Original Article

Anti-tubercular Potency and Computationally-assessed Drug-likeness and Toxicology of Diversely Substituted Indolizines

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ABSTRACT

Background: Several promising compounds against multi-drug-resistant *Mycobacterium tuberculosis* (MTB) are currently in the drug discovery and development pipeline. While it has yet to establish candidature in this pipeline, early results have been promising for the putative anti-mycobacterial potency of the indolizine scaffold. **Methods:** The molecular properties, as well as the Absorption, Disruption, Metabolism, Excretion and Toxicity (ADMET) of indolizines were assessed using the Accelry's Discovery Studio 4.0 client package. **Results:** The current study evaluated the *in vitro* potency of 14 diversely substituted indolizine congeners against H37Rv and multi-drug-resistant strains of *M. tuberculosis.* While all 14 congeners showed potent anti-mycobacterial activity, only three of them had optimal drug-likeness and toxicology, as per *in silico* evaluations. **Conclusion:** The results of the current study identify three indolizine congeners (ethyl 2-methyl-3-(4-methylbenzoyl) indolizine-1-carboxylate (1b)), ethyl 7-acetyl-3-benzoyl-2-methylindolizine-1-carboxylate (3a) and ethyl 7-acetyl-3-benzoyl-2-ethylindolizine-1-carboxylate (3b) with good anti-mycobacterial potency and acceptable drug-likeness and toxicity profiles. Furthermore, the study narrows down the list of indolizine congeners for further evaluation and underscores the importance of computational tools in mitigating the over-utilization of resources and associated costs of drug discovery.

Key words: Multi-drug resistant *Mycobacterium tuberculosis, In silico,* Indolizine, Drug-likeness, pharmacokinetics, Toxicity.

INTRODUCTION

There have been far too many clarion calls to mitigate the burden of tuberculosis.[1-4] While public health measures to ensure the appropriate administration and compliance to anti-tubercular treatment can be effective, challenges to adequate resource allocation continues to obstinately impede the control of multi-drug-resistant tuberculosis.[5-7] Along with repurposed drugs, the available treatment options for multi-drug-resistant tuberculosis have been limited to bedaquiline and Delamanid.[8-10] However, even regimens containing these novel agents and repurposed drugs are expensive and require long-term administration and are thus prone to non-compliance. In addition to effective resource allocation and other measures to clinically manage this infectious disease, the development of

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effective, novel, anti-tubercular agents are an important health priority.[1]

Based on the last 40 years of academic and pharmaceutical industry inventions, only bedaquiline (D1) was the first novel anti-TB drug permitted by the US Food and Drug Administration (US FDA) in December 2012 for the treatment of MDR-TB,[11] while delamanid (D2) was the second anti-TB agent to be approved by the European Medicines Agency in late 2013[12] (Figure 1). It is encouraging to note that numerous novel anti-tubercular agents are currently in the drug development pipeline (Figure 1). These drugs include, GSK 2556286 (D3);[13] TBAJ-587 (D4) from diarylquinoline;[13] TBI-223 (D5) from oxazolidinone;[14] spectinamide 1810 (D6) and spectinomycin analogues,[15] BTZ-043 (D7),[16] GSK 070 and GSK 3036656 (D8) from oxaborole;[13] contezolid (MRX-4/MRX-1) (D9) from oxazolidinone;[17] OPC-167832 (D10), a 3,4-dihyrdocarbostyril derivative;[18] Macozinone (PBTZ169) (D11), a piperazinobenzothiazinone derivative;[19-20] clofazimine (TBI-166) (D12) from riminophenazine;[21] TBA-7371 (D13) from azaindole;[13] and Sutezolid (D14) from oxazolidinone.[22-25]

In continuation of our effort to investigate novel heterocyclic agents for multi-drug resistant anti-tubercular property[26-29] and in addition to the aforementioned chemical structures, the heterocyclic scaffold of indolizine (Figure 2) has shown promising anti-tubercular activity in early experiments.[28,30] While the analgesic,[31] anticancer,[32-33] antidiabetic,[34] antihistaminic,[35] anti-inflammatory,[36-37] antileishmanial,[38] antimicrobial,[39] antimutagenic,[40] antioxidant,[41] antiviral,[42] larvicidal[43-44] and herbicidal[45] activities of this pharmacophore is relatively well-established, data on its anti-tubercular activity are still in accrual.

Recently, we published on the synthesis of indolizine congeners, along with their crystallographic data. In the current article, we present the *in vitro* whole-cell anti-TB activity of 14 indolizine congeners against H37Rv and multi-drug-resistant strains of *M. tuberculosis*. Furthermore, computational tools were used to assess the drug-likeness and toxicological properties of these 14 indolizine congeners. These *in vitro* and *in silico* data are presented here.

In silico assessments are a valuable part of drug-discovery programs, as they circumvent the rising costs and unnecessary utilization of resources and time.[46-49] Though not confirmatory, these computational methodologies do inform and guide the selection of the "most-likely" drug-like candidates. The current study advocates for and employs these computational methods.

MATERIALS AND METHODS

The synthesis of the 14 indolizine congeners used in the current study (Figure 2) was reported earlier.[28,30] In the current study, the minimum inhibitory concentration (MIC) of these 14 indolizine congeners was assessed *in vitro* using the Resazurin microplate assay plate method.[50] Approximately 100 µL of Middlebrook 7H9 broth (BD, Franklin Lakes, New Jersey, USA) was aseptically prepared and dispensed in a 96-well microtiter plate with lids (Lasec, Ndabeni, South Africa). The test compounds were weighed, dissolved in the appropriate solvent and filter sterilized using a 0.2-micron polycarbonate filter. Stock solutions of the test samples were aliquoted into cryovials and stored at –20°C. Then, 100 µL of the test samples was added to each of the wells containing Middlebrook 7H9 broth supplemented with 0.1% casitone, 0.5% glycerol and 10% oleic acid, albumin, dextrose and catalase. The test samples were then serially diluted two-fold directly in the broth of the microtiter plate to reach a desired concentration ranging from 40–0.625 µg/mL.

Inoculums from clinical isolates were prepared fresh from Middlebrook 7H11 agar plates by scraping and

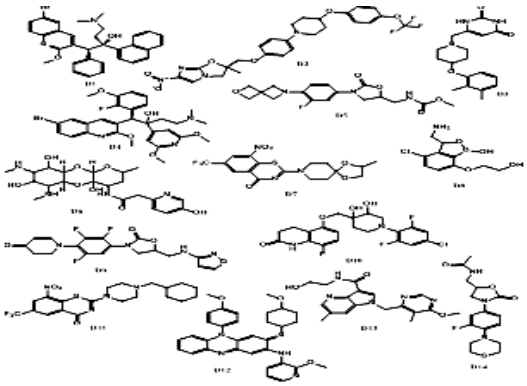


Figure 1:. Chemical Structure of Clinical Approved (D1-D2) and Compounds in the Anti-TB Pipeline (D3-D14).

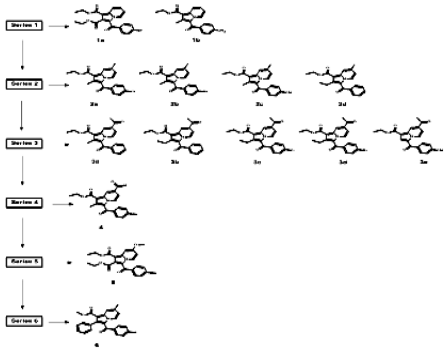


Figure 2: Chemical Structures of Diversely Substituted Indolizine Molecules 1a-b, 2a-d, 3a-e, 4, 5 and 6.

re-suspending loopfuls of colonies into Middlebrook 7H9 broth containing glass beads. The inoculum turbidity was adjusted to a McFarland number 1 standard and was further diluted 1:10 in M7H9 broth prior to being added (100 µL) to each of the test samples and drug-free wells. A growth control and a sterile control were also included for each isolate. Sterile M7H9 broth was added to all perimeter wells to avoid evaporation during incubation. The plate was covered, sealed in a plastic bag and incubated at 37°C. After 8 days of incubation, 30 µL of 0.02% working solution of resazurin salt was inoculated into each microtiter well. The plates were then incubated overnight and read the following day. A positive reaction resulted in a color change from blue to pink, owing to the reduction of resazurin to rezarufin, which confirmed *M. tuberculosis* cell viability/growth, thus resulting in drug resistance. The minimum inhibitory concentrations were defined as the minimum drug concentration to inhibit the growth of the organism with no color changes present in the well.

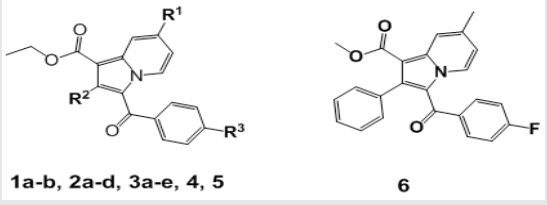
In this study, the molecular properties, as well as the absorption, disruption, metabolism, excretion and toxicity (ADMET) of indolizines were assessed using the online software, Accelry's Discovery Studio 4.0 client package.[51-52] The molecular properties were studied using molecular weight, partition coefficient cLogP, molecular Polar Surface Area (PSA), the number of hydrogen bond acceptors and donors, the number of rings and the number of rotatable bonds. The ADMET Descriptors model utilizes Quantitative Structure-Activity Relationship linear regression-based models to estimate solubility, cytochrome P450 inhibition, liver toxicity, blood-brain barrier (BBB) penetration, human intestinal absorption and Plasma Protein Binding (PPB). The biplot ADMET_PSA_2D and ADMET_AlogP98 developed by Egan *et al.* were used to predict absorption (with 95% and 99% confidence levels).[53] The ellipses region from the biplot ADMET_PSA_2D and ADMET_AlogP98 were employed to define the probability of the absorption levels. The drug distribution was estimated from the BBB, the biplot ADMET_PSA_2D and ADMET_AlogP98 and the PPB level algorithms/models. The cytochrome P450 2D6 model was used to predict potential metabolic processes.

A variety of toxicological endpoints were assessed using the TOPKAT module, which employs cross-validated Quantitative Structure–Toxicity Relationship models. The toxicity profile of the compounds involved screening for Ames mutagenicity, carcinogenicity, developmental toxicity potential, aerobic biodegradability, ocular irritancy, skin sensitizer, skin irritancy and toxicity dose (Carcinogen potency TD_{50}, rat oral lethal dose LD_{50}, rat maximum tolerated dose and rat chronic lowest observed adverse effect level).

RESULTS AND DISCUSSION

Table 1 presents the minimum inhibitory concentration (MIC) of the 14 diversely substituted indolizine analogues against H37Rv and multi-drug-resistant strains of *M. tuberculosis*. Almost all the compounds exhibited potent activity. Table 2 presents the *in silico* ADMET prediction of the bioactive indolizines. The *in silico* ADMET prediction of a *M. tuberculosis* drug currently being evaluated in a clinical trial, reported in Table 3, revealed that the drug-likeness property was almost optimal for drugs D3, D5, D6, D8 and D13, which displayed a lipophilicity cLogP <3.5. All other drugs had a similar drug-likeness property with indolizines. The BBB property indicated that D3, D5, D7–D10, D13 and D15 were less likely to induce adverse effects in the Central Nervous System (CNS), while the remaining were found in the non-defined category. The minority of the drugs was predicted to be the CYP 450 inhibitor (D1, D2 and D4). It was observed that only D6, D7 and D11 were not hepatotoxic. Human intestinal

Table 1: The anti-TB Activity of Diversely Substituted Indolizine Congeners against H37Rv and Multidrug-Resistant Strains of *Mycobacterium tuberculosis*.



Entry	R1	R2	R3	MIC (µg/mL)	
				H37Rv	MDR-MTB
1a	H	$COOCH_2CH_3$	F	8	16
1b	H	CH_3	CH_3	11.3	NA
2a	CH_3	CH_3	Cl	16	NA
2b	CH_3	CH_3	Br	16	32
2c	CH_3	CH_3	CN	16	32
2d	CH_3	CH_2CH_3	H	16	NA
3a	$COCH_3$	CH_3	H	8	20
3b	$COCH_3$	CH_2CH_3	H	5.5	11.3
3c	$COCH_3$	CH_2CH_3	Cl	11	11
3d	$COCH_3$	CH_2CH_3	Br	11	NA
3e	$COCH_3$	H	CN	20	NA
4	CHO	CH_3	Br	4	32
5	OCH_3	$COOCH_2CH_3$	Br	20	NA
6	CH_3	Phenyl	F	30	NA

Table 2: *In silico* ADMET Prediction of Bioactive Indolizines.

Entry	Solubility Level	BBB Level	CYP450 Inhibition	Hepato-toxicity	HIA Level	PPB	AlogP98	PSA 2D
1a	2	2	No	Yes	0	Yes	3.972	75,11
1b	2	1	No	No	0	Yes	4.534	48.88
2a	1	1	Yes	Yes	0	Yes	5.199	48.88
2b	1	0	No	No	0	Yes	5.283	48.88
2c	2	1	No	No	0	Yes	4.416	71.81
2d	1	1	No	No	0	Yes	4.991	48.88
3a	2	2	No	No	0	Yes	3.788	66.18
3b	2	1	No	No	0	Yes	4.244	66.18
3c	2	1	Yes	Yes	0	Yes	4.909	66.18
3d	1	1	No	Yes	0	Yes	4.933	66.18
3e	2	3	No	Yes	0	Yes	3.181	89.11
4	2	1	No	No	0	Yes	4.556	66.18
5	2	2	No	Yes	0	Yes	4.498	84.04
6	1	0	Yes	No	0	Yes	5.772	48.88

Criteria:
Solubility level/ drug-likeness: 0: extremely low/no, 1: very low/ possible, 2: low/yes, 3: good/yes, 4: optimal/yes
BBB level (Blood-brain barrier): 0: very high penetrant, 1: high, 2: medium, 3: low, 4: undefined
HIA level (Human Intestinal Absorption): 0: good, 1: moderate, 2: poor, 3: very poor
PBB: Plasma Protein Binding.

Table 3: *In silico* ADMET Prediction of MTB Drugs in Clinical Trial.

Entry	Solubility Level	BBB Level	CYP450 Inhibition	Hepato-toxicity	HIA Level	PPB	AlogP98	PSA 2D
D1	1	4	Yes	Yes	2	Yes	6.933	44.359
D2	1	4	Yes	Yes	2	Yes	6.452	98,505
D3	3	3	No	Yes	0	No	2.063	72.504
D4	1	4	Yes	Yes	2	Yes	6.109	82.41
D5	3	3	No	Yes	0	No	1.167	80.907
D6	3	4	No	No	3	No	-2.223	177.044
D7	2	3	No	No	0	Yes	3.515	92.659
D8	3	3	No	Yes	0	Yes	1.456	86.031
D9	2	3	No	Yes	0	No	2.54	86.862
D10	2	3	No	Yes	0	Yes	3.066	84.024
D11	1	4	No	No	0	Yes	5.219	78.152
D12	1	4	No	Yes	1	Yes	5.467	76.86
D13	3	3	No	Yes	0	No	1.79	98.987
D14	3	3	No	Yes	0	No	1.639	63.049

Criteria
Solubility level/Druglikeness: 0: extremely low/no, 1: very low/ possible, 2: low/yes, 3: good/yes, 4: optimal/yes
BBB level (Blood-brain barrier): 0: very high penetrant, 1: high, 2: medium, 3: low, 4: undefined
HIA level (Human Intestinal Absorption): 0: good, 1: moderate, 2: poor, 3: very poor
PBB: Plasma Protein Binding.

absorption and PPB were closely related to drug lipophilicity. Drugs D1, D2, D4, D11 and D12 had a cLog *p* value >5 and were predicted to be poorly absorbed into the human intestine. No PPB was observed for D3, D5, D6, D9, D13 and D14 (cLoP <2.5). In general, it can be observed that indolizine derivatives better predicted the ADMET profile than the current *M. tuberculosis* drugs. The absorption profile of indolizines indicated that all compounds presented good absorption in the human intestine. All compounds were found within the red ellipsoid from the biplot ADMET_PSA_2D and ADMET_AlogP98, indicating that 95% of the

compound would be absorbed into the human bloodstream (Figure 3). However, although the compounds presented excellent lipophilicity (High Alog*P*98 value), their solubility was predicted to be low and the drug-likeness property was favorable only for compounds 1a, 1b, 2c, 3a, 3b, 3c, 3e, 4 and 5. It is also noteworthy that none of the compounds violated Lipinski's rule of five (Alog*P*98 ≤5) and they could thus be expected to be orally active. The distribution profile showed that all compounds may interact with the CNS, as indicated by the BBB level; thus, these compounds could readily cross the BBB. However, compounds 1a, 3a, 3e and 5 are less likely to cross the BBB. Considering the biplot ADMET_PSA_2D and ADMET_AlogP98 (Figure 3), all compounds were observed to reside in 95% BBB confidence limit ellipses (Magenta ellipsoid), except for compound 5, which was predicted to be within the 99% confidence level (Blue ellipsoid). This prediction indicates that the indolizines are more likely to penetrate the cell wall of bacteria. This prediction was verified by the *in vitro* experiments.

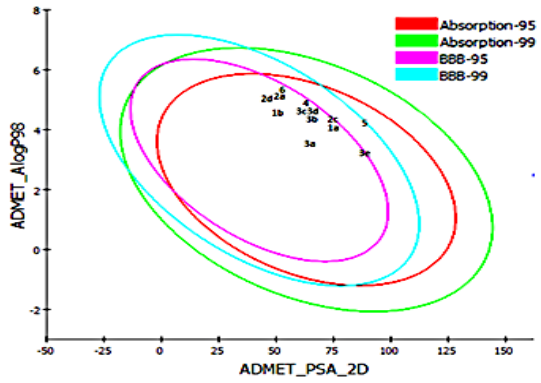


Figure 3: Biplot ADMET_PSA_2D and ADMET_AlogP98 of Investigated Indolizines.

Moreover, all compounds were predicted to bind effectively to the carrier protein in blood plasma to facilitate transportation to the active site. Most of the compounds, except for 2a, 3c and 6, were predicted to be metabolically stable toward cytochrome P450 2D6. The hepatotoxicity prediction revealed that compounds 1b, 2b, 2c, 2d, 3a, 3b, 4 and 6 were not expected to damage the liver. Based on our computational ADMET analysis, compounds 1b, 2c, 3a, 3b and 4, demonstrated favorable ADMET features and were subjected to predictive toxicity assessments using the TOPKAT tool. The assessments included Ames mutagenicity, carcinogenicity, Developmental Toxicity Potential (DTP), skin irritancy, skin sensitizer, ocular irritancy and aerobic biodegradability (Table 4). While none of the compounds appear to have a mutagenic character, all compounds were carcinogenic in male mice models. However, in female mice, it was found that compounds 1b (NTP) and 4 (NTP and FDA) were predicted to be carcinogenic. As for the rat model, it was predicted that all compounds were non-carcinogenic, with some exceptions. For instance, compound 2c is toxic via FDA in male rats. Compounds 3a and 4 were predicted to be toxic for female rats in NTP and FDA, respectively. Based on these aforementioned observations, compound 4 was the most likely compound to induce cancer. The Developmental Toxicity

Table 4: *In silico* Toxicity Prediction of Anti-TB Indolizines.

Entry	AMES Mutagenicity	Carcinogenicity								DTP	Skin Irritancy	Skin Sensitizer	Ocular Irritancy	Aerobic Bio-Degradability
		Mouse Female		Mouse Male		Rat Female		Rat Male						
		NTP	FDA	NTP	FDA	NTP	FDA	NTP	FDA					
1a	No	No	No	Yes	S	No	No	No	No	No	No	Strong	No	No
1b	No	Yes	No	Yes	S	No	No	No	No	No	No	Strong	No	No
2a	No	No	No	Yes	S	No	No	No	No	No	No	Strong	No	No
2b	No	Yes	No	Yes	S	No	S	No	No	No	No	Strong	No	No
2c	No	No	No	Yes	S	No	No	No	M	No	No	Strong	No	No
2d	No	No	No	Yes	N	No	No	No	No	No	No	Strong	No	No
3a	No	No	No	Yes	S	Yes	No	No	No	No	No	Strong	No	No
3b	No	No	No	Yes	M	No	No	No	No	No	No	Strong	No	No
3c	No	No	No	Yes	No	No	No	No	No	No	No	Strong	No	No
3d	No	Yes	No	Yes	No	No	No	No	No	No	No	Strong	No	No
3e	No	No	No	No	S	No	No	No	No	No	No	Strong	No	No
4	No	Yes	S	Yes	S	No	S	No	No	No	No	Strong	No	No
5	No	Yes	No	Yes	No	No	S	No	Yes	No	No	Strong	No	No
6	No	Yes	No	Yes	S	No	No	Yes	No	No	No	Strong	No	No

Table 5: *In silico* Toxicity Dose Prediction of Anti-TB Indolizines.					
Entry	Carcinogen Potency TD 50[a]		Rat Oral LD 50[b]	Rat Maximum Tolerated Dose[c]	Rat Chronical Lowest Observed Adverse Effect Level (LOAEL)[c]
	Mouse	Rat			
1b	125.032	26.2296	3.60695	79.592	15.7891
2c	94.1142	7.41595	1.17908	62.923	76.7282
3a	423.794	125.888	1.51934	65.209	20.8913
3b	287.501	146.503	1.9968	80.701	21.0608
4	41.1384	12.6491	2.0493	61.604	60.0322

[a]: mg/kg body weight/day; [b]: g/kg body weight, [c]: mg/kg body weight

Potential (DTP) evaluation showed that all compounds might not have any alteration effects during the developmental processes. All compounds were expected to be sensitive on the skin; however, they appeared not to irritate the skin and eyes. The indolizines were predicted to be inactive under aerobic cellular conditions.

Additional predictive models, such as carcinogen potency Toxic Dose (TD_{50}), rat oral Lethal Dose (LD_{50}), rat Maximum Tolerated Dose (MTD) and the Observed Adverse Effect Level (LOAEL) in rats, were also examined for selected derivatives 1b, 2c, 3a, 3b and 4 (Table 5). The predicted carcinogen potency TD_{50} revealed that compounds 2c and 4 were susceptible to provoking cancer in both mouse and rat models with a TD_{50} <100 mg/kg/day, while compound 1b may only induce cancer in a rat model. Conversely, compounds 3a and 3b presented less tumorigenic potency. The oral LD_{50} in rats for the various compounds was predicted to be within the range of 1–4 g/kg. As for rat MTD, which ranged from 61–80 mg/kg, it showed that compounds 3a and 3b had MTD values that were less than the TD 50 values, indicating that the clinical sign of cancer induction is less likely to be observed. The predicted adverse effect (LOAEL) demonstrated that compounds 1b, 3a and 3b had a low LOAEL value (≤MTD), suggesting that no toxicity would be observed at the predicted adverse effect dose. Based on LOAEL, compounds 1b, 3a and 3b would not present any signs of adverse effects and toxicity, whereas compounds 2c and 4, which had a TD 50 that was equal to or less than the LOAEL, may potentially increase the risk of cancer. Overall, compounds 1b, 3a and 3b appeared to possess acceptable drug-likeness and safety profiles.

CONCLUSION

The results of the current study indicate that the indolizine scaffold is a promising pharmacophore against multi-drug-resistant *M. tuberculosis*. Among the various diversely substituted indolizine congeners, only indolizines 1b, 3a and 3b appeared to have a favorable safety profile. The promising findings associated with these three agents serves as the rationale to continue investigating these indolizine congeners as part of our ongoing anti-tubercular drug-discovery program.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

ADMET: Absorption, Disruption, Metabolism, Excretion and Toxicity; **BBB:** blood-brain barrier; **CNS:** central nervous system; **DTP:** Developmental Toxicity Potential; **HIA:** Human Intestinal Absorption; LD: Lethal Dose; **LOAEL:** Lowest Observed Adverse Effect Level; **MIC:** Minimum Inhibitory Concentration; **MTB:** *Mycobacterium tuberculosis*; **MTD:** Maximum Tolerated Dose; **NTP:** National Toxicology Program; **PPB:** Plasma Protein Binding; **PSA:** Polar Surface Area; **TD:** Toxic Dose; **US FDA:** US Food and Drug.

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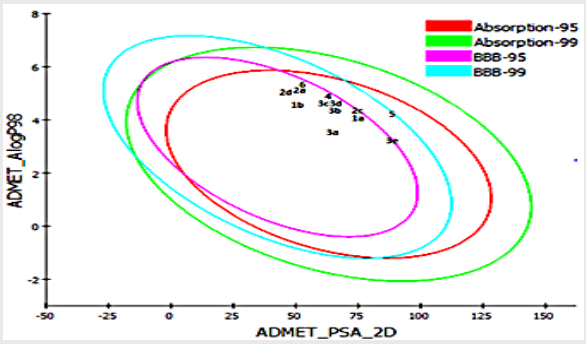
PICTORIAL ABSTRACT



SUMMARY

- Indolizine scaffold is a promising pharmacophore against multi-drug resistant *Mycobacterium tuberculosis*. However, as per *in silico* predictions, only 3 of the 14 diversely substituted indolizines have optimal drug-likeness and toxicological profiles. These *in silico* findings may narrow down the list of indolizine congeners for further evaluation and mitigates the avoidable over-utilization of resources and associated costs of drug discovery.

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