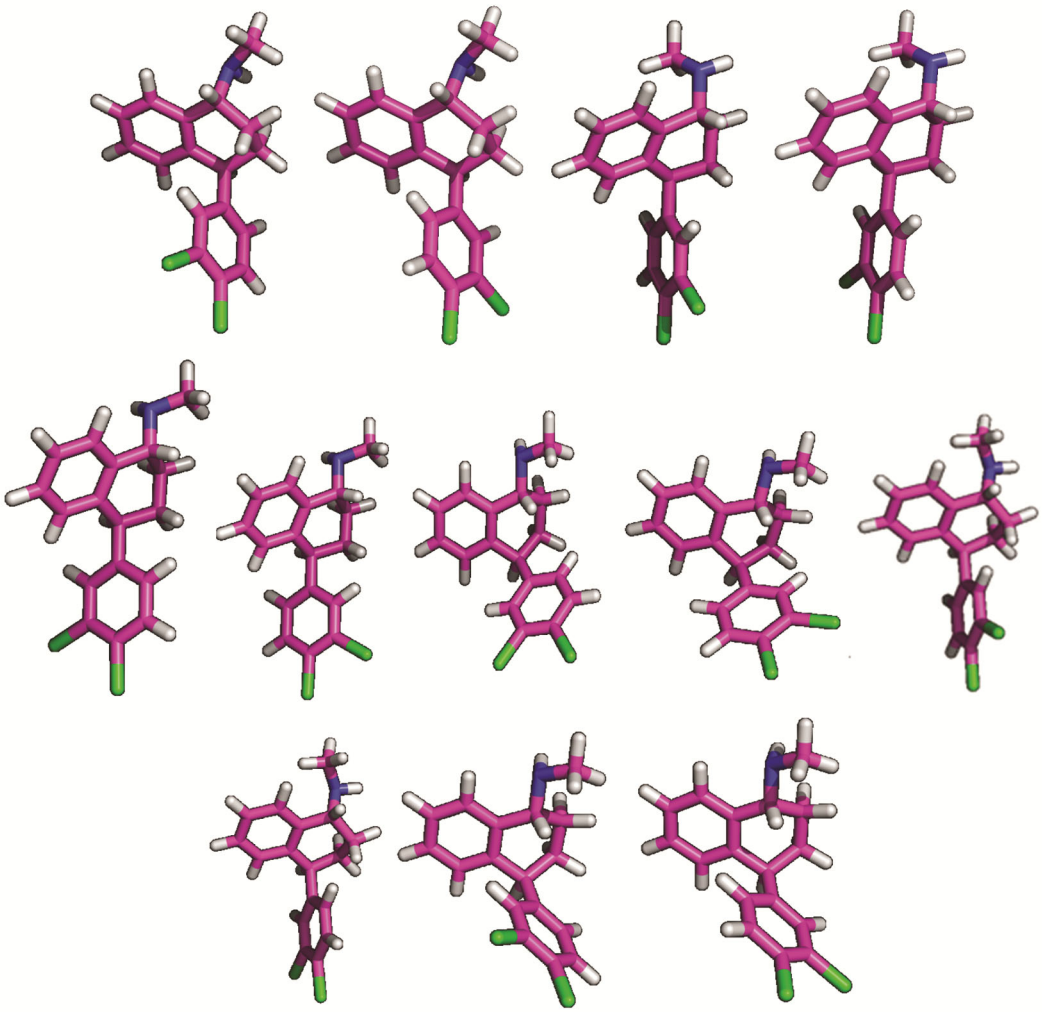


Figure 1 — Conformers generated for Sertraline by Omega 2.4.6 software

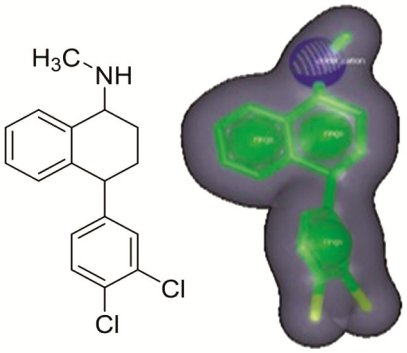


Figure 2 — Model generated by vROCS for sertraline

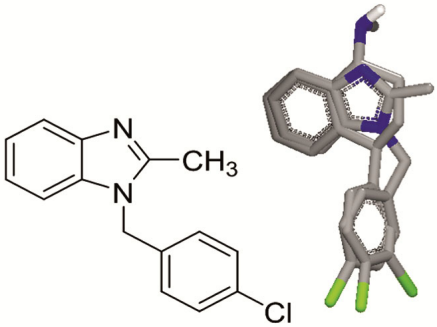


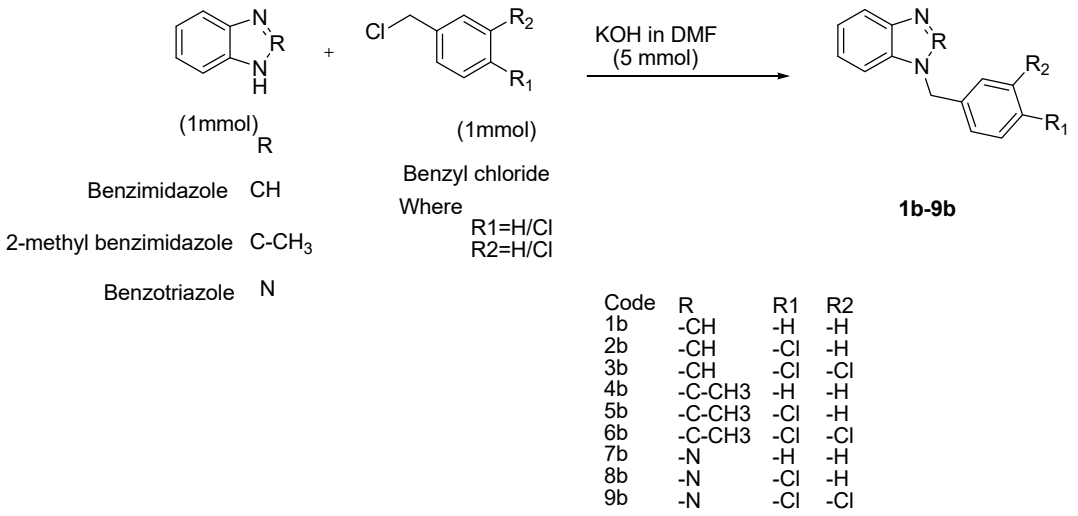
Figure 3 — The possible bioactive conformation of Chlormidazole matching sertraline

Antifungal activity

In the antifungal activity studies (Table II), none of the compounds showed activity against *Candida albicans* but compounds **2b, 3b, 5b** (Chlormidazole) and **6b** showed activity against *Aspergillus niger* with zone of inhibition in the range of 20mm-32mm at 100µg/mL concentration.

Table I — Hit molecules obtained by vROCS taking sertraline as a query molecule

S.No	Molecule	Tanimotto Combo	Shape Tanimotto	Colour Tanimotto	Rank	Therapeutic activity
1	Sertraline	1.93	0.95	0.98	1	Antidepressant
2	Amitriptyline	1.30	0.72	0.57	2	Antidepressant
3	Dosulepin	1.298	0.731	0.567	3	Antidepressant
4	Cyclobenzaprine	1.289	0.729	0.560	4	Antidepressant
5	Nortriptyline	1.286	0.714	0.572	5	Antidepressant
6	Chlorimidazole	1.253	0.850	0.400	6	Antifungal
7	Fluoxetine	1.231	0.660	0.571	7	Antidepressant
8	Adrafinil	1.176	0.751	0.425	8	CNS stimulant
9	Lamotrigine	1.145	0.786	0.358	9	Antidepressant, Anti convulsant
10	Imipramine	1.143	0.673	0.471	10	Antidepressant
11	Trimipramine	1.137	0.704	0.433	11	Antidepressant
12	Benactyzine	1.136	0.661	0.475	12	Antidepressant
13	Desipramine	1.132	0.661	0.471	13	Antidepressant
14	Zotepine	1.131	0.652	0.479	14	Antipsychotic
15	Prothipendyl	1.126	0.708	0.418	15	Antipsychotic
16	Clomipramine	1.123	0.653	0.471	16	Antidepressant
17	Proquazone	1.120	0.757	0.363	17	Anti-inflammatory
18	Tramadol	1.112	0.702	0.410	18	Narcotic analgesic
19	Duloxetine	1.111	0.607	0.505	19	Antidepressant
20	Medifoxamine	1.104	0.761	0.343	20	Antidepressant



Scheme I — Scheme for the synthesis of benzylated benzimidazole/benzotriazoles **1b-9b**

Anti TB activity

The synthesized compounds (1b-9b) were tested for antiTB activity against MtbH37Rv bacilli strain by MABA Assay and percentage inhibition values were determined by MTT Assay and Axenic assay using MtbH37Ra bacilli strain (Table III). Among the synthesized compounds, all the compounds from **1b-6b** showed excellent anti TB activity with an MIC 3.12-1.6 µg/mL, percentage inhibition in the range of 80%-86% by MTT Assay and 70%-76% inhibition by Axenic assay. None of the benzylated benzotriazole (7b-9b) compounds showed anti TB activity below 25µg/mL.

Discussion

Drug Bank 5.0 represents a compilation of over 8261 molecules including FDA-approved and experimental drugs. It is therefore particularly useful in conducting virtual screening experiments aimed at searching for drug repurposing. As these are FDA approved drugs, comprehensive data is available on their bioactivity and toxicity profiles. If a drug is

Table II — Anti bacterial activity and anti fungal results of the compounds **1b-9b**

Compd	Antibacterial activity		Antibacterial activity		Antifungal activity	
	Zone of inhibition (mm)		MIC(µg/mL)		Zone of inhibition (mm)	
	SA	*EC*	*SA*	*EC*	*AN*	*CA*
1b	–	–	>15	>15	–	–
2b	10	10	15	15	32	–
3b	10	8	15	15	23	–
4b	–	–	>15	>15	–	–
5b	–	–	>15	>15	32	–
6b	–	–	>15	>15	20	–
7b	–	–	>15	>15	–	–
8b	14.5	–	10	–	–	–
9b	–	–	>15	>15	–	–
Rifampicin	27	27	5	5	–	–
Methanol	–	–	Turbid	Turbid	–	–
Ketoconazole	–	–	–	–	35	38

SA-Staphylococcus aureus (NCIM 2122), EC-*Escherichia coli* (NCIM 2137),
AN–*Aspergillusniger* (NCIM 652), CA- *Candida albicans* (NCIM 3102)

Table III — Anti TB activity results of the compounds **1b-9b**

Compd	Anti TB activity		
	MABA assay (µg/mL)	MTT assay (% inhibition)	Axenic assay (% inhibition)
	MtbH37Rv	*Mtb H37Ra*	*MtbH37Ra*
1b	1.6	86	76
2b	1.6	85	74
3b	1.6	86	75
4b	3.12	81	70
5b	1.6	84	74
6b	3.12	80	72
7b	50	59	55
8b	50	56	53
9b	25	74	63
Benzimidazole	25	69	53
2-Methyl benzimidazole	50	54	49
Benzotriazole	25	51	48
Pyrazinamide	3.125	–	–
Streptomycin	6.25	–	–
Ciprofloxacin	3.125	–	–
Isoniazid(std)	–	89	92
Rifampicin(std)	–	84	83
Control	–	–	–

found to possess clinically significant antitubercular activity, it may be repurposed with a relatively less clinical trial burden. Structure optimizations can be done easily as synthetic procedures and starting materials are available. Information on Quantitative Structure-Activity Relations, mechanism of action and toxicity data for the approved drug is also available. This work may result in finding alternative applications for approved drugs and help in repositioning drugs with least efforts and expenditure.

In our earlier study, we found significant anti TB activity for sertraline (MIC-1.6 µg/mL). Sertraline is a very popular antidepressant drug with annual sales of over 86 million dollars. Hence, is not suitable for repurposing as an anti TB drug. Hence we intended to use its shape based pharmacophoric model to screen the existing approved drug molecules.

Chlormidazole is one of the HIT molecules ranked 6 in the screening by the models generated with 12 conformations of sertraline. It is the first "azole"

containing drug to show antifungal activity[16].It was totally replaced by better azole antifungals like fluconazole. Due to this reason, Chlormidazole was selected as a lead molecule for optimization studies. To probe into activity profile of this pharmacophore, we planned to synthesize benzylated benzimidazole, 2-methylbenzimidazole and benzotriazole derivatives for bioactivity screening.

The formation of the synthesized compounds (1b-9b) was confirmed by the IR spectrum which had shown characteristic signals for benzimidazole/ benzotriazole (NH, 3440-3340 cm^{-1}; C=N, 1685-1607 cm^{-1}), Aromatic ring (C=C, 1567-1450 cm^{-1}; Ar C-H , 3094-3010 cm^{-1}; C-H str, 2976-2980 cm^{-1}). The ^{1}H NMR of the compounds (1b-9b) showed characteristic signals of the protons present on C-8 of the methylene linkage at δ 5.32- δ 5.27 (2H, s) for benzimidazoles and at δ 5.83- δ 5.75 (2H, s) for benzotriazoles. The protons in the aromatic rings appeared in the range of δ 8.08 - δ 7.10.In the ^{13}C NMR spectrum, characteristic signals of the carbon present on C-8 of the methylene linkage at a range of δ 45.55- δ 49.00 (for benzimidazoles) and δ 51.91- δ 50.93 (for benzotriazoles). The carbon present on C-2 of the benzimidazole ring appeared in the range δ 142.27- δ 152.35. For the compounds (4b-6b) on C-2a characteristic carbon signal for the methyl carbon was observed at a range of δ 13.67- δ 14.05. The ^{13}C NMR spectral details are with good agreement with the ^{1}H NMR of the compounds. HMBC correlation data confirmed that benzylation has undergone on N1 of the benzotriazoles. Mass spectral studies of the synthesized compounds are in agreement with their molecular weight and molecular formula. Results of the elemental analysis and spectral studies confirmed structure and purity of the products obtained.

In summary, Chlormidazole was found to have potent anti Tb activity with MIC 1.6 µg/mL. The activity profile of synthesized compounds revealed that, benzimidazole ring and benzylation as essential for anti Tb activity. Addition of chlorines to the benzylic moiety increased the antimicrobial spectrum of the compound leading to reduced selectivity.

Materials and Methods

Compound uniformity and reaction monitoring were done by pre coated silica gel TLC (thin layer chromatography) plates (Silica gel 60 F254, Merck) using appropriate mobile phase and with the help of iodine chamber. Melting points were recorded using EZMELT 120 (Stanford Research Systems, USA) and are uncorrected. Elemental analysis experiments were conducted using Carlo Erba elemental analyzer. The IR spectra were obtained using BRUKER FT-IR (software-OPUS 6.4) spectrometer and the values were expressed in cm^{-1}. The ^{1}H NMR, ^{13}C NMR spectra of the compounds were recorded in DMSO-d_6 or $CDCl_3$ with BRUKER AVANCE 400MHz NMR spectrometer (software-Topspin 3.2) using TMS as an internal standard and the chemical shifts were expressed in δ (ppm). The mass spectra were obtained using Agilent 6410QQQ LC-MS (ESI-MS) spectrometer (software-Mass Hunter B.03.01).

Experimental Section

Computational methods

Preparation of database

DrugBank5.0 database with a compilation of 5783 FDA-approved small molecule drugs were used for our study[11]. The structures present in the Drug Bank database processed by using OMEGA 2.4.6 module to generate all possible 3D conformers (maximum conformations 200, cut-off 5 Kcal between two conformations).

Preparation of Shape Model for Sertraline and Virtual Screening of Drug Bank database

vROCS 3.1.2 module (Open Eye Scientific Software: Santa Fe, NM, 2014. www.eyesopen.com) was used for the preparation of query and performing virtual screening. The query for our search, sertraline was prepared by following the default protocol and all the important structural features including hydrophobic, hydrogen bond donor/acceptor and aromatic rings were considered. All the molecules from drug bank data base were screened against sertraline model. Ligand alignment in the shape model was evaluated by using COMBO Score (CS). Twenty drugs were selected based on CS score (>1.00) and structural diversity. *In vitro* antimicrobial and antitubercular activity screening was performed on these drugs, which procured from commercial vendors and from our standard drug collection.

Synthetic methods

Synthesis of benzylated benzimidazoles 1b-6b

To a suspension of KOH (0.5g, 5mmol) in DMF (5 mL), a solution of benzimidazole (1.0 g, 1mmol) in DMF was added and the mixture was refluxed for half an hour. The corresponding benzyl chloride (1.0 mL, 1mmol) was added drop wise and the reaction mixture

was further refluxed for 2Hrs. After the completion of the reaction it was cooled to ambient temperature. Water was added to the solution and was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. Purification of the crude product with hexane washings rendered almost pure l-benzyl benzimidazoles[12].

Synthesis of benzylated benzotriazoles 7b-9b

Benzotriazole was synthesized by reacting OPDA, sodium nitrite and acetic acid via diazotization process at one of the amine groups. Benzylation was done by using the similar protocol as benzimidazoles. Purity of the synthesized compounds was obtained by recrystallization from hexane or column chromatography.

Characterization Data

1-Benzyl-1*H*-benzo[*d*]imidazole, 1b: Pale white color powder, yield: 42.61%, Mol.wt: 208.10, mp: 113.6°C. IR(KBr) υmax: cm-1: 3440-NH (str), 3010 -CH (str), 2911-Ali CH (str), 1609 -C=N (str), 1493 -Ar C=C (str), 1446-C-C (str), 1264- C-N (str) ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.05-δ 7.11 (m, 10 H, Im-H, Ar-H): δ 5.32 (s, 2H, CH_2).^{13}C NMR (DMSO-d_6, 100MHz): δ 48.08 (CH_2-C): δ 144.70 (Im-C): δ 144.04, 137.44, 134.12, 129.16, 128.19, 127.85, 122.85, 122.04, 119.95, 111.17 (Ar-C). ESI MS *m/z*: 209 $[M+1]^+$.Anal. calcd for $C_{14}H_{12}N_2$; Calculated: C 80.76, H 5.76, N 13.46; found: C 80.74, H 5.81, N 13.45.

1-(4-Chlorobenzyl)-1*H*-benzo[*d*]imidazole, 2b: White colour powder, yield: 31.70%, Mol.wt: 242.06, mp: 71.1°C. IR (KBr) υ max: cm^{-1}: 3340 -NH (str), 3050 -CH (str), 2850-Ali CH (str), 1615 -C=N (str), 1493 -Ar C=C (str), 1450-C-C (str), 1282 -C-N (str), 747-C-Cl. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.94-7.11 (m, 9H, Im-H, Ar-H): δ 5.33 (s, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100MHz): δ 49.00 (CH_2-C): δ 142.27 (Im-C): δ 139.47, 134.72, 132.96, 132.69, 129.45, 128.79, 124.53, 124.12, 119.04, 110.75(Ar-C), ESI MS *m/z*: 243 $[M+1]^+$. Anal. calcd for $C_{14}H_{11}ClN_2$; Calculated: C 69.40, H 4.54, N 11.56; found: C 69.28, H 4.57, N 11.54.

1-(3,4-Dichlorobenzyl)-1*H*-benzo[*d*]imidazole, 3b: White colour powder, yield: 38.54%, Mol.wt: 276.02, mp: 129°C. IR (KBr) υ max: cm^{-1}: 3410 -NH (str), 3031 -CH (str), 2850 -Ali CH (str), 1607 -C=N (str), 1493 -Ar C=C (str), 1450 -C-C (str), 1362 -C-N (str), 746 -C-Cl. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.96-6.96 (m, 8H, Im-H, Ar-H): δ 5.32 (s, 2H, CH_2). ^{13}C NMR (DMSO-d_6, 100MHz): δ 46.82 (CH_2-C): δ 144.71 (Im-C): δ 144.01, 138.60, 131.42, 130.02, 128.25, 123.07, 122.25, 120.06, 111.07 (Ar-C), ESI MS *m/z*: 277 $[M+1]^+$. Anal. calcd for $C_{14}H_{11}Cl_2N_2$; Calculated: C 60.86, H 3.62, N 10.14; found: C 60.67, H 3.64, N 10.11.

1-Benzyl-2-methyl-1*H*-benzo[*d*]imidazole, 4b: Pale yellow solid, yield: 41.66%, Mol.wt: 222.12, mp: 115.6°C. IR (KBr) υ max: cm^{-1}: 3450 -NH (str), 3032 -CH (str), 2945 -Ali CH (str), 1669 -C=N (str), 1353 -C-H (bend), 1450 -Ar C=C (str), 1400 -C-C (str), 1288 -C-N (str). ^{1}H NMR ($CDCl_3$, 400MHz): δ 7.73-7.04 (m, 9H, Ar-H): δ 5.30 (s, 2H, CH_2): δ 2.55 (s, 3H, CH_3),^{13}C NMR (DMSO-d_6, 100MHz): δ 13.79 (CH_3-H): δ 47.22 (CH_2-C): δ 151.74 (Im-C): δ 141.47, 135.52, 135.12, 129.08, 128.49, 128.06, 127.49, 126.97, 126.25, 122.69, 122.47, 118.82, 109.54 (Ar-C), ESI MS *m/z*: 223 $[M+1]^+$. Anal. calcd for $C_{15}H_{14}N_2$; Calculated: C 81.03, H 6.30, N12.60; found: C 81.05, H 6.35, N 12.60.

1-(4-Chlorobenzyl)-2-methyl-1*H*-benzo[*d*]imidazole, 5b: yellow solid, yield: 41.20%, Mol.wt: 256.73, mp: 120°C. IR (KBr) υ max: cm^1: 3455- NH (str), 3054- CH (str), 2945-Ali CH (str), 1618 -C=N (str), 1489 -Ar C=C, 1450 -C-C (str), 1219 -C-N (str). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.09-7.03 (m, 8H, Ar-H): δ 5.34 (s, 2H, CH_2): δ 2.86 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6, 100MHz): δ 13.67 (CH_3-H): δ 46.71 (CH_2-C): δ 151.48 (Im-C): δ 149.99, 140.73, 139.60, 134.71, 134.12, 133.77, 133.24, 132.37, 131.29, 129.69, 129.43, 129.35, 128.59, 128.27, 127.63, 125.52, 125.10,123.13, 122.95, 120.16, 118.66, 113.92, 109.57 (Ar-C), ESI MS *m/z*: 257 $[M+1]^+$. Anal.calcd for $C_{15}H_{13}ClN_2$; Calculated: C 70.11, H 5.06, N 10.09; found: C70.18, H 5.10, N 10.91.

1-(3,4 Dichlorobenzyl)-2-methyl-1*H*-benzo[*d*] imidazole, 6b: White solid,yield: 38.72%, Mol.wt: 290.18, mp: 117.2°C.IR (KBr) υ max: cm^{-1}: 3485 -NH (str), 3032 -CH (str), 2850-Ali CH (str), 1611 -C=N (str), 1498 -Ar C=C, 1398 -C-N (str), 1131 -C-C (str), 732 -C-Cl. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.75-6.82 (m, 7H, Ar-H): δ 5.27 (s, 2H, CH_2): δ 2.57 (s, 3H, CH_3), ^{13}C NMR (DMSO-d_6, 100MHz): δ 14.05 (CH_3-H): δ 45.55 (CH_2-C): δ 152.35 (Im-H): δ 142.84, 138.72, 131.81, 131.52, 129.22, 127.36, 122.31, 122.01, 118.88, 110.40 (Ar-C), ESI MS *m/z*: 291 $[M+1]^+$. Anal. calcd for $C_{15}H_{12}Cl_2N_2$; Calculated: C 62.03, H 4.13, N9.64; found: C61.87, H4.15, N 9.62.

1-Benzyl-1*H*-benzo[*d*], [1, 2, 3]triazole, 7b: White solid,yield: 30.06%, Mol.wt: 209.10, mp: 116°C. IR (KBr) υ max: cm^{-1}: 3425 -NH (str), 3063-CH (str), 2945 -Ali CH (str), 1490 -C-C (str), 1449-Ar C=C, 1364- C-N (str). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.06-7.25 (m, 9H, Ar-H): δ 5.83 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100MHz): δ 40.60 (CH_2-C): δ 145.80, 13.34, 133.14, 129.27, 128.55, 128.21, 127.92, 124.52, 119.69, 111.15 (Ar-C), ESI MS *m/z*: 210 [M+1]. Anal. calcd for $C_{13}H_{11}N_3$; Calculated: C 74.60, H 5.26, N 20.08; found: C 74.62, H 5.30, N 20.08.

1-(4-Chlorobenzyl)-1*H*-benzo[*d*][1,2,3]triazole, 8b: White solid, yield: 36.76%, Mol.wt: 243.06, mp: 95.2°C. IR (KBr) υ max: cm^{-1}: 3443 -NH (str), 3060 -CH (str), 2941 -Ali CH (str), 1492 -Ar C=C, 1488 -C-C (str), 1336 -C-N (str), 757 -C-Cl. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.08-7.19 (m, 8H, Ar-H): δ 5.81 (s, 2H, CH_2).^{13}C NMR ($CDCl_3$, 100MHz): δ 51.91 (CH_2-C): δ 146.36, 134.52, 133.24, 129.27, 129.05, 127.70, 124.18, 120.30, 109.64 (Ar-C), ESI MS *m/z*: 244.06 $[M+1]^+$.Anal. calcd for $C_{13}H_{10}ClN_3$; Calculated: C 64.17, H 4.11, N 17.27; found: C 64.07, H 4.14, N 17.24.

1-(3,4-Dichlorobenzyl)-1*H*-benzo[*d*][1,2,3]triazole, 9b: White solid, yield: 34.40%, Mol.wt: 277.02, mp: 107.1°C.IR (KBr) υ max: cm^{-1}: 3420- NH (str), 3070 -CH (str), 2945 -Ali CH (str), 1496 -Ar C=C, 1493 -C-C (str), 1349 -C-N (str), 754 -C-Cl. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.08-7.05 (m, 7H, Ar-H): δ 5.75 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$, 100MHz): δ 50.93 (CH_2-C): δ 146.20, 134.86, 133.30, 132.90, 132.63, 131.07, 129.51, 127.90, 126.81, 124.30, 120.29, 109.27 (Ar-C), ESI MS *m/z*: 278 $[M+1]^+$. Anal. calcd for $C_{13}H_9Cl_2N_3$; Calculated: C 56.31, H 3.24, N15.16; found: C 56.14, H 3.26, N 15.11.

Biological Activity

All the compounds, **1b-9b** were screened for their anti microbial activity using agar well diffusion method and anti tubercular activity using MABA assay,Axenic Assay and MTT Assay.

Antimicrobial activity

MIC and MBC determination for antibacterial activity

MIC of the compounds was generally determined by nutrient broth dilution method[13]. The stock solution of 1mg/mL concentration was prepared by dissolving the compounds (1b-9b) in methanol. From this stock solution, different concentrations of 100µg/mL, 75µg/ mL, 50µg/ mL, 25µg/ mL, 15µg/mL, 10µg/mL and 5µg/mL solutions were prepared. This sterilized nutrient broth was then poured into sterilized boiling tubes each 9mL by volume. Then every broth solution was added with 0.5 mL of a bacterial inoculum of strains *Escherichia coli* (NCIM 2137) and *Staphylococcus aureus* (NCIM 2122). To these boiling tubes, 1mL of diluted compounds (1b-9b) were added and incubated at 37°C for 24hrs.The clear solution without turbidity indicates no growth and indicates MIC of the compound.

The MBC test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MBC is determined using a series of steps, undergoing after completion of Minimum Inhibitory Concentration (MIC) test. To determine the MBC, the dilution representing the MIC and at least two of the more concentrated test product dilutions are taken. Agar media was plated in and 1mL of the solution from above tubes was added to it and incubated at 37°C for 24hrsto determine viable CFU/mL. The MBC is the lowest concentration that demonstrates a pre-determined reduction (such as 99.9%) in CFU/mL when compared to the MIC dilution[14].

In vitro antitubercular activity screening

MABA Assay

The compounds were screened for antitubercular activity on MtbH37Rv (ATCC 27294) using Micro plateAlamar Blue Assay (MABA)[15]. In brief, growth on Lowenstein-Jensen (LJ) medium was suspended in sterile Middlebrook 7H9 broth supplemented with 0.2%glycerol and 10%OADC (oleate-albumin-dextrose-catalase) enrichment and a 1:20 dilution used as the inoculum for MABA. The 96 wells plate received 100 µL of the Middlebrook7H9 broth and serial dilution of compounds was made directly on the plate. The final drug concentrations tested were 0.8 to100 µg/mL. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this, 25µL of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween80 added to the plate and incubated for 24 hrs. DMSO-d_6 was added as a co-solvent to facilitate poorly soluble compounds. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration, which prevented the colour change from blue to pink.

MTT Assay

The compounds (1b-9b) were further evaluated by undergoing MTT Assay (cell proliferation Assay)

against Mtb H37Ra (MTCC 300). In this method, the calculated amount of cell suspension was added to the required amount of sauton media. 10mL of the above solution was added in 25mL of McCartney bottles. Then test sample was added into the respective bottles (in duplicates). Control bottles (only cells plus Sauton, without inhibitor) and then bottles of standard drugs (Isoniazid and Rifampicin) are separated. These were incubated for 10 days at 37°C. After 10 days incubation, 1mL culture from each 10days culture bottles was taken in another bottle separately. Yellow MTT (dimethyl thiazolyl diphenyl tetrazolium bromide) dye (5mg/mL), 200 µL was added to 1mL cell culture and incubated for 4 hrs at 37°C. Then 1mL of lysis buffer {50% formamide+20% SDS (sodium dodecyl sulfate)} was added in each tube for dissolving the crystals formed. After overnight incubation, the resulting purple solution was spectrophotometrically measured at 570 nm. The increase in absorbance indicates anincrease in cell number by the formation of MTT formazan.

Axenic Assay

The compounds synthesized (1b-9b) were further evaluated by Axenic culture method on Mtb H37Ra (MTCC 300). In this method, the calculated amount of cell suspension was added to the required amount of Sauton media. 10mL of the above solution was added in 25 mL of McCartney bottles. Then test sample was added into the respective bottles (in duplicates), control bottles (only cells plus sauton, without inhibitor) and then standard bottles (Isoniazid and Rifampicin). These are incubated for 10 days at 37°C and OD was taken at 540 nm.

Axenic culture assay:

$$\% \text{ Inhibition} = (\text{control-test})/\text{control}*100$$

Conclusion

In conclusion, our shape based screening and ligand optimization studies could successfully found anti TB activity in chlormidazole (**5b**, MIC 1.6µg/mL) which can be repurposed. Removal of chlorines from the benzylic moiety improved selectivity as observed in **1b**and **4b,** which showed selective potent anti TB activity (MIC 1.6µg/mL) and free from antimicrobial activity at 100µg/mL. The synthesized compounds **2b, 3b, 5b** and **6b** showed potent anti TB activity along with antifungal activity against fungal strain *Aspergillus niger*.

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