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Research paper

Hybrid triazoles: Design and synthesis as potential dual inhibitor of growth and efflux inhibition in tuberculosis

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ABSTRACT

Efflux inhibition is proven bacterial machinery responsible for removal of bacterial wastage including antibiotics. Recently, efflux inhibitors (EI) have been tested with encouraging results as an adjuvant therapy for treatment of tuberculosis (TB). Although, EI have emerged as innovative approach of treatment for multi drug resistant (MDR) & extensively drug resistant tuberculosis (XDR-TB), toxicity profile limits their wider use. To address this issue, we have attempted synthesizing hybrid molecules those results by combining known EI and triazole. This synthesis was aimed to arrive at structure that possesses pharmacophore from known EI. Synthesized molecules were evaluated as growth inhibitors (GI) and Efflux inhibitor of TB initially against *Mycobacterium smegmatis* mc^2155. Pharmacologically active compounds were then tested for their cytotoxicity to further narrow down search. Most active compounds 144, 145, 154 and 163 were then tested for their GEI action against *Mycobacterium tuberculosis* (Mtb). Synthesized compounds were also tested for their synergistic action with first line and second line anti-TB drugs and ethidium bromide (EtBr). We arrived at compound 135 as most potent dual inhibitor of tuberculosis.

1. Introduction

Currently, the global armamentarium of antituberculosis drugs is insufficient to address the growing populations of patients with multi-drug resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis*. According to World Health Organization (WHO), tuberculosis (TB) is the second leading cause of death from an infectious disease. Recent databases suggested 9 million new cases and 1.5 million deaths owing to this infection, including 360,000 deaths among HIV-positive people [1]. It is also stated that one-third of the world's population is infected with latent TB and 10% of which is expected to develop active TB at some point in their lives [2].

Stringent supervision, consistent supply and drug intake fidelity is a must during entire anti-tuberculosis program to be successful and to avoid recrudescence and resistance. Emergence of multi and extensively drug resistant (MDR & XDR) TB has frustrated researchers further.

Above mentioned scenario recommends drug discovery to be synergistic; approach to target newer molecular mechanism and novel NCE which can be combination of pharmacophores of earlier proven entities. Current drug pipeline lacks this innovation [3–5] making "so called" newer NCE vulnerable to mutation based resistance (MBR).

Reports suggests that along with MBR, efflux pump also plays vital role in development of resistance by Mtb [6–8]. Intrinsic resistance of *M. tuberculosis* to standard antimicrobial therapies has been attributed primarily to its lipid-rich cell wall composition which limits drug penetration. Efflux pumps, which reduce the passage of antimicrobials across the bacterial cell wall, have been identified as an additional mechanism of resistance. The recent identification of clinical isolates of resistant *M. tuberculosis* that have an efflux component as part of their resistance phenotype represents an exciting new field in tuberculosis therapeutics. Along-with detoxification of intracellular metabolites and cellular homeostasis [9], efflux pumps does contribute for intrinsic and acquired drug resistance in many bacterial pathogens. Usually they confer low-to-intermediate level of resistance; however, the constant pressure of subinhibitory concentrations of the antibiotic promotes the selection of spontaneous mutants [9]. Based on the literature, we state efflux pumps are crucial in a) low level direct

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drug resistance, b) high level indirect (through effluxing drug from within cell) drug resistance, c) they are effectors of the innate drug-resistance machinery and d) if efflux pumps are inhibited, improvement or restoration of activity of old drug is observed.

Thioridazine [10–15], chlorpromazine [16,17] and Verapamil [18] have shown efflux pumps as area of opportunity in anti-tubercular agents but have not evolved toward clinical usage due to potential toxicities. The development of agents with low toxicity profiles that target drug efflux pumps may be clinically useful in reducing the global threat of MDR and XDR tuberculosis. Based on recent reports [19–22], we have embarked with present series to identify and evaluate novel compounds for their dual inhibitory action; growth and efflux inhibition (GEI).

2. Results and discussion

2.1. Chemistry

Chemical class of triazole is cost effective nucleus which has proven its commercial viability. Thus by undertaking present series, we can state to have a cost effective drug candidate. The synthetic route was followed as reported (Fig. 2) [23].

As detailed literature discussing SAR on efflux inhibitors as anti-TB is lacking, we initiated our synthesis based on Ligand-based Drug Design approach. Two of most studied efflux inhibitors were identified for drug designing, Thioridazine (TZ) and Verapamil (VP) wherein TZ is also explored in depth for its enhancer activity along-with other anti-TB agents [24,25]. Phenothiazine ring is crucial in conferring neuroleptic activity for TZ while efflux systems are known to affine to basic amino group. Given these premises, we embarked at a structure which reflects parts of these two pharmacophores with couple of variations. The triazole core is well known privileged nucleus in recent drug discovery as it's a nifty heterocycle with a wide range of activities like antibacterial, antifungal, antiviral and also antimycobacterial. Many reports are available confirming fused or linked triazole emerging as novel antitubercular agent [26–29]. Most of the studies have also suggested amino and mercapto position's availability for extensive variation for modulation of activity. Thus combination of hybrid design linked with triazole was finalized to arrive at dual inhibitors. (Fig. 1)

Target structure we have designed was combination of 3 precursors viz., protected triazole (4), a chalcone (7) and heterocycle. In brief, initially hydrazide (1) was synthesized by reacting carbon disulfide with hydrazine hydrates. Bromo acetic acid was then treated with hydrazine (2) to produce 4-amino-3-bromomethyl-5-mercapto-(1,2,4)-triazole (3). This conversion did not yield products for several time at the addition was tricky. After several trial and error we identified root cause is the way and speed of addition of reactants. Slow, drop wise addition of bromo acetic acid over 30 min to hydrazine provided compound 3. Amino and mercapto groups which served as reactive terminals were then protected by Boc anhydride and benzyl bromide respectively to furnish protected triazole (4). Second precursor, chalcone (7) was prepared by reacting 2-hydroxy acetophenone (5) with 2,4-dichlorobenzaldehyde (6). These triazolyl-chalocones (8) were then cyclized using hydroxylamine and further deprotected at amino and thio function by Pd/C and then TFA/DCM to furnish compound PDST121. This compound has shown good inhibition against primary screening. This compound is under further screening at our lab and currently out of scope of this manuscript. Meanwhile, it was thought worthwhile to develop SAR related to PDST121.

In attempt to do so, we first reacted free amino group and converted to substituted amide (136–138). On observing promising activity of amide compounds, it was further decided to alter reactive thiol group. As initial transformation, esterification was performed to produce PDST139-141. As these compounds shown detrimental effects on inhibitory action, program for ester linkage was scrapped and instead chain elongation strategy was considered. Starting substitutions with smallest linkage, methyl group we extended to ethyl, propyl and n-butyl which have shown betterment of activity. We also synthesized compounds having *a*. appendage with double bond, *b*. branching instead of elongation and *c*. ring insertion but were shown diminishing activity. Thus to develop a concluding SAR for this series, we synthesized PDST142-168 and were evaluated. Most of the conversion were smooth except mentioned ones and gave products with high purity.

2.2. Pharmacology

An urgent need for effective alternative anti-TB treatment, especially for the severe destructive and disseminated forms of TB initiated attempts to design and develop compounds with anti-mycobacterial activity. Current manuscript reveals first report wherein a hybrid molecule is coupled with triazole that are aimed as potent and rationally designed TB-GEI (growth and efflux inhibitor). Total of 33 compounds were synthesized in this experiment and all underwent inhibitory assay. All the compounds were initially tested against *Mycobacterium smegmatis* mc^2155 reference strain to achieve faster and cheaper results. Molecules, which have shown inhibitory activity better than TZ were further tested for cytotoxicity. Compounds with better inhibition and lesser cytotoxicity were then assessed on *M. tuberculosis* H37rv and further for synergistic profile with first and second line anti-tubercular agents.

2.2.1. Preliminary inhibitory assessment

2.2.1.1. Growth inhibition of M. smegmatis mc^2155. As mentioned earlier, we have observed promising dual inhibitory activity of PDST121 from our in-house cluster of compounds. To further explore SAR of this compound, we initiated current synthetic

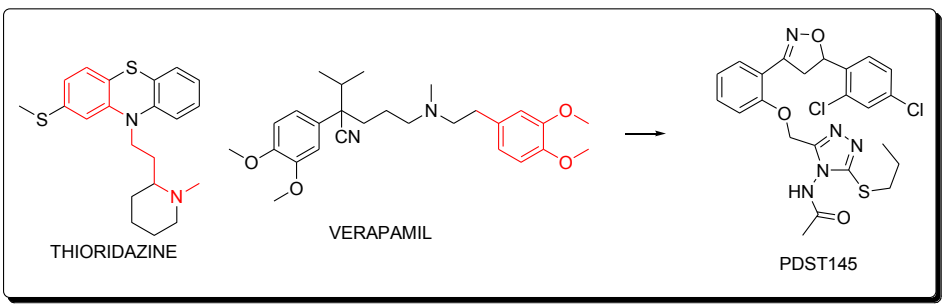

Fig. 1. Identification of molecular fragments in generating hybrid molecule.

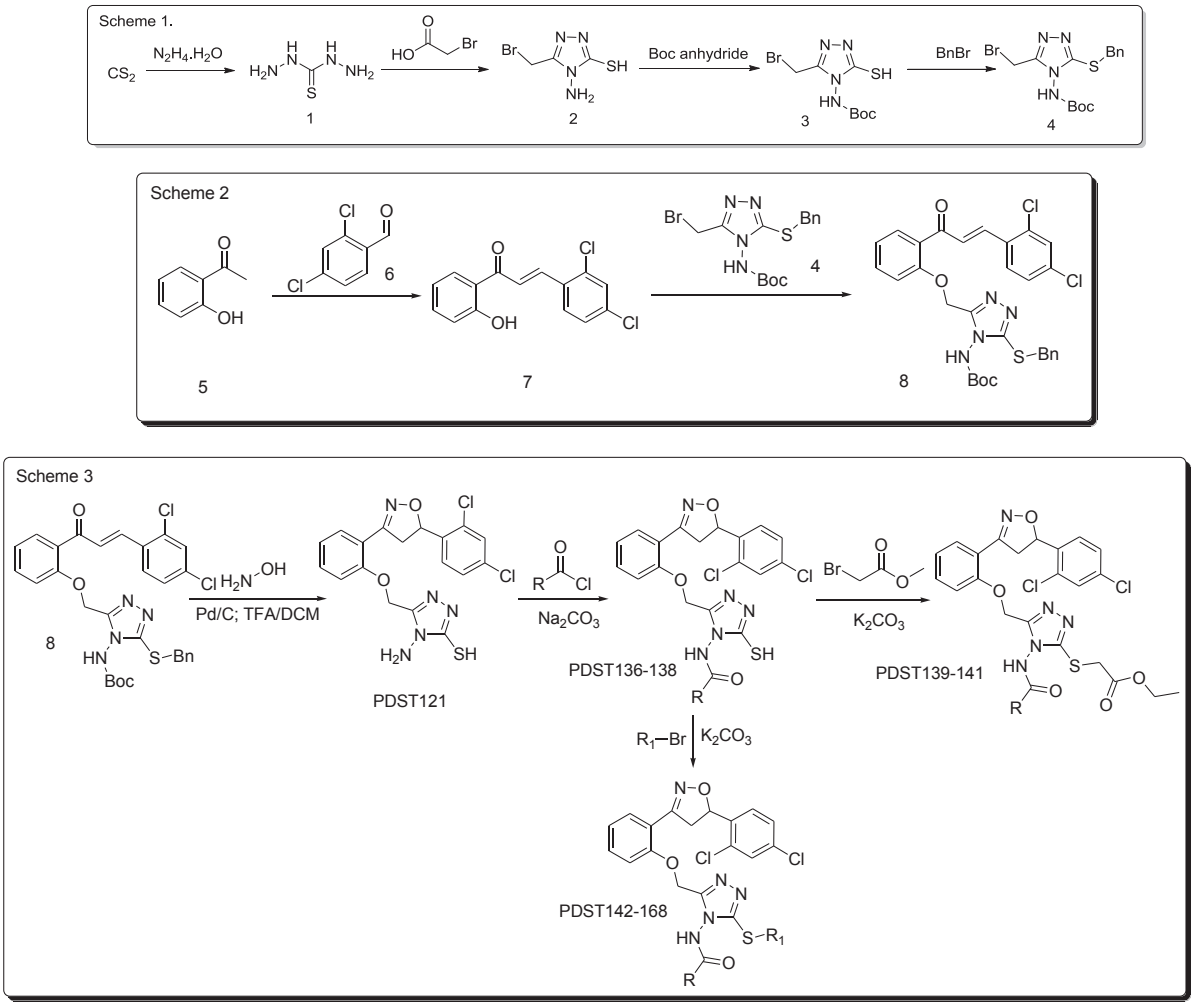

Fig. 2. Route of synthesis of **PDST136–168**.

scheme. As our strategy, we initiated exploring the structure-activity relationship of the **PDST** series to determine its potential for progression as a drug candidate. Therefore, we designed, synthesized and tested a number of analogs in this process. In systematic modification, we initially applied amidation at amino group represented by R, while the second variation was esterification and then chain elongation at thiol group which was represented by R_1.

Evaluation of SAR initiated with examining effect of amidation at amino group of traizole. To begin with, three segments of varied length and bulk were tried examples are methyl, phenyl and chloromethyl carbonyl groups. This was undertaken to define the structure activity relationships influencing potency of carbon length and bulkiness of group. Striking with good inhibition, all three compounds suggested amino substitution was allowed but none of them was as active as parent compound PDST121. Thus it was thought to test substitution of thiol group keeping amino group intact. This attempt showed complete loss of activity, suggesting, thiol alone cannot be substituted. The results are not included here and considered to be out of scope of this manuscript.

Introduction of ester at the mercapto is well known to increase anomeric stability, modulate ring pucker and binding affinity as well as enhance pharmacokinetic properties [30,31]. Thus it was thought worthwhile to synthesize molecule with ester linkage (139–141) but to our disappointment, this substitution led to diminishing activity. Diminished activity of 139–141 suggests steric clash between the ester appendages and a putative protein responsible for uptake or association.

Next set of compounds synthesized was to check whether the chain variation at mercapto can be substituted with improvement in activity. Strategy to design this series was based on overall 2D and 3D-analysis of chemical structures of these derivatives that showed electron-donating groups induced an enhancement in the activity. Especially smaller alkyl groups were observed to be critical for better inhibition. Attempt to test derivative with critical alkyl chain length was encouraging, showing good inhibition. Besides, substitution with ethyl acetoacetate, branching and phenyl group seems to decrease antimycobacterial activity.

Out of three series of nine compounds, one with methyl have shown better activity while with phenyl group shown lesser than methyl and series with chloromethyl carbonyl have shown least inhibition. Thus we could state entities with modification with methyl substitutions have shown better inhibition than TZ and can be critical.

To further comment on structural consequences, we state compound 142–150 with link alteration have shown two most promising analogs. Compound 145 is exceptionally potent inhibiting growth as well as efflux compared to TZ which is 5 fold better. The structural modification here stated now have prompted requirement of methyl group attachment to phenyl ring as favored

substitution. Due to the reduced solubility and the low anti-mycobacterial activity, it was not possible to determine the exact MIC values for some compounds such as 139, 148, 150 & 165.

2.2.1.2. Efflux inhibition. After determining their MICs the compounds were tested for their capability to inhibit efflux of EtBr from *M. smegmatis* mc^{2}155 cells by real-time fluorometry, using TZ and VP as controls (Table 1, relative final fluorescence (RFF) values). According to a widely used protocol for this kind of assay, not to compromise the cellular viability, all of the compounds were tested at ½ of their MIC. For those compounds for which the exact MIC could not be determined (above reported), the last concentration value that could be technically determined was considered ½ of the MIC. Although SAR was developed with limited compounds, still we can concise our results on SAR of efflux inhibitory effect. From these initial SAR studies based on 5 series, we determined that the key substituents for activity enhancement are: a acetamido group at the amino position and a two or three carbon chain at the thiol position of the triazole ring. We used this knowledge to further identify their effect on *M. tuberculosis* H37Rv strain and then to observe synergistic effect, if any (Table 2).

2.2.1.3. Cytotoxicity. Compounds 136, 137, 138, 142, 144, 145, 149, 151, 153, 154, 158, 160, 163 & 167 were emerged as most active compounds of the series. These were evaluated against human-monocyte derived macrophages to assess their ex vivo cytotoxicity toward eukaryotic cells (Table 1).

We here noticed that, with some of the compounds i.e., 136, 137, 138, 142, 149, 151, 153, 158, 160 & 167 have been proved to be toxic when compared with TZ. Compounds 154 and 163, (IC_{50} 14.8 μg/mL and 28.7 μg/mL, respectively) are 2–3-fold less cytotoxic than TZ

Table 2
Screening of anti-tubercular and efflux inhibitory activity of selected compounds against *M. tuberculosis* H37Rv.

ID	*M. tuberculosis* H37Rv	
	MIC (μg/mL)[a]	REF ± SD[b]
144	8	2.17 ± 0.07**
145	4	8.75 ± 0.06***
154	32	0.36 ± 0.05
163	64	0.32 ± 0.03
TZ	16	0.25 ± 0.04
VP	128	1.98 ± 0.03**

[a] Determined by microdilution.
[b] Relative final fluorescence (RFF) based on accumulation of EtBr at 0.5 μg/mL; the results are presented as the average of three independent assays plus standard deviation (±SD). The results were considered significant when *P < 0.05 and highly significant when**P < 0.01 and***P < 0.001.

Table 1
Anti-tubercular and efflux inhibitory activity of PDST136-168 against *M. smegmatis* mc^2 155 and their toxicity index towards human monocyte derived macrophages.

ID	R	R_1	*M. smegmatis* mc^2 155		IC_{50}[c] μg/mL
			MIC (μg/mL)[a]	REF ± SD[b]	
PDST121	–	–	2	13.26 ± 0.69***	87.3
PDST136	$-CH_3$	–	8	2.18 ± 0.16**	1.3
PDST137	$-C_6H_5$	–	32	0.98 ± 0.078*	1.7
PDST138	$-COCH_2Cl$	–	16	0.77 ± 0.073*	3.2
PDST139	$-CH_3$	–	>64[d]	0.33 ± 0.045	ND
PDST140	$-C_6H_5$	–	64	0.18 ± 0.088	ND
PDST141	$-COCH_2Cl$	–	128	0.29 ± 0.031	ND
PDST142	$-CH_3$	$-CH_3$	32	0.81 ± 0.069*	2.7
PDST143	$-CH_3$	$-CH_2COCH_2COOEt$	512	0.42 ± 0.043	ND
PDST144	$-CH_3$	$-C_2H_5$	2	12.65 ± 0.76***	9.7
PDST145	$-CH_3$	$-C_3H_7$	1	14.71 ± 0.81***	127.6
PDST146	$-CH_3$	$-CH_2CH=CH_2$	256	0.27 ± 0.065	ND
PDST147	$-CH_3$	$-CH=CH-CH_3$	512	0.38 ± 0.042	ND
PDST148	$-CH_3$	$-CH_2-CH(CH_3)-CH_3$	>256[d]	0.11 ± 0.022	ND
PDST149	$-CH_3$	$-(CH_2)_3-CH_3$	32	0.84 ± 0.093*	2.6
PDST150	$-CH_3$	$-C_6H_5$	>512[d]	0.16 ± 0.039	ND
PDST151	$-C_6H_5$	$-CH_3$	32	0.89 ± 0.032*	2.2
PDST152	$-C_6H_5$	$-CH_2COCH_2COOEt$	512	0.25 ± 0.082	ND
PDST153	$-C_6H_5$	$-C_2H_5$	4	2.97 ± 0.56**	1.8
PDST154	$-C_6H_5$	$-C_3H_7$	2	9.06 ± 0.28***	14.8
PDST155	$-C_6H_5$	$-CH_2CH=CH_2$	128	0.29 ± 0.046	ND
PDST156	$-C_6H_5$	$-CH=CH-CH_3$	128	0.24 ± 0.047	ND
PDST157	$-C_6H_5$	$-CH_2-CH(CH_3)-CH_3$	512	0.36 ± 0.052	ND
PDST158	$-C_6H_5$	$-(CH_2)_3-CH_3$	64	0.81 ± 0.028*	2.5
PDST159	$-C_6H_5$	$-C_6H_5$	256	0.23 ± 0.041	ND
PDST160	$-COCH_2Cl$	$-CH_3$	32	0.93 ± 0.018*	2.7
PDST161	$-COCH_2Cl$	$-CH_2COCH_2COOEt$	128	0.48 ± 0.074	ND
PDST162	$-COCH_2Cl$	$-C_2H_5$	512	0.37 ± 0.065	ND
PDST163	$-COCH_2Cl$	$-C_3H_7$	4	2.91 ± 0.52**	28.7
PDST164	$-COCH_2Cl$	$-CH_2CH=CH_2$	256	0.45 ± 0.087	ND
PDST165	$-COCH_2Cl$	$-CH=CH-CH_3$	>512[d]	0.21 ± 0.082	ND
PDST166	$-COCH_2Cl$	$-CH_2-CH(CH_3)-CH_3$	1024	0.10 ± 0.021	ND
PDST167	$-COCH_2Cl$	$-(CH_2)_3-CH_3$	32	0.82 ± 0.023*	2.8
PDST168	$-COCH_2Cl$	$-C_6H_5$	128	0.53 ± 0.064	ND[e]
TZ			31	0.88 ± 0.1*	5.5
VP			776	2.20 ± 0.12**	51.33

[a] Determined by microdilution.
[b] Relative final fluorescence based on accumulation of EtBr at 0.25 μg/mL; the results are presented as the average of three independent assays plus standard deviation (±SD). The results were considered significant when *P < 0.05 and highly significant when**P < 0.01 and***P < 0.001.
[c] Index of Cytotoxicity (IC) determined in human monocyte derived macrophages.
[d] Due to the reduced solubility of the compounds it was not possible to test at higher concentrations.
[e] Not determined.

(IC_{50} = 5.5 μg/mL), whereas compounds 144 & 145 (IC_{50} = 87.9 μg/mL and 122.4 μg/mL) were found to be safe towards human macrophages. Overall, these preliminary data clearly suggest that the replacement of the phenothiazine core with other ring systems allows maintaining the efflux inhibitory activity with the possibility to decrease the cytotoxicity. Hence, compounds 144, 145, 154 & 163 that have shown better inhibition and less cytotoxicity than TZ were selected for further inhibitory profile against *M. tuberculosis* H37Rv strain.

2.2.2. Secondary evaluation of growth and efflux inhibition on *M. tuberculosis H37Rv*

As a second phase in establishing TB-GEI effect of selected compounds 144, 145, 154 & 163 we here determined antimycobacterial activity against pan susceptible *M. tuberculosis* H37Rv strain, followed by evaluation of efflux inhibitory action.

On evaluating MIC for above compounds, we were taken to surprise by results. Two out of four 144 & 145 (MIC = 8 μg/mL & MIC = 4 μg/mL) shown far better results than TZ while other two, 154 & 163 (MIC = 32 μg/mL & MIC = 64 μg/mL) have shown less activity. In order to explore EI, we used 154 & 163 using the same process as that of *M. smegmatis* while for 144 & 145 we have performed the essay using ½ MIC. This has ruled out any possibility of bias in activity profiling. As a result, we observed that 154 & 163 have displayed equipotency to that of TZ while 144was3 fold active and 145 was 5 fold active than TZ. Overall activity profile has weaken when compared with *M. smegmatis*. This can be explained by stating the fact that *M. smegmatis* has faster metabolism when compared to *M. tuberculosis* H37Rv making it more susceptible to efflux inhibition. Although the molecules have maintained relativity, overall activity profile can be seen down-regulated. We can still state that 144 & 145 reigns supreme as most potent inhibitors.

2.2.3. Tertiary evaluation: synergistic effect

Confident with dual inhibition of 144, 145, 154 & 163 we went ahead with evaluating synergism of these entities with first line (Isoniazide, INH; rifampicin, RIF) and second line (Amikacin, AMK; Ofloxacin, OFX) antitubercular drugs. The MICs of INH, RIF, AMK and OFX were calculated in the absence and in the presence of scalar sub-inhibitory concentrations of each compound against *M. tuberculosis* H37Rv, that is known to have intrinsic and readable efflux activity of these anti-TB drugs [32] (Table 3).

To begin with, compounds 144, 145, 154 & 163 were tested at ½ MIC followed by two-dimensional broth microdilution checkerboard assay [33] which is used to calculate magnitude of synergism. All the four compounds displayed highest level of synergism at ½ MIC in both antibiotics as well as Ethidium bromide (EtBr). Here, the MIC values of antibiotic and EtBr have observed to decrease more than 64–128 folds. Compound 145 have shown promising synergism in general, even at concentration of 0.25 μg/mL in case of RIF. Compound 145 also imparted synergistic action with second line antibiotics at 2 μg/mL increasing potency by 128 folds. It is also noted that compound 145 also reflected synergistic activity with TZ on efflux of EtBr by 64 folds at 8 &4 μg/mL while it also produced synergistic action at even 2 μg/mL. Thus we can look at compound 145 as potent dual inhibitor which is better than TZ devoid of any CNS related side effects. This compound can further be explored to study its potency at nanomolar level.

Compound 144 have shown promising synergistic action with RIF and INH but it failed to reflect its potency in case of EtBr. It was frustrating to notice and state that compound 144 have displayed better antimycobacterial activity but not efflux inhibition. Thus we cannot call it as dual inhibitor. Compounds 154 and 163 have shown good effect at their ½ MIC but failed to display better inhibition at further lesser concentration.

3. Experimental

3.1. Chemistry

The melting points were recorded on electrothermal apparatus and are uncorrected. ^{1}H NMR spectra on a Bruker Avance 500 MHz and ^{13}C NMR spectra on Briker Avance300 MHz instrument using DMSO-d_6 as solvent using TMS as internal standard; the chemical shifts (δ) are reported in ppm and coupling constants (J) are given in Hertz. Signal multiplicities are represented by s, d, t, ds, dd, m, tt, and br. Mass spectra (m/z) were recorded on an ESI-TOF mass spectrometer (Bruker Micro TOF), and reported mass values are within the error limits of 5 ppm mass units. Elemental analysis was performed on a Heracus CHN-Rapid Analyser. Analysis indicated by the symbols of the elements of functions was within ±0.4% of the theoretical values. The purity of the compounds was checked on silica gel coated Al plates (Merck).

3.1.1. Synthesis of (3-Bromomethyl-5-mercapto-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **3**

To a solution of 4-amino-5-bromomethyl-4*H*-[1,2,4]triazole-3-thiol[12] (15 g, 0.071 mmol), in dichloromethane (150 mL), was added triethylamine (10.87 g, 0.107 mmol), drop wise, at 0 °C. The reaction mixture was stirred for 10 min. Then, di-tert-butyl dicarbonate (18.77 g, 0.086 mmol) was added to reaction mixture at 0 °C. After 30 min, reaction mixture was allowed to stir at room temperature overnight. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford colorless oily product.

(3-Bromomethyl-5-mercapto-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **3.** Yield 78%; mp = 176–181 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.40–1.43 (s, 9H, CH_3), 4.55–4.57 (s, 2H, CH_2Br), 8.30–8.34 (bs, 1H, NH), 12.83 (s, 1H, SH); ^{13}CNMR (300 MHz, DMSO-d_6) δ 23.5, 29.7 (for 3 carbons), 80.1, 155.6, 163.1, 168.7; HRMS [ESI]: calculated for $C_8H_{13}BrN_4O_2S$ [M^+]: 309.183; Found: 309.156.

3.1.2. Synthesis of (3-Benzyl sulfanyl-5- bromomethyl-[1,2,4] triazol-4-yl)-carbamic acid tert-butyl ester **4**

To a solution of ((3-bromomethyl-5-mercapto-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester 915 g, 0.084 mmol) in *N,N*-dimethyl formamide (75 mL), was added potassium carbonate (15.4 g, 0.111 mmol) at room temperature. After stirring for 10 min, Benzyl bromide (9.96 g, 0.058 mmol) was added to reaction mixture at room temperature. The resulting mixture was allowed to stir at room temperature for next 4 h. Progress of the reaction was monitored by TLC. After completion of reaction, the contents were poured on to ice-cold water (375 mL) and stirred for 15 min. It was then extracted with ethyl acetate (3X150 mL). Organic layer was washed with water (150 ml), dried over anhydrous Na_2SO_4, filtered, concentrated and purified by column chromatography using silica gel, to get yellowish oily product.

(3-Benzyl sulfanyl-5- bromomethyl-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **4**. Yield 69%; mp = 158–163 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.41–1.45 (s, 9H, CH_3), 4.21–4.24 (s, 2H, CH_2), 4.52–4.54 (s, 2H, CH_2Br), 7.08–7.17 (m, 5H, ArH), 8.32–8.35 (bs, 1H, NH); ^{13}CNMR (300 MHz, DMSO-d_6) δ 23.4, 29.5 (for 3 carbons), 38.4, 80.3, 127.4, 128.7, 128.8, 139.5, 127.8, 127.9, 148.7, 154.9, 163.4; HRMS [ESI]: calculated for $C_{15}H_{19}BrN_4O_2S$ [M^+]: 399.306; Found: 399.328.

3.1.3. Synthesis of 3-(2,3-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one **7**

2,3-Dichlorobenaldehyde (0.072 mmol) was added to a suspension of 1-(2-hydroxyphenyl)ethanone (5.0 g, 0.036 mmol), in

Table 3
Evaluation of the synergistic effect and determination of the modulation factor (MF) of the selected compounds with first- and second-line drugs against *M. tuberculosis* H37Rv.

ID	Test Conc[a]	MIC (μg/mL) against *M. tuberculosis* H37Rv (MF)[g]				
		INH[b]	RIF[c]	AMK[d]	OFX[e]	EtBr[f]
No compd		0.1	1	2	2	12.5
144	16	0.00156 (↓64)	0.0156 (↓64)	0.0312 (↓64)	0.0312 (↓64)	0.390 (↓32)
	8	0.025 (↓4)	0.0312 (↓32)	0.25 (↓8)	1	1.56 (↓8)
	4	0.1	0.125 (↓8)	1	1	6.25
	2	0.1	0.25 (↓4)	1	1	12.5
	1	0.1	0.5	1	1	12.5
	0.5	0.1	0.5	1	1	12.5
	0.25	0.1	1	1	2	12.5
145	8	0.00078 (↓128)	0.0156 (↓64)	0.0312 (↓64)	0.0156 (↓128)	0.195 (↓64)
	4	0.00312 (↓32)	0.0156 (↓64)	0.25 (↓8)	0.0156 (↓128)	0.195 (↓64)
	2	0.0125 (↓8)	0.0156 (↓64)	0.25 (↓8)	0.0312 (↓64)	0.781 (↓16)
	1	0.05	0.0156 (↓64)	1	0.25 (↓8)	6.25
	0.5	0.1	0.0312 (↓32)	1	1	12.5
	0.25	0.1	0.0312 (↓32)	1	1	12.5
	0.12	0.1	0.0625 (↓16)	1	1	12.5
154	64	0.05	0.0312 (↓32)	1	1	6.25
	32	0.1	0.125 (↓8)	1	1	12.5
	16	0.1	0.25 (↓4)	2	2	12.5
	8	0.1	0.25 (↓4)	2	2	12.5
	4	0.1	0.25 (↓4)	2	2	12.5
	2	0.1	0.25 (↓4)	2	2	12.5
	1	0.1	0.5	2	2	12.5
163	128	0.00156 (↓64)	0.0156 (↓64)	0.125 (↓16)	0.625 (↓32)	0.390 (↓32)
	64	0.1	0.25 (↓4)	1	1	6.25
	32	0.1	0.5	1	1	12.5
	16	0.1	0.5	1	1	12.5
	8	0.1	0.5	1	1	12.5
	4	0.1	0.5	1	2	12.5
	2	0.1	0.5	1	2	12.5
TZ	8	0.00156 (↓64)	0.0156 (↓64)	0.0312 (↓64)	0.0312 (↓64)	0.195 (↓64)
	4	0.0125 (↓8)	0.5	0.25 (↓8)	1	1.562 (↓8)
	2	0.1	0.5	1	1	3.125 (↓4)
	1	0.1	0.5	1	1	6.25
	0.5	0.1	0.5	1	1	6.25
	0.25	0.1	0.5	1	2	12.5
	0.12	0.1	1	1	2	12.5
VP	256	0.0125 (↓8)	0.125 (↓8)	0.25 (↓8)	0.25 (↓8)	0.781 (↓16)
	128	0.05	0.5	1	1	3.12 (↓4)
	64	0.1	0.5	1	1	6.25
	32	0.1	0.5	1	2	12.5
	16	0.1	0.5	1	2	12.5
	8	0.1	1	1	2	12.5
	4	0.1	1	2	2	12.5

The modulation factor (MF) was calculated with the following formula.$MF = \frac{MIC_{antibiotic}}{MIC_{combination}}$
The modulation factor reflects a reduction of the MIC values of a given antibiotic in the presence of an inhibitor and was considered significant when MF ≥ 4 (≥four-fold reduction). Allexperiments were repeated at least three times.
[a] Concentration at which the compound was tested (in μg/mL).
[b] Isoniazid.
[c] Rifampin.
[d] Amikacin.
[e] Ofloxacin.
[f] Ethidium bromide; TZ, thioridazine; VP, verapamil.
[g] Modulation factor.

ethanol (50 mL). The mixture was stirred at room temperature overnight. Progress of reaction was monitored by TLC. After completion of reaction, the contents were poured on to ice cold water and acidified with hydrochloric acid (2M) till pH~4. Solid thus obtained was filtered, dried and purified by recrystallization from ethanol to obtain 3-(substituted-phenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one.

3-(2,3-Dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one **7**. Yield 73%; mp = 122–127 °C;^{1}H NMR (500 MHz, DMSO-d_6) δ 4.92–4.93 (s, 1H, OH), 6.92–6.96 (d, 1H, ArH), 7.02–7.05 (m, 1H, ArH), 7.09–7.12 (d, 1H J = 4 MHz, ArH), 7.16–7.19 (d, 1H J = 4 MHz, ArH), 7.24–7.26 (s, 1H, ArH), 7.32–7.37 (d, 1H J = 2 MHz, CH=CH), 7.41–7.44 (m, 1H, ArH), 7.67–7.69 (d, 1H, ArH), 8.15–8.19 (d, 1H J = 2 MHz, CH=CH);^{13}CNMR (300 MHz, DMSO-d_6) δ 116.6, 121.6, 121.8, 122.9, 126.7, 129.5, 130.5, 131.4, 131.6, 132.8, 134.6, 136.2, 145.4, 161.6, 189.5; HRMS [ESI]: calculated for $C_{15}H_{10}Cl_2O_2$[M^+]: 293.145; Found: 293.127.

*3.1.4. Procedure for synthesis of {3-Benzyl sulfanyl-5-[2-(3-2,3-dichlorophenyl-acryloyl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-carbamic acid tert-butyl ester **8***

To a solution of 3-(2,3-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one **7** (0.013 mmol) in *N,N*-dimethyl formamide (20 mL) was added potassium carbonate (2.22 g, 0.016 mmol) at room temperature. After stirring for 10 min, a solution of (3-Benzyl sulfanyl-5- bromomethyl-[1,2,4]triazol-4-yl)-carbamic acid tert-butyl ester **4** in *N,N*-dimethyl formamide (10 mL), was added to reaction mixture at room temperature. The resulting mixture was

allowed to stir at room temperature overnight. Progress of the reaction was monitored by TLC. After completion of reaction, the contents were poured on to ice cold water, stirred for 15 min. It was then filtered under suction, dried under high vacuum to get off white solid product.

{3-Benzyl sulfanyl-5-[2-(3-2,3-dichlorophenyl-acryloyl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-carbamic acid tert-butyl ester **8**. Yield 68%; mp = 135–139 °C;^{1}H NMR (500 MHz, DMSO-d_6) δ1.42-1.46 (s, 9H, CH_3), 4.23–4.25 (s, 2H, CH_2), 5.23–5.27 (s, 1H, CH_2), 6.91–6.95 (d, 1H, ArH), 7.01–7.06 (m, 6H, ArH), 7.10–7.13 (d, 1H J = 4 MHz, ArH), 7.17–7.20 (d, 1H J = 4 MHz, ArH), 7.25–7.27 (s, 1H, ArH), 7.33–7.36 (d, 1H J = 2 MHz, CH=CH), 7.42–7.44 (m, 1H, ArH), 7.68–7.70 (d, 1H, ArH), 8.17–8.21 (d, 1H J = 2 MHz, CH=CH), 8.30–8.35 (br, 1H, NH); ^{13}CNMR (300 MHz, DMSO-d_6) δ 28.5 (3 carbon), 38.2, 62.5, 79.2, 114.8, 121.2, 121.4, 121.7, 126.8, 127.3, 127.7, 127.9, 128.7, 128.8, 129.3, 130.4, 130.8, 131.1, 132.7, 134.8, 135.5, 139.8, 145.3, 148.6, 151.5, 154.6, 161.3, 189.4; HRMS [ESI]: calculated for $C_{30}H_{28}Cl_2N_4O_4S$ [M^+]: 611.539; Found: 611.524.

3.1.5. Synthesis of PDST121

3.1.5.1. Cyclization. {3-Benzyl sulfanyl-5-[2-(3-2,4-dichlorophenyl-acryloyl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-carbamic acid tert-butyl ester **8** (0.0018 mol) and hydroxyamine(0.0036 mol) were heated in triethylamine (15 mL). As soon as, the solution starts bumping (10–15 min), heating was stopped. Te reaction mixture was cooled and poured onto ice-cold water. The product thus obtained was filtered, washed with water and recrystallized from ethanol to give Boc and benzyl protected **PDST121**.

3.1.5.2. Debenzylation. One of the Boc and benzyl protected **PDST121** (0.0012 mol) was dissolved in Methanol (10 mL) and 10% Pd/C (50%wet) catalyst (2 times) was added into this solution. The resulting mixture was kept in shaker under hydrogen pressure of 80psi for 4 h. Progress of the reaction was monitored by TLC. After completion of reaction, reaction mixture was filtered through celite under suction, washed with methanol. Filtrate and washings were mixed together and concentrated under reduced pressure, dried under high vacuum to get colorless oily debenzylated but Boc protected **PDST121** product.

3.1.5.3. Boc deprotection. One of the Boc protected **PDST121** (0.0009 mol) was dissolved in dichloromethane (2.5 mL). This solution was cooled to −10 °C. Trifluoro acetic acid (2.5 mL) was added slowly, drop wise to this solution at −10 °C over a period of 10 min. The resulting mixture was allowed to stir at −10 °C for 1 h. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mass was poured onto Petroleum ether (50 mL), stirred for 5 min, decanted. This process was repeated thrice. The thick oily material was then poured onto diethyl ether (50 ml), stirred for 15 min, decanted. This process was also repeated thrice. Finally, the solid obtained was dried under high vacuum to give **PDST121**.

5-((2-(5-(2,4-dichlorophenyl)-4,5-dihydroisoxazol-3-yl)phenoxy) methyl)-4-amino-1,2,4-triazole-3-thiol **PDST121.** Yield 55%; mp = 147–152 °C; White solid;^{1}H NMR (500 MHz, DMSO-d_6) δ 3.03–3.05 (d, 1H, CH_2), 3.32–3.34 (d, 1H, CH_2), 4.55–4.56 (t, 1H, CH), 4.91 (s, 2H, NH_2), 5.21 (s, 2H, CH_2), 6.82–6.85 (m, 2H, ArH), 7.04–7.05 (d, 1H, ArH), 7.05–7.07 (d, 1H, ArH), 7.15–7.17 (m, 1H, ArH), 7.21 (s, 1H, ArH), 7.51–7.55 (m, 1H, ArH), 12.5 (s, 1H, SH),; ^{13}CNMR (300 MHz, DMSO-d_6) δ 41.8, 62.2, 71.5, 114.5, 117.1, 121.4, 127.3, 130.0, 130.1, 130.5, 131.2, 133.9, 134.7, 135.4, 151.4, 156.4, 160.3, 167.3; HRMS [ESI]: calculated for $C_{18}H_{15}Cl_2N_5O_2S$ [M^+]: 435.0324; Found: 435.0342.

3.1.6. N-(3-((2-(5-(2,4-dichlorophenyl)-4,5-dihydroisoxazol-3-yl) phenoxy)methyl)-5-mercapto-4H-1,2,4-triazol-4-yl)substituted-amide ***PDST136-PDST138***

Mixture of triazole **PDST121** (0.01 mol) and Na_2CO_3 (1 mol) in ethanol was treated drop-wise with an equimolar amount of the acid/acetyl chloride at 0 °C, which was stirred for 30–45 min. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford white product. The precipitate was then filtered, washed thoroughly with water and crystallized to yield **PDST136-PDST138**.

3.1.7. N-(3-((2-(5-(2,4-Dichlorophenyl)-4,5-dihydroisoxazol-3-yl) phenoxy)methyl)-5-((ethoxycarbonyl)methylthio)-4H-1,2,4-triazol-4-yl)substituted-amide ***PDST139-PDST141***

A solution of triazoles **PDST136-PDST138** (0.01 mol), (0.01 mol) of K_2CO_3 and ethyl 2-bromoacetate (0.01 mol) was prepared. The reaction was stirred at 40 °C for 2 h. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford white product. The precipitate was then filtered, washed thoroughly with water and crystallized to yield **PDST139-PDST141**.

3.1.8. N-(3-((2-(5-(2,4-Dichlorophenyl)-4,5-dihydroisoxazol-3-yl) phenoxy)methyl)-5-(substituted-thio)-4H-1,2,4-triazol-4-yl) substituted-amide ***PDST142-PDST168***

A solution of triazoles **PDST136-PDST138** (0.01 mol), (0.01 mol) of K_2CO_3 and alkyl halide (0.01 mol) was prepared. The reaction was stirred at 40 °C for 4 h. Progress of the reaction was monitored by TLC. After completion of reaction, the reaction mixture was washed with water (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford white product. The precipitate was then filtered, washed thoroughly with water and crystallized to yield **PDST142-PDST168**. (See Supplementary information)

3.2. Pharmacology

3.2.1. Strains, growth conditions and reagents

Two mycobacterial strains differed in virulence were used primarily in this study: *M. smegmatis* mc^{2}155 ATCC700084 and *M. tuberculosis* H37Rv ATCC27294T. *M. tuberculosis* strains were maintained in Middlebrook broth medium. Mycobacteria-specific Middlebrook 7H9 and 7H10 media were obtained from Difco (Detroit, MI, USA) supplemented with 0.2% glycerol, 0.05% Tween 80 and 10% ADS supplement. Thioridazine (TZ), verapamil (VP), isoniazid (INH), rifampicin (RIF), amikacin (AMK), ofloxacin (OFX), EtBr, phosphate-buffered saline (PBS), and glucose were purchased from Sigma–Aldrich (India). All solutions were prepared in deionized water, except rifampicin and the PDST101-135, which were prepared in DMSO. All solutions were prepared on the day of the experiment.

3.2.2. Determination of minimum inhibitory concentration

For *M. smegmatis* the determination of the MICs of synthesized compounds, the efflux inhibitors TZ and VP, and the efflux substrate ethidium bromide were conducted by the 96-well broth microdilution method. Briefly, *M. smegmatis* was grown in MB7H9 supplemented with 10% OADC at 37 °C with shaking until an OD_{600} of 0.8. The inoculum was prepared by adjusting the culture to a density corresponding to 0.5 McFarland standard diluted to 1:100. Aliquots of 0.1 mL were transferred to each well of the 96-well plate containing 0.1 mL of each compound at concentrations prepared

from two-fold serial dilutions in MB7H9. The inoculated plates were sealed in plastic bags, incubated at 37 °C and the results registered after three days of incubation. Growth controls with no drug and a sterility control were included in each assay. Two hundred microliters of sterile deionized water was added to all outer-perimeter wells of the 96-well plates to reduce evaporation of the medium during the incubation. The MIC was defined as the lowest concentration of compound that inhibited visible mycobacterial growth. *M. tuberculosis* was grown in MB7H9 plus 10% OADC supplement at 37 °C until an OD_{600} of 0.8, without stirring. The inoculum was prepared by diluting the bacterial cultures in MB7H9/OADC to a final density of approximately 10^5 cells/ml. The MICs of analogues, TZ, VP, and ethidium bromide, were determined by a tetrazolium microplate-based assay with slight modifications. Briefly, aliquots of 0.1 ml of inoculum were transferred to each well of the plate that contained 0.1 ml of each compound at concentrations prepared from two-fold serial dilutions in MB7H9/OADC medium. Growth controls and a sterility control were included in each assay. The outer perimeter wells of the plates were filled with two hundred microliters of sterile deionized water to reduce evaporation of the medium during the incubation. The inoculated plates were sealed in plastic bags and incubated at 37 °C during seven days. After the seven days of incubation, MTT was added to each well to a final concentration of 2.5% and the plates incubated overnight. The bacterial viability was registered for each well based on the MTT color change and the MIC was defined as the lowest concentration of compound that totally inhibited bacterial growth (no color change) [34]. The assays were performed in triplicate.

3.2.3. Evaluation of synergistic effect with selected antibiotics and ethidium bromide by broth microdilution checkerboard assay

The synergistic effect of synthesized analogs and that of the efflux inhibitors TZ and VP in combination with INH, OFX, AMK, RIF and EtBr was evaluated by two dimensional checkerboard assays. *M. tuberculosis* was grown in MB7H9 medium supplemented with 10% OADC at 37 °C, until an OD_{600} of 0.8. Briefly, the 96-well microdilution plates were inoculated with a suspension of the strain diluted to yield a density of 10^5 CFU/ml. Ethidium bromide or the antibiotics were two-fold serial diluted in MB7H9 supplemented with OADC from column 3 to column 11 of each plate assay. Twofold dilutions of compounds, TZ or VP were then added to rows 2 to 6 of each plate. Column 1 and 2 contains the negative and positive control, respectively. Two hundred microliters of sterile deionized water was added to the outer-perimeter wells of the plates to decrease evaporation of the medium during incubation. The plates were sealed and incubated at 37 °C for seven days. After this period, MTT was added, the plates were re-incubated overnight, and the results interpreted as described above. The synergistic effect of TZ analogues and efflux inhibitors on each antibiotic and ethidium bromide was evaluated through the determination of fractional inhibitory concentrations (FIC), according to the formula: $FIC_{compound} = MIC_{compound}$ in the presence the antibiotic/EtBr divided by $MIC_{compound}$ alone. The FIC were interpreted adapting the criteria established by Pillai et al. to one variable as follows: FIC $\leq$0.25, synergism; FIC > 0.25 < 2, indifference and FIC $\geq$ 2, antagonism. All assays were carried out in triplicate.

3.2.4. Evaluation of efflux inhibitory activity of compounds by real-time fluorometry

The EtBr accumulation and efflux by the mycobacterial strains was assessed on a real-time basis using a fluorometric method, as previously described*M. smegmatis* and *M. tuberculosis* were grown as described above, with the addition of 0.05% Tween 80 to the growth medium. (i) 3.2.4.1. Accumulation assays.

After reaching an OD_{600} of 0.8, the cells were collected by centrifugation at 2940 x g for 3 min, the pellet washed in PBS, and the OD_{600} of the suspension adjusted to 0.8 with PBS. To determine the concentration of EtBr at which there is an equilibrium between the influx and efflux of EtBr the assays where performed in the presence of increasing concentrations of EtBr. The assays were prepared to a final volume of 0.1 mL containing 0.05 mL of the cellular suspension (final OD_{600} of 0.4) plus 0.05 mL of EtBr solutions to final concentrations of 0.125, 0.25, 0.5, 1, 2, and 3 mg/L. To assess the effect of analogues and that of the efflux inhibitors TZ and VP on EtBr accumulation, the assays were performed in a final volume of 0.1 mL containing 0.05 mL of the cellular suspension (final OD_{600} of 0.4) and 0.05 mL of a solution containing the EtBr concentration previously selected (0.25 mg/L for *M. smegmatis* and 0.5 mg/L for *M. tuberculosis*) and the compound to be tested to a final concentration of 1/2 their MIC, in order to not compromise the cellular viability. In all assays, was included a control containing solely EtBr at the equilibrium concentration selected previously. The assays were conducted in a Vimta labs, Hyderabad, India at 37 °C, and the fluorescence acquired at 530/585 nm at the end of every 60 s, during 60 min. The activity of analogues, TZ, and VP on the accumulation of EtBr was evaluated by the relative final fluorescence (RFF) index according to the formula: $RFF = (RF_{treated} - RF_{Untreated})/RF_{untreated}$, were $RF_{treated}$ corresponds to the fluorescence at the last time point of the EtBr accumulation curve (minute 60) in the presence of a compound; and the RFuntreated corresponds to the fluorescence at the last time point of the EtBr accumulation curve of the control tube containing only EtBr.

3.2.4.1. Efflux assays. After reaching an OD_{600} of 0.8, the cells were collected by centrifugation at 2940 x g for 3 min, the pellet washed in PBS, and the OD_{600} of the suspension adjusted to 0.4 with PBS. Cell suspensions were then exposed to conditions that promote maximum EtBr accumulation, i.e. EtBr at the equilibrium concentration; presence of the efflux inhibitor that caused maximum accumulation (VP for both strains) at 1/2 of the MIC; and incubation at 25 °C during 1 h. Following, EtBr loaded cells were centrifuged at 4860 x g during 5 min and resuspended in PBS to a final OD_{600} of 0.8. Efflux assays were prepared to a final volume of 0.1 mL containing 0.05 mL of the cellular suspension (final OD_{600} of 0.4) plus 0.05 mL of analogues, TZ, and VP at 1/2 of their MIC with and without glucose at a final concentration of 0.4%, to energize the cells. In all assays, control conditions containing only cellular suspension and cellular suspension plus glucose (condition of maximum efflux) were included. Fluorescence was measured in the VRotor-Gene™ 3000 at 37 °C, at the end of every 30 s during 30 min. Efflux activity was quantified by comparing the fluorescence data obtained under conditions that promote efflux (presence of glucose and absence of efflux inhibitor) with the data from the control in which the mycobacteria are under conditions of no efflux (presence of an inhibitor and no energy source). Thus, the relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr-loaded cells (cells plus VP). Statistical analysis of the data was carried out using Student's t-test. A P value < 0.05 was considered statistically significant (two-tailed tested).

3.2.5. Cytotoxicity screening

In vitro cytotoxicity assays were evaluated against human monocyte-derived macrophages. Blood was collected from healthy volunteers and peripheral blood mononuclear cells isolated by Ficoll–Paque Plus (Vimta Labs, Hyderabad, India) density gradient centrifugation. Monocytes were differentiated into macrophages during seven days in macrophage medium containing RPMI-1640 medium with 10% fetal calf serum (FCS), 1% GlutaMA, 1 mM sodium pyruvate, 10 mM HEPES at pH 7.4, 100 IU/ml penicillin and

100 μg/ml streptomycin (Gibco, Life Technologies), and 20 ng/ml M-CSF (Vimta Labs, Hyderabad, India) and incubated at 37 °C in 5% CO_2medium was added at day 4 post isolation. The effect of the TZ analogues was evaluated by using the vital dye Alamar Blue (Molecular Probes, Life Technologies) following the manufacturer'sindications. Briefly, 5×10^4 atm. Freshcells were seeded in 96-well microplates, treated with the compounds and then incubated at 37 °C in a 5% CO_2atmosphere. After 3 days of treatment, cell viability was assessed. Briefly, 10% Alamar Blue was added to each well and incubated during 4 h at 37 °C and 5% CO_2

Fluorescence was measured with a 540/35 excitation filter and a 590/20 emission filter in a Synergy HT multi-mode microplate reader (BioTek® Instruments, Inc, Vermont, USA). The IC_{50} corresponds to the highest concentration of compound at which 50% of the cells are viable relative to the control.

3.2.6. Intracellular synergy assay

Human monocyte-derived macrophages were infected with M. tuberculosis H37Rv at a multiplicity of infection (MOI) 1:1 and were allowed to uptake the bacteria for 3 h. After, the cells were washed three times with PBS to remove all non-internalized bacteria. Afterwards, the compounds were added to the infected macrophages at the desired concentrations in combination with INH or RIF. At day three post-infection, cells were lysed with 0.05% Igepal (Sigma–Aldrich). Serial dilutions of the lysate were placed on MB7H11/10% OADC medium. Colony forming units were counted upon three weeks of incubation at 37 °C. INH and RIF were used at ½ of its MIC (0.05 and 0.5 μg/ml, respectively).

4. Conclusion

Although there are reports on efflux inhibitors role in controlling drug resistance, not much of efforts have been made by medicinal chemists. Marketed or recently identified efflux inhibitors (e.g. TZ) poses threat of side effect and toxicity limiting their use as adjuvant or dual inhibitor. This is first report aimed at synthesizing hybrid molecule of TZ, VP and triazole and their evaluation as dual inhibitors. Hit compound 135 have found to show very less toxicity compared to TZ towards human macrophages (16 folds). This compound also displayed higher growth inhibition in both *M. tuberculosis* H37Rv & *M. smegmatis* mc^2155. Compound 135 have demonstrated its synergistic effect with first line and second line antitubercular agents. Considering the data on inhibition of EtBr efflux, coupled to the antimycobacterial activity, it might be claimed that the synergetic activity is due to its ability to hamper the intrinsic mycobacterial drug efflux. Altogether, these findings provide a solid base to further investigate compound 135 as a booster of the antimycobacterial chemotherapy when associated with first- and second-line drugs, by virtue of its capability to block the intrinsic efflux activity of mycobacteria. This might represent a lateral approach toward the cure of TB.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.10.054.

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