Received: 29 August 2022 | Revised: 3 November 2022 | Accepted: 12 December 2022

DOI: 10.1111/cbdd.14197

RESEARCH ARTICLE

WILEY

Myoinositol and methyl stearate increases rifampicin susceptibility among drug-resistant *Mycobacterium tuberculosis* expressing Rv1819c

Christy Rosaline Nirmal[1] | **Sam Ebenezer Rajadas**[1] | **Mahizhaveni Balasubramanian**[1] | **Sucharitha Kannappan Mohanvel**[1] | **Muthu Sankar Aathi**[2] | **Saravanan Munishankar**[1] | **Naresh Babu Chilamakuru**[3] | **Kannan Thiruvenkadam**[1] | **Ajith Kumar Pandiya Raj**[1] | **Ramalingam Paraman**[4] | **Azger Dusthackeer**[1]

[1]ICMR-National Institute for Research in Tuberculosis, Chennai, India

[2]Ajeenkya DY Patil University, Pune, Maharashtra, India

[3]Raghavendra Institute of Pharmaceutical Education and Research (RIPER), Andhra Pradesh, India

[4]National Institute of Pharmaceutical Education and Research (NIPER), Hajipur, India

Correspondence
Azger Dusthackeer, ICMR-National Institute for Research in Tuberculosis, Chennai, India.
Email: azger@nirt.res.in

Present address
Sam Ebenezer Rajadas, Department of Biotechnology, VSB Engineering College, Karur, India

Funding information
Indian Council of Medical Research

Abstract

The alarming increase in multidrug resistance, which includes Bedaquiline and Delamanid, stumbles success in Tuberculosis treatment outcome. *Mycobacterium tuberculosis* gains resistance to rifampicin, which is one of the less toxic and potent anti-TB drugs, through genetic mutations predominantly besides efflux pump mediated drug resistance. In recent decades, scientific interventions are being carried out to overcome this hurdle using novel approaches to save this drug by combining it with other drugs/molecules or by use of high dose rifampicin. This study reports five small molecules namely Ellagic acid, Methyl Stearate, Myoinositol, Rutin, and Shikimic acid that exhibit synergistic inhibitory activity with rifampicin against resistant TB isolates. In-silico examinations revealed possible blocking of Rv1819c—an ABC transporter efflux pump that was known to confer resistance in *M. tuberculosis* to rifampicin. The synergistic anti-TB activity was assessed using a drug combination checkerboard assay. Efflux pump inhibition activity of ellagic acid, myoinositol, and methyl stearate was observed through ethidium bromide accumulation assay in the drug-resistant *M. tuberculosis* clinical strains and recombinant *Mycobacterium smegmatis* expressing Rv1819c in coherence with the significant reduction in the minimum inhibitory concentration of rifampicin. Cytotoxicity of the active efflux inhibitors was tested using in silico and ex vivo methods. Myoinositol and methyl stearate were completely non-toxic to the hematological and epithelial cells of different organs under ex vivo conditions. Based on these findings, these molecules can be considered for adjunct TB therapy; however, their impact on other drugs of anti-TB regimen needs to be tested.

KEYWORDS

drug resistance, efflux inhibitors, efflux pump, Rv1819c, tuberculosis

1 | INTRODUCTION

Tuberculosis (TB) is a chronic disease that is enlisted in the second position of the top 10 fatal infectious diseases next to HIV/AIDS. India, being a country with a high TB prevalence is determined to become a TB-free nation by 2025 ahead of the global target of 2035 fixed by the World Health Organization (WHO; Shrivastava & Shrivastava, 2021; World Health Organization, 2019). The growing incidences of drug resistance especially multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB is a threat to achieving this target (Liu et al., 2021). Globally in the year 2019 nearly half a million people were identified with rifampicin (RMP) resistant TB (RR-TB; World Health Organization, 2020a), which includes 78% of MDR-TB cases (World Health Organization, 2020b).

The first drug to be approved by the FDA to treat MDR TB in 45 years is bedaquiline in 2017 (Ghajavand et al., 2019). Despite being unsafe for cardiac patients, 68 countries had adapted bedaquiline to treat MDR−/rifampicin resistant-TB, since it reduces the duration of the TB treatment (da Silva et al., 2016). However, *Mycobacterium tuberculosis* (Mtb) has gained efflux-mediated resistance against bedaquiline also (Rodrigues et al., 2013). Mutation in the drug target genes drives the pathogen to become resistant to anti-TB drugs by expelling the anti-TB through efflux pumps outside the Mtb cell wall (da Silva et al., 2016; Nikaido, 2001; Song & Wu, 2016). The major facilitator super family (MFS), ATP-binding cassette (ABC), resistance-nodulation and cell division (RND), and small multidrug resistance (SMR) families of efflux pumps are found in Mtb (Viveiros et al., 2012). The resistance mechanisms exhibited by efflux pumps are synergistic with a permeable reactive barrier to lessen the passage of antimicrobials across the bacterial membrane (Caminero et al., 2017). Several Macrophage-induced Mtb efflux pump proteins are also reported previously that ensure Mtb intracellular survival (Cosma et al., 2003; Gupta et al., 2014). Thus, efflux pumps are viewed as an acceptable target to handle resistance menace (Amaral et al., 2007). The efflux pump inhibitors (EPIs) reduce the drug efflux by blocking the efflux pumps and thereby increase the drug bioavailability. EPIs have been considered as adjunct molecules along with antibiotics and have been taken to human trials (Sharma et al., 2019).

These EPIs subsequently reverse Mtb drug resistance and produce synergistic effects with first-line anti-TB drugs (Pule et al., 2016; Shepard, 1957). Moreover, in a recent report by Remm et al. (2022), the efflux pump inhibitors dissipate the proton motive force leading to a decrease in the energy of all active membrane transporters (Remm et al., 2022). Verapamil, protonophores, and thioridazine are a few of these EPIs which were reported to have inhibitory activity on mycobacterial efflux systems (Amaral et al., 2007). Remarkably, a 2- to 16-fold reduction in resistance against bedaquiline and clofazimine was observed in the presence of verapamil (Camacho-Corona et al., 2008). Rv1819c, belonging to the ABC transporter family is one such EP that is made of six transmembrane domains. Rv1819c codes for putative ABC transporter with negative surfaced cavity specifically for long-chain fatty acid transportation including Vitamin B12 and drugs like bleomycin (Ramón-García et al., 2007; Rempel et al., 2020). It acts as an importer of Vitamin B12 which functions as a core element in the Mycobacterial pathway. Rv1819c knockout was shown to confer up to 32-fold resistance to bleomycin when compared to wild type H37RV (Gopinath et al., 2013). Over expression of Rv1819c was observed in several clinical isolates when treated with rifampicin (Gupta et al., 2010). It is also associated with chronic TB infection and Mtb survival within the host during the treatment regimen (Domenech et al., 2009). It was also reported to get over-expressed at five folds in Mtb H37Rv (virulent strain) in contrast to H37Ra (avirulent strain) denoting its relationship with Mtb virulence (Målen et al., 2011). Its ciprofloxacin effluxing activity has been previously proven in the heterologous host *Escherichia coli* BL21-AI (Mazando et al., 2017). Within the macrophages Mtb survival is based on the ability to inhibit phagosomes. The genes present in Mtb mutants that are unable to inhibit the phagosomes showed homologues to MDR efflux pumps and with the enzymes which synthesis lipids. There is evidence that MDR ABC transporters involves in transportation of lipids. The efflux pump Rv1819c may involve in lipid transportation within the exterior of the bacterium (Tarling et al., 2013). Hence, targeting Rv1819c with potent inhibitors is expected to down-regulate the efflux pump activity of Rv1819c besides limiting the TB progression (Rodrigues et al., 2013). This study is an attempt to test the EP inhibiting activity of five EPIs namely ellagic acid, methyl stearate, myoinositol, rutin, and shikimic acid against Mtb efflux pump, Rv1819c through in silico and in vitro methods.

2 | MATERIALS AND METHODS

2.1 | Bacterial cultures and strains used

Middle brook 7H9 medium enriched with oleic acid, albumin, dextrose, and catalase (OADC; 7H9) was used to grow *Mtb* and *Mycobacterium smegmatis*. Luria-Bertani (LB) medium is used for *E. coli* DH5α. Two MDR-TB (NIRT-001, NIRT-002) and one XDR-TB (NIRT-003) isolates that were used for the study were retrieved from the NIRT repository of clinical Mtb isolates. These

isolates were characterized by AFB smear microscopy, solid-culture (egg-based LJ media- MIC method for drug susceptibility testing [DST]), and also by liquid culture (MGIT960 based culture), and the respective DST systems. *E. coli* DH5α competent cells (Novagen) used in the gene cloning were propagated according to the manufacturer's instructions. The antibiotics kanamycin (35 μg/ml) and ampicillin (100 μg/ml) were used for screening the transformants. PCR primers 5′-GGAAT TCTTGGGCCCGAAATTGTTTAAGC-3′ (forward) and 5′-CCGGAATTCTTGGGCCCGAAATTGTTTAAGC-3′ (reverse) specific to Rv1819c were procured from GCC Biotech Chennai.

2.2 | Rv1819c expression analysis in Mtb drug-resistant strains

The expression of Rv1819c in MDR clinical isolates was analyzed through Quantitative Real-Time PCR (qRT-PCR). RNA was extracted using the TRIZOL method from mid-log phase cultures of *M. tuberculosis* H37Rv and MDR-TB clinical isolates. These cultures were grown in the presence and absence of sub-inhibitory concentration of RMP (1/4 of the MIC). The cDNA was synthesized from the isolated RNA, by following the manufacturer's protocol using the Verso cDNA kit. RNase-free DNase was used to remove chromosomal DNA contamination. Primers for the Mtb 16s RNA were used as the internal control. The qRT-PCR was carried out in Thermo scientific ABI-7500. The experiment was performed in triplicates.

2.3 | Homologous overexpression of Mtb Rv1819c in *M. smegmatis* mc^2 155

The expression of Rv1819c in *M. smegmatis* mc^2 155 was carried out as described earlier (Mazando et al., 2017). Initially, the Rv1819c gene was PCR amplified from the *Mtb* chromosome and transformed to *E. coli* DH5α using the pMV261 shuttle vector. The transformed cells were selected by antibiotic screening by spreading them on LB agar plates that were incorporated with Kanamycin at a final concentration of 35 μg/ml. The recombinant pMV261-Rv1819c was then electroporated into *M. smegmatis* mc^2 155 competent cells that were prepared as described elsewhere (Venkatesh et al., 2003). In brief, the freshly prepared competent cells (400 μl) were electroporated with 200 to 400 ng of recombinant pMV261-Rv1819c. The transformation was confirmed through antibiotic screening, PCR (using Rv1819c gene-specific primers), and also by sequencing methods.

2.4 | In silico selection of lead compounds against efflux pump Rv1819c

Active efflux inhibitors from the plant sources were retrieved from duke's database and were used for virtual screening. In silico lead compound identification was performed using Maestro (version 9.1), LigPrep (version 9.1), Phase (version 9.1), Protein Preparation, High Throughput Virtual Screening, Glide, and Induced Fit docking of Schrodinger.

The active site was predicted using site map prediction tool, and grid was generated in the potential active site. Docking analysis was performed by using the Maestro v9.1 (Friesner et al., 2006). The receptor Rv1819c was subjected to docking and the active compounds from the database were optimized with the OPLS_2005 force field. Different ligand conformations were generated. The compounds with more than a 1.0 fitness score were docked with Rv1819c.

Lipinski's rule of five drug-likeness of the compounds was predicted using the Swiss ADME tool to access the bioavailability of the identified lead inhibitors (Daina et al., 2017). Based on the glide score and the drug-likeness property the compounds were selected for the in vitro validation.

2.5 | Determination of minimum inhibitory concentration by micro broth dilution method

Minimum inhibitory concentration (MIC) of RMP, piperine (known EPI), and the selected EPIs against two MDR, and one XDR clinical isolates were determined using the broth micro-dilution method, as described elsewhere (do Amaral et al., 2015; Makane et al., 2019). In brief, the mid-log phase Mtb clinical cultures (~10^5/ml) were exposed to a range of concentrations of the test molecules in 96-well microtiter plates containing a lid. The dilution of test molecules was made using Middlebrook 7H9 media. Culture control (10^5 and 10^2/ml) and solvent control (DMSO) were also included in the assay. Post 5 days of incubation at 37°C, the plates were observed through an inverted light microscope for Mtb growth in the form of serpentine chords. The least concentration that completely inhibited the Mtb growth was considered as its MIC. All the tests were performed in triplicates.

Similarly, MICs of EPIs, isoniazid (INH), and RMP were determined against recombinant Rv1819c in *M. smegmatis* mc^2 155 as described previously (Caviedes et al., 2002). In Brief, the culture was grown in 7H9 at 37°C until its OD_{600} reaches 0.5. This culture suspension was further diluted 1000 times in 7H9 broth. In a 96-well plate, 100 μl of this

culture suspension was dispensed and exposed to a range of concentrations of test molecules. The plates were incubated at 37°C for 48 h. The wells with bacterial turbidity were considered as positive growth, whereas the one with transparent clear media was taken as negative. The corresponding drug concentration to the last negative well was considered as MIC of that particular test molecule. All the experiments were carried out in triplicates.

2.6 | Checkerboard titration assay

Checkerboard broth dilution assay or drug-combination assay was performed as described elsewhere to determine the synergism between selected EPIs with RMP. Piperine a known efflux pump inhibitor was used as a control (do Amaral et al., 2015; Makane et al., 2019). The two-fold dilutions of each of the drugs with all possible combinations starting with its corresponding MICs were prepared using 7H9 broth. The culture inoculum size (Mtb MDR, XDR clinical isolate, and Rv1819c recombinant *M. smegmatis* mc^2 155), controls (culture control and solvent control) and incubation were the same as it was in the MIC determination.

2.7 | Ethidium bromide accumulation assay

Ethidium bromide accumulation assay was carried out for the recombinant *M. smegmatis* mc^2 155 expressing Rv1819c on a real-time basis as described elsewhere to assess the efflux pump inhibition (Rodrigues et al., 2013). Recombinant *M. smegmatis* mc^2 155 Rv1819c was grown in 7H9 with OADC enrichment at 37°C until the culture reaches OD_{600} of 0.8. Cultures were centrifuged at 3000*g* for 15 min, and the pellet was re-suspended in phosphate buffer saline (PBS; pH 7.4), and adjusted to OD_{600} of 0.1. To each well of 96-well black plates, 100 µl of this culture suspension was added. Ethidium Bromide at the final concentration of 1 µg/ml was added. The efficiency of the lead compounds in accumulating EtBr was determined by adding 10 µl of each of the EPIs at a final concentration of 0.5× of its MICs. Fluorescence intensity was measured using a SpectraMax multimode reader (Spinco Biotech; 530 nm excitation and 590 nm emission) for 30 at 5 min intervals. The test was performed in triplicates. An unpaired *t*-test was used to find the statistical significance between the treated and untreated groups. The accumulation of EtBr is expressed in terms of the percentage of fluorescence at each time point using the formula given below.

$$\text{Percentage increase in Fluorescence} = \left[\left(Ft_{25} - Ft_0\right) / Ft_0\right] \times 100 \quad (1)$$

whereas, Ft_{25}, Fluorescence at 25th minute and Ft_0, Fluorescence at 0th minute.

2.8 | Assessment of cytotoxicity of the selected efflux pump inhibitors

The cytotoxicity of these lead molecules was assessed by both in silico and ex vivo-based approaches.

2.8.1 | In silico-QSAR and toxicity analysis

The drugability and toxicity of selected active compounds were predicted using the Derek Nexus module of Stardrop software (Optibrium; Camacho-Corona et al., 2008) as described elsewhere (Morris et al., 2009). This module assists to predict the relationship between the molecule structure and toxicity by evaluating endpoints like mutagenicity, hepatotoxicity, and cardio toxicity.

2.8.2 | Ex vivo cytotoxicity testing

Cytotoxicity of EPIs was tested on the established cell lines namely Vero cells, HepG2 cells, and THP1 using resazurin assay (Rodríguez-Corrales & Josan, 2017), whereas the same was done in peripheral blood mononuclear cells (PBMCs). The toxicity of the identified EPIs was initially tested on PBMCs, by trypan blue exclusion assay (Fishwild et al., 1991). The PBMCs were separated from the human peripheral blood using Hi-Sep 1.077 solution (HiMedia) and suspended in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Vero cells were propagated in DMEM media supplemented with 10% FBS. HepG2 cells were grown in MEM with 10% FBS. THP1 cells were grown in RPMI1640 medium with 10% FBS. No antibiotics were added to the medium for all the cells. Each well of the 96-well plate (Tarsons) was loaded with 10^4 cells and treated with selected EPIs at the concentration of 2×, 1×, and 0.5× of their MIC as determined previously. Untreated cells were kept as control and the assay was done in triplicates. The treated and untreated control cells were incubated at 37°C in the presence of 5% CO_2. Post 24 h of incubation, the cells were treated with 0.1% resazurin. After 24 h of resazurin treatment, plates were read using a multimode reader (Spinco Biotech) at 530 nm excitation and 590 nm

emission. The percentage of cell viability was estimated using the formula given below (Equation 3.). All the experiments were done in triplicates.

$$\text{Percentage of viability} = \left\{ \frac{(\text{Number of viable cells in test})}{\text{Number of viable cells in untreated control}} \right\} \times 100 \quad (2)$$

$$\text{Percentage of viability} = \left\{ \frac{(\text{Fluorescence units in test})}{\text{Fluorescence units in untreated control}} \right\} \times 100 \quad (3)$$

2.8.3 | Hemolytic activity testing

Cytotoxicity of the selected active EPIs was also tested through a hemolytic activity test as described elsewhere (Greco et al., 2020). In brief, washed RBCs that are free of complement proteins were diluted ten times in PBS (pH-7.0) and are exposed to the EPIs at the concentration of 2×, 1×, and 0.5× of its MIC as determined through the checkerboard assay. RBCs exposed to distilled water were kept as positive control while untreated RBCs suspended in the PBS were considered as a negative control. Post 2 h of incubation at 37°C, the cells were centrifuged at 500*g* for 10 min. The optical density of the supernatant was measured at 541 nm. The assay was carried out in triplicates and the percentage of hemolysis was estimated using the formula given below,

$$\text{Percentage of Hemolysis} = \left\{ \frac{\text{Absorbance of test} - \text{absorbance of negative control}}{\text{Absorbance of positive control} - \text{absorbance of negative control}} \right\} \times 100$$

3 | RESULTS

3.1 | Rv1819c expression analysis in Mtb drug-resistant strains

The qRT-PCR was used to measure mRNA levels of Rv1819c in *M. tuberculosis* H37Rv and the MDR clinical isolate grown in the presence and absence of RMP (1/4 the MIC). Data are the means of three independent experiments. The qRT-PCR analysis revealed that there was a five-fold increase in the expression level of Rv1819c in selected MDR clinical isolate when compared with RMP un-induced and *M. tuberculosis* H37Rv (Figure 1).

3.2 | In silico screening to identify lead compounds against efflux pump Rv1819c

The active site was identified using sitemap for Rv1819c protein without the co-crystal molecule. The sitemaps 1–5 were identified with site scores 1.213, 0.981, 0.956, 0.818, and 0.664. Among these sitemaps, 1 was selected due to its higher size 529, Volume 1399.44, exposure 0.58, phobic region 0.370, philic region 1.176, don/acc 1.200. The active site's receptor grid was generated using sitemap 1 entry with XYZ coordinates 97.63, 64.4, 154.36, and the box length used in *X*, *Y*, and *Z* was selected 20A° to completely fit the sitemap 1 in the grid box. Rv1819c with predicted active sites were subjected to High Throughput Virtual Screening, followed by SP and XP docking with a set of compounds from the duke's database. Top five compounds based on the Glide score, Glide energy and amino acid interactions were selected. For each ligand, 10 confirmations were calculated and finally one best pose per ligand with post-docking minimization by OPLS4 force field was selected for the compounds. Table 1 shows the chemical names of the five lead compounds, along with their binding energy, ligand efficiency, and hydrogen bond (H-bond) interaction with Rv1819c. The structures and the interaction of efflux inhibitors are given in Figure S1.

3.3 | Drug likeness analysis for selected efflux inhibitors against Rv1819c

The selected five compounds were subjected to drug-likeness screening, which includes molecular weight, H-bond donors, H-bond acceptors, log P (octanol/water), log P Caco (cell permeability), log Kp (skin permeability), human oral absorption, and their positions according to Lipinski's rule of five. The selected compounds except rutin exhibited values in the acceptable ranges (Cloete et al., 2018) in more than five of the parameters tested (Table 2), thereby indicating the potential of the lead compounds to bind with efflux pump protein Rv1819c.

3.4 | Transformation of Rv1819c into *M. smegmatis*

The Rv1819c gene (1.9 kbp) from *M. tuberculosis* was initially transformed into *Escherichia coli* DH5α strain using pMV261 expression-shuttle vector. Previously, *Rv1819c* was ligated with pJET vector using EcoRI and Hind III multiple cloning site to amplify. It was then ligated to pMV261 expression vector containing Mtb promoter. The pMV261-Rv1819c was isolated from the recombinant *Escherichia coli* DH5α and electroporated into the competent cells of *M. smegmatis* mc^2 strains. The recombinant

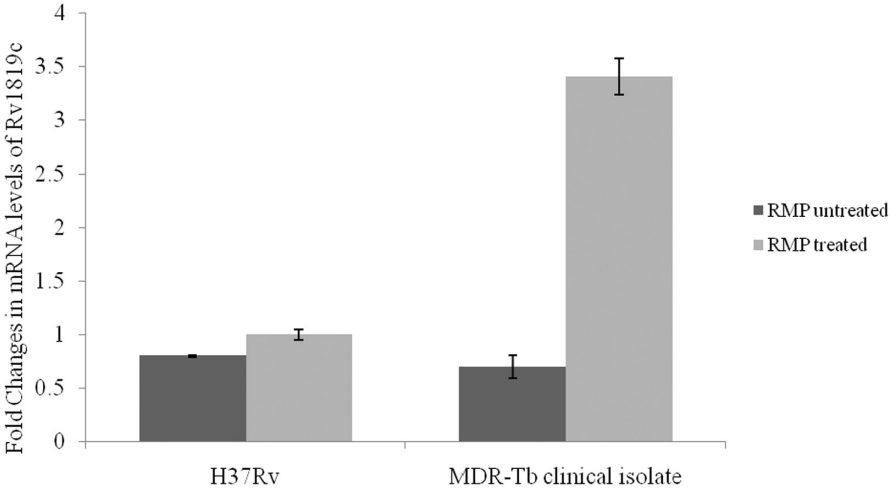

FIGURE 1 Analysis of mRNA transcriptional levels of - Rv1819c in H37Rv and MDR-TB clinical isolates.

TABLE 1 Binding energy and ligand efficiency score of docked complexes for Rv1819c with identified molecules

Compounds	Binding energy (kcal/mol)	Ligand efficiency (kcal/mol)	Interaction residues		Distance between residues (A°)
			Protein atom	Ligand atom	
Ellagic acid	−4.71	−0.21	LEU38: O	H	2.0
			LEU38: O	H	2.2
Methyl stearate	−3.45	−0.16	ARG14:N	O	2.9
			ALA15:N	O	3.4
			PHE16:N	O	3.4
Myoinositol	−2.17	−0.18	ASN387: O	H	1.7
			ASN387: O	H	2.0
			ASN387: O	H	2.1
Rutin	−3.65	−0.08	ASN240: O	H	1.9
			VAL351: O	H	1.9
Shikimic acid	−6.86	−0.57	ARG42: N	O	3.0
			TYR43: O	H	2.8
			ARG56: N	O	2.5
			ARG56: N	O	3.1
			ARG56: N	O	3.2

strains were screened by colony PCR and confirmed by sequencing. The sequencing data and the cloning images were given as supplementary data.

3.5 | MIC determination for selected efflux pump inhibitors and RMP individually against Mtb clinical isolate and the recombinant Rv1819c

MICs of RMP against two MDR-TB, one XDR-TB isolate, and recombinant *M. smegmatis* mc^2 155 Rv1819c were determined at the concentration range of 1 to 128 μg/ml. Similarly, the MICs for the selected molecules were determined at the ranging between 100 and 1000 μg/ml. The MIC of the RMP and the inhibitors are listed in Table 3. The two MDR-TB clinical isolates showed MIC of >128 and 32 μg/ml against RMP. The selected XDR TB isolate has a MIC of 8 μg/ml against RMP. For the selected inhibitors, the MIC varied between 700 and 1000 μg/ml against MDR and XDR TB clinical isolates. RMP showed a MIC of 8 and 0.5 μg/ml against recombinant-Rv1819c and wildtype *M. smegmatis*, respectively. INH did not show any inhibition against recombinant-Rv1819c and wild type *M. smegmatis* (Table 3). The inhibitor showed a higher MIC against recombinant-Rv1819c in comparison with the clinical isolates tested.

3.6 | Combinative anti-TB effect of both RMP and selected lead molecules against the drug-resistant isolates

The efficacy of the selected efflux inhibitors with RMP was studied against MDR, XDR TB clinical isolates, and recombinant Rv1819c. The MIC of RMP was determined in the presence of increasing concentrations of selected efflux

TABLE 2 Lipinski's rule of five for ADME analysis of the compounds

Compounds	Molecular weight (g/mol) <500	Lipophilicity (MLog*P*) <5	Hydrogen bond donors <5	Hydrogen bond acceptors <10
Ellagic acid	302.19	0.41	4	8
Methyl state	298.50	4.91	0	2
Myoinositol	180.16	−3.16	6	6
Rutin	610.52	−3.89	10	16
Shikimic acid	174.15	−1.43	4	5

TABLE 3 Individual MIC and In vitro synergistic rifampicin MIC lowering potential of selected efflux inhibitors

Sl. No.	Clinical isolates used	Compound ID (Duke's database)	Individual MIC of selected compounds (μg/ml)	Test concentration of selected compounds with RMP (μg/ml)	MIC of RMP	
					Without lead inhibitors (μg/ml)	With lead inhibitors (μg/ml)
1	MDR-TB isolate NIRT-001 RMP MIC tested range 128 to 0.25 μg/ml	Piperine	25	25	>128	32
		Ellagic acid	1000	1000		32
		Methyl stearate	100	100		0.5
		Myo inositol	50	50		32
		Rutin	1000	1000		64
		Shikimic acid	900	900		32
2.	MDR-TB isolate NIRT-002 RMP MIC tested range 32 to 0.0075 μg/ml	Piperine	25	25	32	8
		Ellagic acid	900	900		1
		Methyl stearate	100	100		0.0078
		Myo inositol	50	50		2
		Rutin	1000	1000		1
		Shikimic acid	800	800		0.5
3.	XDR-TB isolate NIRT-003 RMP MIC tested range 8 to 0.003 μg/ml	Piperine	25	25	8	8
		Ellagic acid	900	900		0.01
		Methyl stearate	200	200		0.0078
		Myo inositol	100	100		8
		Rutin	900	900		4
		Shikimic acid	700	700		8
4	recombinant *Mycobacterium smegmatis* expressing Rv1819c	Piperine	25	25	8	8
		Ellagic acid	100	100		8
		Methyl stearate	80	80		0.015
		Myo inositol	100	100		0.5
		Rutin	50	50		4
		Shikimic acid	100	100		1

pump inhibitors by the broth chequerboard method in microtiter plates. The 2-fold serial dilutions of RMP, ranging from 0.03 to 128 μg/ml were tested in combination with selected efflux pump inhibitors based on the MIC of the compounds (100–1000 μg/ml). Piperine ranging from 1.56 to 25 μg/ml was tested in combination with RMP. The MIC of RMP got reduced by more than 4- to 8-fold in the presence of efflux pump inhibitors (Table 3). The resistance conferred by MDR and XDR clinical isolates got reversed in the presence of the selected efflux inhibitors used in this study. In particular, methyl stearate at 100 μg/ml drastically decreased MIC of RMP from 128 to 0.5 μg/ml in isolate 1, 32 to 0.0078 μg/ml in the isolate 2 and 8 to 0.0078 μg/ml in the isolate 3. A similar kind of MIC reduction was observed

when the EPIs were treated with recombinant Rv1819c in *M. smegmatis* (wildtype MIC for RMP in *M. smegmatis* was 0.5 μg/ml whereas in the recombinant it was 8 μg/ml). The decrease in the MICs of RMP in combination with the selected molecules was more prominent even though the selected EPIs on their own did not show any significant antibacterial activity in the lower range.

3.7 | Ethidium bromide accumulation assay

The efflux pump inhibitory efficiency of the selected lead compounds was validated against recombinant Rv1819c.

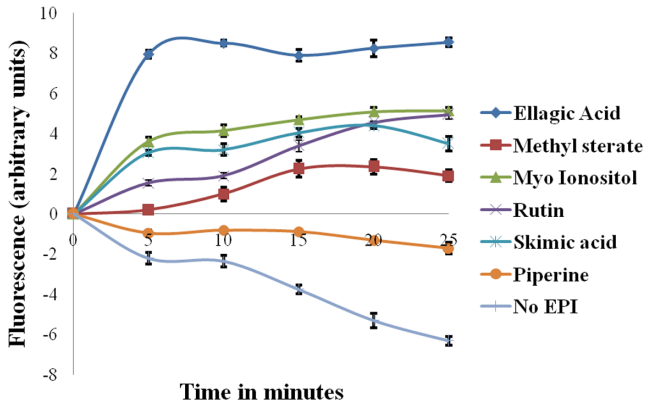

FIGURE 2 Effect of selected lead compounds as efflux pump inhibitors on the accumulation of ethidium bromide in the recombinant *Mycobacterium smegmatis* expressing Rv1819c. The efflux inhibitors were used at ½ the MIC and ethidium bromide at 1 μg/ml concentration. ($p < .01$. when compared to piperine).

Positive or high fluorescence values denote the increased accumulation of EtBr in the treated cells than in the untreated cells.

The recombinant *M. smegmatis* expressing Rv1819c was exposed to EtBr in the presence of the identified EPIs ellagic acid, shikimic acid, methyl stearate, myoinositol, rutin, and piperine—a known inhibitor individually. Recombinant bacterial cells without identified EPIs were considered a negative control. The compounds (ellagic acid, methyl stearate, myoinositol, rutin,) tested showed increased accumulation of EtBr inside the cells when compared with the control bacterial cells that were left untreated with EPIs molecules (p-value of <.01; Figure 2). A complete blockage of Rv1819c with an increase in the percentage of fluorescence was seen in ellagic acid when compared to other compounds and known efflux inhibitor piperine. myoinositol, methyl stearate, and rutin also showed increased accumulation of ethidium bromide indicating the inhibition of the efflux pump activity of Rv1819c.

3.8 | Cytotoxicity of identified efflux pumps inhibitors

The in silico toxicity and the drugability of the new efflux pump inhibitors predicted using Star Drop software showed that methyl stearate, myoinositol, and shikimic acid are safe with non-toxic and non-mutagenic effects. The ellagic acid was predicted to be highly toxic and hence excluded from further cytotoxicity studies (Figure 3).

The MIC of the EPI identified for the MDR-TB isolate NIRT-001 is used for the toxicity assay. The 2×, 1×, and

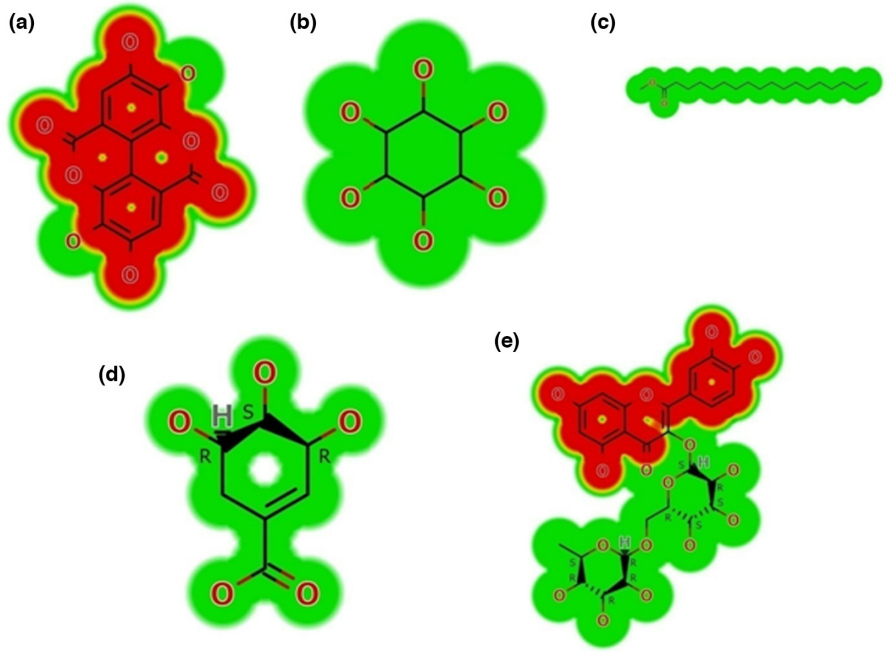

FIGURE 3 STAR DROP software interactive designer for the selected active molecules (a) Ellagic acid, (b) Myoinositol, (c) Methyl stearate, (d) Shikimic acid, (e) Rutin. The green color indicates that molecules are non-toxic and red color indicates the toxicity.

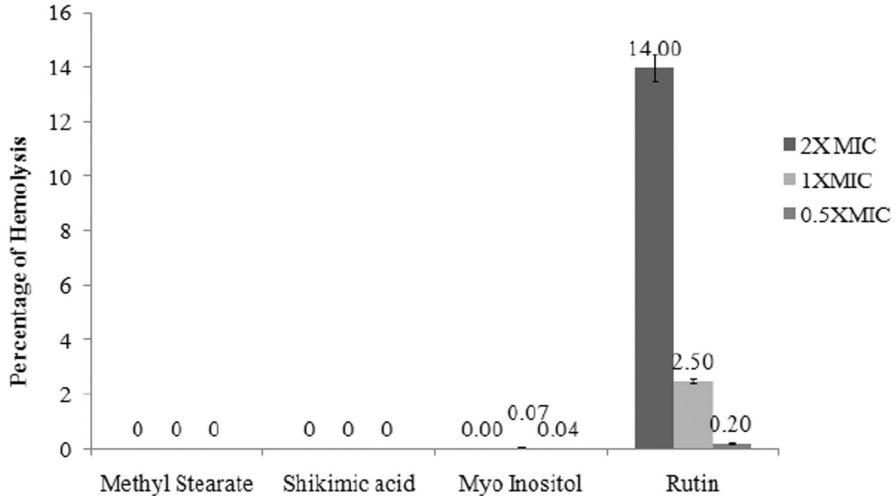

FIGURE 4 Cytotoxicity of selected efflux pump inhibitors on the PBMCs. Untreated PBMCs were used as a control.

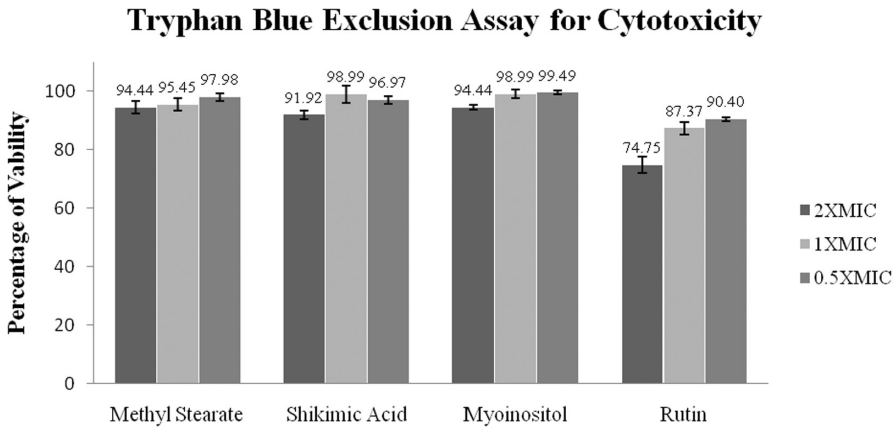

FIGURE 5 Hemolytic activity of selected active compounds. RBCs treated with distilled water and PBS was considered as positive and negative control, respectively.

0.5× MIC used for the toxicity assay are as follows: ellagic acid 2000, 1000, 500 μg/ml, methyl stearate 200, 100, 50 μg/ml, myoinositol 100, 50, 25 μg/ml, rutin 2000, 1000, and 500 μg/ml, shikimic acid 1800,900, and 450 μg/ml and piperine 50, 25, and 12.5 μg/ml.

No hemolysis was observed in the hemolytic assay in the presence of methyl stearate and shikimic acid at 2× MIC. The compound myoinositol stands next with 0.07% of cell lysis when compared with the control. Rutin, which showed minimal toxicity in STAR DROP analysis caused 14% of RBC lysis at 2× MIC while it showed only 2.5% lysis at 1× MIC (Figure 4).

The EPIs: methyl stearate, myoinositol, rutin, and shikimic acid were found to be non-toxic to PBMCs with more than 90% viability (Figure 5). As shikimic acid's MIC was high and rutin showed hemolysis, we excluded it from further cytotoxicity studies. The cytotoxicity of selected EPIs along with piperine a known EPI was further tested on Vero, THP1, and HepG2 cells belonging to different cell origins. Parallel with previous findings on PBMCs, both methyl stearate and myoinositol sustained the viability (90%) of the treated cells at 0.5× and 1×. However, limited toxicity of (40%–50%) was observed at 2× MIC on THP1 and HepG2 cells contradicting the 90% viability of Vero cells at the same concentration (Figures 6–8). The findings from the in silico, in vitro, and ex vivo studies are in coherence with each other stating that these molecules methyl stearate and myoinositol are potentially safe EPIs.

4 | DISCUSSION

The increasing prevalence of MDR strains has resulted in major health issues with high morbidity and mortality rate. In surge upon finding the novel strategies to address TB drug resistance, there is an increased interest to impede drug efflux activity among MDR-TB isolates using EPIs. EPIs have been known to reduce the level of resistance from high to low in the resistant Mtb phenotypes (Amaral et al., 2007; Machado et al., 2012). These EPIs should effectively bind to and inhibit efflux pumps that are present in the Mtb membrane as integral proteins (Verma et al., 2018). This study targets Rv1819c, an efflux pump belonging to the ABC transporter family, and was attempted to identify, screen, and validate plant-based EPIs. In drug-resistant strains, the sub-inhibitory concentration of RMP triggers the expression of efflux-associated genes (Li et al., 2015). Likewise, our study showed a noteworthy

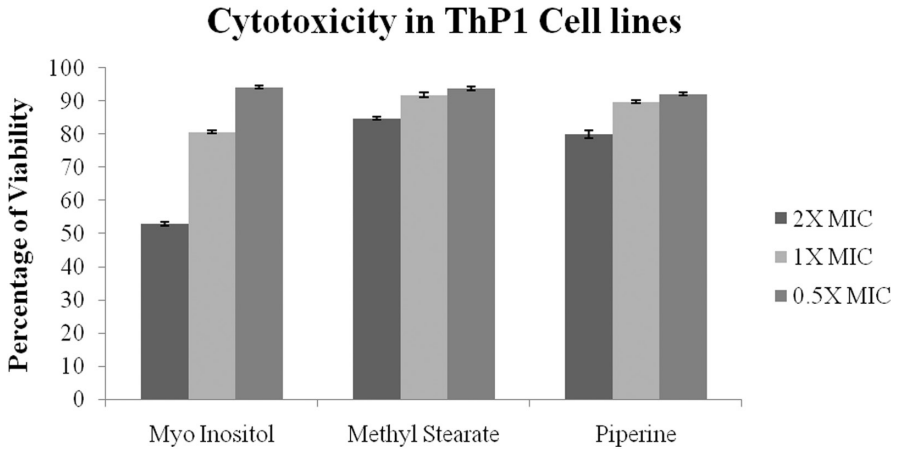

FIGURE 6 Cytotoxicity of selected efflux pump inhibitors on the THP1 cells.

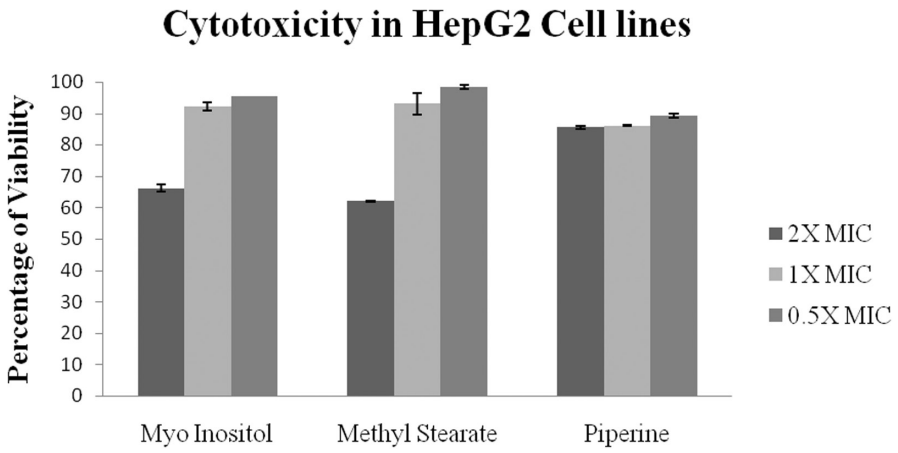

FIGURE 7 Cytotoxicity of selected efflux pump inhibitors on the HepG2 cells.

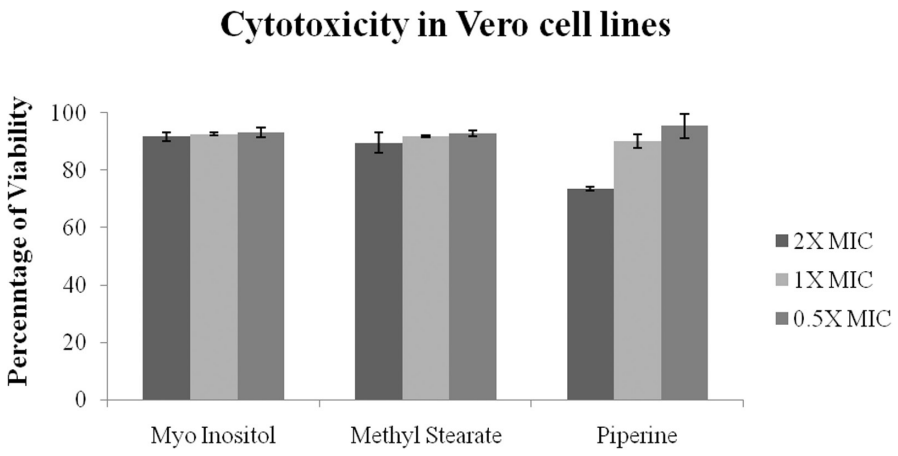

FIGURE 8 Cytotoxicity of selected efflux pump inhibitors on the Vero cells.

5-fold increased expression of Rv1819c when compared to RMP un-induced MDR isolate and *M. tuberculosis* H37Rv (Figure 1). This highlights that Rv1819c could play a significant role in helping the MDR-TB to survive in the presence of RMP. Hence, this EP could be considered a potential target to strike MDR-TB. Previously, Mazando et al., have shown a reduction in the ciprofloxacin efflux by blocking Mtb Rv1819c using *Mammea africana* plant extracts in recombinant *Corynebacterium glutamicum*. In this study, to further ensure its RMP effluxing activity, we transformed *M. smegmatis* mc^2 155 with the Rv1819c gene from the MDR-TB strain using the pMV261 shuttle vector. The recombinant *M. smegmatis* mc^2 155 expressing Rv1819c also showed increased resistance to RMP to 8 μg/ml similar to the XDR-TB clinical isolate used in this study (Table 3).

To fish out possible efflux pump inhibitors, particularly against Rv1819c, we did in silico-based High Throughput Virtual Screening and selected five plant-based compounds ellagic acid, methyl stearate, myoinositol, rutin, and shikimic acid out of 84 initially screened molecules based on the glide score. When tested in vitro, these selected putative efflux pump inhibitors individually could

inhibit Mtb MDR and XDR clinical isolates only at higher concentrations ranging between 700 and 1000 μg/ml and much higher in the recombinant *M. smegmatis* expressing Rv1819c (Table 3). Yet, MIC of RMP got effectively reduced by many folds in the presence of EPIs. Especially, methyl stearate, myoinositol, shikimic acid, and ellagic acid caused a drastic drop in the RMP MIC in all the strains tested. However, ellagic acid did not behave similarly when tested upon the recombinant *M. smegmatis* expressing Rv1819c (Table 3). Piperine a known inhibitor also did not inhibit the Rv1819c efflux pump. Even though rutin and ellagic acid caused more accumulation of EtBr depicting its EPI potential, these compounds are found to be toxic in both in silico and in vitro studies (Figures 3–5). Methyl stearate and myoinositol are known to be present in plants like *Ophiorrhiza rugosa*, *Lantana camara*, and *Euphorbia hirta* and vegetable oils (Adnan et al., 2019), which can be purified and could be a potentially promising drug candidate for being efflux pump inhibitors. Methyl stearate reduced the MICs of RMP considerably below that of its critical concentration (1 μg/ml) in all the three drug-resistant clinical isolates of Mtb, whereas both methyl stearate and myoinositol were able to reduce in such a manner against the recombinant *M. smegmatis* mc^2 155. Our study is the first to identify the role of methyl stearate and myoinositol as potent EPIs for Rv1819c that reduces the MIC of RMP so that the possibility of reintroducing RMP into the MDR and XDR regimen remains high. Upregulation of Rv1819c in the presence of a reduced concentration of RMP decrease in the MICs among the isolates of MDR and XDR TB (Table 3), and expression studies provide preliminary evidence that the EPIs identified in this study reduce the MICs of RMP by inhibiting Rv1819c.

Blocking of Rv1819c can lead to the reduced accumulation of lipid bodies in the tubercle bacilli (Gopinath et al., 2013) and hence these identified EPIs could bring about this beneficial consequence in addition. EPIs are known to decrease the energy of all active membrane transporters (Remm et al., 2022) and this adds more efficiency in retaining the rifampicin thereby making it eliminate the pathogen at low levels. Moreover, the positively charged EPIs selected in this study were proved to be with high receptor binding energy with Rv1819c, based on the docking results, which also contributes to the increased efficacy of these molecules, since this protein is known to be substrate-specific (Remm et al., 2022). Further validation of the selected putative Rv1819c efflux inhibitors is to be done on more MDR/XDR-TB clinical strains with resistance to bedaquiline and other second-line anti-TB drugs with a wide range of MIC. The non-inclusion of Rv1819c gene knockouts is another limitation of this study to claim that methyl stearate and myoinositol reduce the RMP MIC by blocking Rv1819c.

In conclusion, methyl stearate and myoinositol from plant sources are cytologically safe while blocking the Rv1819c and reducing the MIC of rifampicin in MDR and XDR-TB clinical isolates. This finding opens up the window of opportunities to reintroduce one of the first-line anti-TB drugs (RMP) into the regimen for treating MDR-TB and XDR-TB patients subject to a positive outcome on safety and efficacy in the uninfected and infected animal models.

ACKNOWLEDGMENTS

The shuttle vector pMV261 used in the study was a kind gift from Prof. H. Shakila, Chairperson, School of Biotechnology, Madurai Kamaraj University. The authors thank the entire staff of the Department of Bacteriology ICMR-National Institute for Research in Tuberculosis, Chennai, India. CRN and SER thank ICMR, India for Post-Doctoral Research Fellowship.

FUNDING INFORMATION

The authors CRN and SER thank the Indian Council of Medical Research India, for providing financial support for Post-Doctoral Research (3/1/3/PDF (16)/−2017-HRD 3 (16th batch)), (3/1/3/PDF (16)/−2019-HRD 3 (21st batch)).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Azger Dusthackeer https://orcid.org/0000-0002-3616-3675

REFERENCES

Adnan, M., Chy, N. U., Kamal, A. T. M. M., Barlow, J. W., Faruque, M. O., Park, C. H., & Cho, D. H. (2019). Investigation of the biological activities and characterization of bioactive constituents of Ophiorrhiza rugosa var. prostrata (D.Don) & Mondal leaves through in vivo, in vitro, and in silico approaches. *Molecules*, *24*(1367), 1–24. https://doi.org/10.3390/molecules24071367

Amaral, L., Martins, M., & Viveiros, M. (2007). Enhanced killing of intracellular multidrug-resistant *Mycobacterium tuberculosis* by compounds that affect the activity of efflux pumps. *The Journal of Antimicrobial Chemotherapy*, *59*(6), 1237–1246. https://doi.org/10.1093/jac/dkl500

Camacho-Corona, M. D. R., Ramirez-Cabrera, M. A., Santiago, O. G., Garza-Gonzalez, E., Palacios, I. D. P., & Luna-Herrera, J. (2008). Activity against drug resistant-tuberculosis strains of plants used in Mexican traditional medicine to treat tuberculosis and other respiratory diseases. *Phytotherapy Research*, *22*(1), 82–85. https://doi.org/10.1002/ptr.2269

Caminero, J. A., Piubello, A., Scardigli, A., & Migliori, G. B. (2017). Bedaquiline: How better to use it. *European Respiratory*

Journal, *50*(5), 1701670. http://erj.ersjournals.com/content/50/5/1701670.abstract, https://doi.org/10.1183/13993003.01670-2017

Caviedes, L., Delgado, J., & Gilman, R. H. (2002). Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, *40*(5), 1873–1874. https://pubmed.ncbi.nlm.nih.gov/11980982, https://doi.org/10.1128/jcm.40.5.1873-1874.2002

Cloete, R., Kapp, E., Joubert, J., Christoffels, A., & Malan, S. F. (2018). Molecular modelling and simulation studies of the *Mycobacterium tuberculosis* multidrug efflux pump protein Rv1258c. *PLoS One*, *13*(11), e0207605. https://doi.org/10.1371/journal.pone.0207605

Cosma, C. L., Sherman, D. R., & Ramakrishnan, L. (2003). The secret lives of the pathogenic mycobacteria. *Annual Review of Microbiology*, *57*, 641–676. https://doi.org/10.1146/annurev.micro.57.030502.091033

da Silva, P. E. A., Machado, D., Ramos, D., Couto, I., Von Groll, A., & Viveiros, M. (2016). Efflux pumps in mycobacteria: Antimicrobial resistance, physiological functions, and role in pathogenicity. In X.-Z. Li, C. A. Elkins, & H. I. Zgurskaya (Eds.), *Efflux-mediated antimicrobial resistance in bacteria: Mechanisms, regulation and clinical implications* (pp. 527–559). Springer International Publishing. https://doi.org/10.1007/978-3-319-39658-3_21

Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, *7*, 42717. https://doi.org/10.1038/srep42717

Demitto, F. D. O., do Amaral, R. C. R., Maltempe, F. G., Siqueira, V. L. D., Scodro, R. B. D. L., Lopes, M. A., Caleffi-Ferracioli, K. R., Canezin, P. H., & Cardoso, R. F. (2015). In vitro activity of rifampicin and verapamil combination in multidrug-resistant *Mycobacterium tuberculosis*. *PLoS One*, *10*(2), e0116545. https://doi.org/10.1371/journal.pone.0116545

Domenech, P., Kobayashi, H., LeVier, K., Walker, G. C., & Barry, C. E., III. (2009). BacA, an ABC transporter involved in maintenance of chronic murine infections with *Mycobacterium tuberculosis*. *Journal of Bacteriology*, *191*(2), 477–485. https://doi.org/10.1128/JB.01132-08

Fishwild, D. M., Staskawicz, M. O., Wu, H. M., & Carroli, S. F. (1991). Cytotoxicity against human peripheral blood mononuclear cells and T cell lines mediated by anti-T cell immunotoxins in the absence of added potentiator. *Clinical and Experimental Immunology*, *86*(3), 506–513. https://doi.org/10.1111/j.1365-2249.1991.tb02961.x

Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., Sanschagrin, P. C., & Mainz, D. T. (2006). Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *Journal of Medicinal Chemistry*, *49*(21), 6177–6196. https://doi.org/10.1021/jm051256o

Ghajavand, H., Kargarpour Kamakoli, M., Khanipour, S., Pourazar Dizaji, S., Masoumi, M., Rahimi Jamnani, F., Fateh, A., Yaseri, M., Siadat, S. D., & Vaziri, F. (2019). Scrutinizing the drug resistance mechanism of multi- and extensively-drug resistant *Mycobacterium tuberculosis*: Mutations versus efflux pumps. *Antimicrobial Resistance & Infection Control*, *8*(1), 70. https://doi.org/10.1186/s13756-019-0516-4

Gopinath, K., Moosa, A., Mizrahi, V., & Warner, D. F. (2013). Vitamin B12 metabolism in *Mycobacterium tuberculosis*. *Future Microbiology*, *8*(11), 1405–1418. https://doi.org/10.2217/fmb.13.113

Greco, I., Molchanova, N., Holmedal, E., Jenssen, H., Hummel, B. D., Watts, J. L., Håkansson, J., Hansen, P. R., & Svenson, J. (2020). Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. *Scientific Reports*, *10*(1), 13206. https://doi.org/10.1038/s41598-020-69995-9

Gupta, A. K., Katoch, V. M., Chauhan, D. S., Sharma, R., Singh, M., Venkatesan, K., & Sharma, V. D. (2010). Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. *Microbial Drug Resistance*, *16*(1), 21–28. https://doi.org/10.1089/mdr.2009.0054

Gupta, S., Cohen, K. A., Winglee, K., Maiga, M., Diarra, B., & Bishai, W. R. (2014). Efflux inhibition with verapamil potentiates bedaquiline in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *58*(1), 574–576. https://pubmed.ncbi.nlm.nih.gov/24126586, https://doi.org/10.1128/AAC.01462-13

Li, G., Zhang, J., Guo, Q., Wei, J., Jiang, Y., Zhao, X., Zhao, L.-L., Liu, Z., Lu, J., & Wan, K. (2015). Study of efflux pump gene expression in rifampicin-monoresistant *Mycobacterium tuberculosis* clinical isolates. *The Journal of Antibiotics*, *68*(7), 431–435. https://doi.org/10.1038/ja.2015.9

Liu, Q., Yang, D., Qiu, B., Martinez, L., Ji, Y., Song, H., Li, Z., & Wang, J. (2021). Drug resistance gene mutations and treatment outcomes in MDR-TB: A prospective study in eastern China. *PLOS Neglected Tropical Diseases*, *15*(1), e0009068. https://doi.org/10.1371/journal.pntd.0009068

Machado, D., Couto, I., Perdigão, J., Rodrigues, L., Portugal, I., Baptista, P., Veigas, B., Amaral, L., & Viveiros, M. (2012). Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLoS One*, *7*(4), e34538. https://doi.org/10.1371/journal.pone.0034538

Makane, V. B., Krishna, V. S., Krishna, E. V., Shukla, M., Mahizhaveni, B., Misra, S., Chopra, S., Sriram, D., Dusthackeer, V. N. A., & Rode, H. B. (2019). Synthesis and evaluation of alpha-aminoacyl amides as antitubercular agents effective on drug resistant tuberculosis. *European Journal of Medicinal Chemistry*, *164*, 665–677. https://doi.org/10.1016/j.ejmech.2019.01.002

Målen, H., De Souza, G. A., Pathak, S., Søfteland, T., & Wiker, H. G. (2011). Comparison of membrane proteins of *Mycobacterium tuberculosis* H37Rv and H37Ra strains. *BMC Microbiology*, *11*, 18. https://pubmed.ncbi.nlm.nih.gov/21261938, https://doi.org/10.1186/1471-2180-11-18

Mazando, S., Zimudzi, C., Zimba, M., Pym, A., & Mason, P. (2017). High Rv1819c efflux pump gene expression in persistent *Mycobacterium tuberculosis* clinical isolates. *Journal of Applied Biosciences*, *115*, 11465–11475.

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, *30*(16), 2785–2791. https://pubmed.ncbi.nlm.nih.gov/19399780, https://doi.org/10.1002/jcc.21256

Nikaido, H. (2001). Preventing drug access to targets: Cell surface permeability barriers and active efflux in bacteria. *Seminars in Cell & Developmental Biology*, *12*(3), 215–223. https://doi.org/10.1006/scdb.2000.0247

Pule, C. M., Sampson, S. L., Warren, R. M., Black, P. A., van Helden, P. D., Victor, T. C., & Louw, G. E. (2016). Efflux pump inhibitors: Targeting mycobacterial efflux systems to enhance TB therapy. *The Journal of Antimicrobial Chemotherapy*, *71*(1), 17–26. https://doi.org/10.1093/jac/dkv316

Ramón-García, S., Martín, C., De Rossi, E., & Aínsa, J. A. (2007). Contribution of the Rv2333c efflux pump (the Stp protein) from *Mycobacterium tuberculosis* to intrinsic antibiotic resistance in *Mycobacterium bovis* BCG. *The Journal of Antimicrobial Chemotherapy*, *59*(3), 544–547. https://doi.org/10.1093/jac/dkl510

Remm, S., Earp, J. C., Dick, T., Dartois, V., & Seeger, M. A. (2022). Critical discussion on drug efflux in *Mycobacterium Tuberculosis*. *FEMS Microbiology Reviews*, *46*(1), fuab050. https://doi.org/10.1093/femsre/fuab050

Rempel, S., Gati, C., Nijland, M., Thangaratnarajah, C., Karyolaimos, A., de Gier, J. W., Guskov, A., & Slotboom, D. J. (2020). A mycobacterial ABC transporter mediates the uptake of hydrophilic compounds. *Nature*, *580*(7803), 409–412. https://doi.org/10.1038/s41586-020-2072-8

Rodrigues, L., Villellas, C., Bailo, R., Viveiros, M., & Ainsa, J. A. (2013). Role of the Mmr efflux pump in drug resistance in *Mycobacterium Tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *57*(2), 751–757. https://doi.org/10.1128/AAC.01482-12

Rodríguez-Corrales, J. Á., & Josan, J. S. (2017). Resazurin live cell assay: Setup and fine-tuning for reliable cytotoxicity results. In I. M. Lazar, M. Kontoyianni, & A. C. Lazar (Eds.), *Proteomics for drug discovery: Methods and protocols* (pp. 207–219). Springer New York. https://doi.org/10.1007/978-1-4939-7201-2_14

Sharma, A., Gupta, V. K., & Pathania, R. (2019). Efflux pump inhibitors for bacterial pathogens: From bench to bedside. *The Indian Journal of Medical Research*, *149*(2), 129–145. https://pubmed.ncbi.nlm.nih.gov/31219077, https://doi.org/10.4103/ijmr.IJMR_2079_17

Shepard, C. C. (1957). Growth characteristics of tubercle bacilli and certain other mycobacteria in HeLa cells. *The Journal of Experimental Medicine*, *105*(1), 39–48. https://pubmed.ncbi.nlm.nih.gov/13385405, https://doi.org/10.1084/jem.105.1.39

Shrivastava, J. P., & Shrivastava, A. (2021). Scenario of tuberculosis in India. *Journal of Lung, Pulmonary & Respiratory Research*, *8*(2), 24–25.

Song, L., & Wu, X. (2016). Development of efflux pump inhibitors in antituberculosis therapy. *International Journal of Antimicrobial Agents*, *47*(6), 421–429. https://doi.org/10.1016/j.ijantimicag.2016.04.007

Tarling, E. J., de Aguiar Vallim, T. Q., & Edwards, P. A. (2013). Role of ABC transporters in lipid transport and human disease. *Trends in Endocrinology and Metabolism*, *24*(7), 342–350. https://doi.org/10.1016/j.tem.2013.01.006

Venkatesh, J., Kumar, P., Krishna, P. S. M., Manjunath, R., & Varshney, U. (2003). Importance of uracil DNA glycosylase in *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*, G+C-rich bacteria, in mutation prevention, tolerance to acidified nitrite, and endurance in mouse macrophages. *The Journal of Biological Chemistry*, *278*(27), 24350–24358. https://doi.org/10.1074/jbc.M302121200

Verma, P., Tiwari, M., & Tiwari, V. (2018). In silico high-throughput virtual screening and molecular dynamics simulation study to identify inhibitor for AdeABC efflux pump of *Acinetobacter baumannii*. *Journal of Biomolecular Structure & Dynamics*, *36*(5), 1182–1194. https://doi.org/10.1080/07391102.2017.1317025

Viveiros, M., Martins, M., Rodrigues, L., Machado, D., Couto, I., Ainsa, J., & Amaral, L. (2012). Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs. *Expert Review of Anti-Infective Therapy*, *10*(9), 983–998. https://doi.org/10.1586/eri.12.89

World Health Organization. (2019). *Global tuberculosis report 2019—Executive summary*. https://www.who.int/tb/publications/global_report/tb19_Exec_Sum_12Nov2019.pdf?ua=1

World Health Organization. (2020a). *Tuberculosis and COVID-19*. Retrieved July 27, 2020, from https://www.who.int/teams/globaltuberculosis-programme/covid-19

World Health Organization. (2020b). *Compendium of TB/COVID-19 studies*. Retrieved August 5, 2020, from https://www.who.int/teams/global-tuberculosis-programme/covid-19/compendium

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nirmal, C. R., Rajadas, S. E., Balasubramanian, M., Mohanvel, S. K., Aathi, M. S., Munishankar, S., Chilamakuru, N. B., Thiruvenkadam, K., Pandiya Raj, A. K., Paraman, R., & Dusthackeer, A. (2023). Myoinositol and methyl stearate increases rifampicin susceptibility among drug-resistant *Mycobacterium tuberculosis* expressing Rv1819c. *Chemical Biology & Drug Design*, *101*, 883–895. https://doi.org/10.1111/cbdd.14197