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Dodecanoic acid & palmitic acid disarms rifampicin resistance by putatively targeting mycobacterial efflux pump Rv1218c

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***Background & objectives*: Drug-resistant tuberculosis (TB) jeopardizes the treatment process with poor outcomes. Efflux pumps (EPs) belonging to the ABC transporter family in *Mycobacterium tuberculosis* confer resistance to rifampicin (RMP) besides genetic mutations thus serving as a target for a potential adjunct therapeutic inhibitory molecule. Rv1218c is one such pump that was previously reported to be active in multidrug-resistant TB clinical isolates.**

***Methods*: In this study, the inhibition potential of Rv1218c-EP was tested on 8 molecules that were shortlisted by *in silico* methods. These molecules were subjected to the minimum inhibitory concentration (MIC) determination, checkerboard drug combination assay, ethidium bromide-DNA binding assay, and *in vitro* and *ex vivo* cytotoxicity assay.**

***Results*: Based on the outcome of the study, two molecules dodecanoic acid (DA) and palmitic acid (PA) were found to be potential enough to decrease the MIC of RMP by 8 to 1000 folds against multidrug-resistant clinical isolates and Rv1218c expressing recombinant *Mycobacterium smegmatis*.**

***Interpretation & conclusions*: These molecules were also found to reduce the time taken by RMP to kill these drug-resistant *Mycobacteria* to 48 h, unlike control isolates that survived more than 240 h of RMP exposure. The functional concentration of both molecules was non-toxic to the epithelial and blood mononuclear cells. With further comprehensive scientific validation, PA and DA could be recommended as adjunct therapeutic molecules with first-line anti-TB drugs to treat drug-resistant TB.**

Key words Adjunct therapy - efflux inhibitors - efflux pump - rifampicin-resistance - Rv1218c - tuberculosis

The success rate of anti-tuberculosis (TB) treatment will reportedly decline from 83 per cent to 56 per cent if the infection is due to drug-resistant *Mycobacterium tuberculosis* (MTB)[1]. During rifampicin (RMP)

resistance, the non-synonymous mutations that alter MTB RNA polymerase[2] are common rather than deletions and insertion mutations[3]. Clinical isolates which are resistant to RMP have a higher chance to become isoniazid (INH) resistant and make RMP resistance a potential indicator of multi-drug resistance (MDR) TB[4,5]. Nearly 95 per cent of RMP resistance is due to spontaneous mutations in the 81 base pair regions of the *rpoB* gene[6,7]. The rest five per cent RMP resistance exhibits no evidence of any mutation and offers strong evidence of the role of other mechanisms, particularly chromosome-mediated drug efflux pumps (EPs) that export various molecules outside the bacterial cells[8]. Rv3065, belongs to the family of resistance-nodulation-division transporters and, is an important efflux of pyrrole and pyrazolone molecules. It is related to the susceptibility to RMP, INH, erythromycin, tetracycline, aminoglycosides and fluoroquinolones and has been reported in Indian MDR-TB isolates[9]. Knockout studies on Rv3065, Rv1258, Rv0849 and Rv1819c showed increased susceptibility to drugs in the pyrrole class compared to knockout mutant strains[10]. The efflux gene Rv2938 encodes an inner membrane protein of an EP system in MTB and has been reported to confer drug resistance in some MDR strains[11]. The Rv1819c gene codes for a putative ABC transporter that has a negatively charged cavity and specializes in transporting long-chain fatty acids, vitamin B12 and drugs such as bleomycin[12,13]. According to studies by Gopinath *et al*[14], Rv1819c functions as a channel for the entry of vitamin B12, which plays a crucial role in the mycobacterial pathway. Being an ABC transporter, Rv1819c may also be involved in the transportation of lipids within the bacterium[15]. Rv2209 is required for virulence and is involved in the cell wall synthesis. The overexpression of Rv2477 and Rv2209 has been observed in response to stress from ofloxacin in MTB[16]. These findings highlight the importance of monitoring the emergence of drug resistance in MTB populations and the role that EP genes play in this process. Further research is needed to fully understand the mechanisms and interactions between these genes and other factors that contribute to drug resistance in MTB.

Certain EPs were reported to be specific to RMP mono-resistance[17]. However, the function of other active drug EPs which cause RMP resistance is yet to be explored. MDR pathogens are known to overexpress these EPs to facilitate a dip in the intracellular drug concentration. To date, five families of EPs were identified, including the ABC transporter family, which was identified to be one of the causes of RMP resistance[10,18]. Rv1218c EP is one such ABC transporter family pump of MTB that was reported to efflux RMP, novobiocins, pyrazolones, biarylpiperazines, bisanilino pyrimidines, pyrroles and pyridines[19]. Any loss in function of Rv1218c consequently lowered the minimum inhibitory concentration (MIC) of these drugs by increasing the drug bioavailability and intracellular accumulation by eight-fold[10]. Owing to the efflux-mediated drug resistance, more light has been shed on EP inhibitors (EPI) in the recent years to augment the anti-TB regimen[20], with a primary interest in plant-based secondary metabolites. Verapamil is one such EPI that was approved by the Food and Drug Administration. However, it is incapable to inhibit a wide range of EPs and is not successful in phenotypic levels[21]. This study aimed to evaluate the efflux inhibiting potency of selected bioactive compounds from a plant-based database showing a positive combined inhibition with RMP against MDR-TB clinical isolates and in recombinant *Mycobacterium smegmatis* expressing Rv1218c. *In silico* and *in vitro* validation of efflux inhibitory activity by these molecules against the EP Rv1218c was done.

Material & Methods

This study was conducted at the department of Bacteriology, ICMR-National Institute for Research in Tuberculosis (NIRT), Chennai, India, from January 2018 to November 2020. The approval by the Institutional Ethics Committee was obtained before starting the study.

Bacterial cultures and strains used: Middlebrook 7H9 medium supplemented with oleic acid (Ol), albumin, dextrose and catalase (OADC) (7H9) (BD Biosciences, catal) was used to grow by eight-fold and *M. smegmatis* while Luria-Bertani (LB) medium (Himedia, India) was used for *Escherichia coli* DH5α. Two MDR-TB clinical isolates (NIRT-001 and NIRT-002) from the ICMR-NIRT repository were used in the study. These isolates were well characterized by phenotypical, bacteriological and genetic methods such as line probe assay. The vector system pMV261 and *E. coli* DH5α competent cells (Novagen) were used as per the manufacturer's instructions for the molecular techniques.

Statistical analysis: All experiments were carried out in triplicate. Statistical analysis was carried out using GraphPad Prism 5 software for Windows version 5.03. (*www.graphpad.com*). Significant differences between

treated and untreated control groups were found using the unpaired t test. $P<0.05$ was considered significant.

In silico homology modelling and protein structure prediction: Based on the protein sequence of the EP gene Rv1218c, the 3D protein structure was predicted using I-TASSER, which is a hierarchical approach to predict the protein structure based on a known functional template[22]. The model with low confirmation was selected as a final structure for screening the lead molecules.

Ligand preparation: A total of 25,000 biologically active small molecules were retrieved from Duke's database (*www.phytochem.nal.usda.gov*). These were geometrically minimized for liquid simulations using the optimized potentials. PDBQT file was created in MOL2 format, followed by the addition of hydrogen atoms and AM1-BCC charges by applying the AMBER force field that increases the accuracy of the ligand molecules[23].

Protein preparation: Protein preparation and refinement were performed using Gasteiger charges, in which the following reactions occur, namely removal of water molecules, addition of hydrogen atoms, bond orders assignment hydroxyl group orientation and optimization. Finally, using default constraint 0.3 Å RMSD, energy minimization was carried out. The modelled protein structure of the EP was used as the receptor and the active sites for the proteins were predicted using the online free software SCFBio, (IIT, Delhi). The centre of the receptor grid was generated for the active site residues.

Screening of compounds against Rv1218c using mastero: The Mastero v9.1(22) software was used to perform the docking analysis, using the Rv1218c receptor and compounds optimized with an OPLS_2005 force field. Multiple conformations of the ligands were generated, and compounds with a fitness score higher than 1.0 were selected for docking with Rv1218c.

Analysis of Rv1218c transcriptional levels in multi-drug resistance-tuberculosis (MDR-TB) clinical isolates: The transcription level of Rv1218c was analyzed by quantitative real-time-PCR method by using H37Rv as a reference strain. MTB H37Rv and MDR clinical isolates were grown to the mid-log phase in the presence and absence of sub-inhibitory concentration of RMP (1/4 the MIC). Since RMP at its MIC will lead to inhibition of MTB growth, the test strains were exposed to sub-inhibitory MIC to ensure its survival and induction of the EPs. RNA was extracted using the TRIZOL reagent and DNA contamination was removed using RNase-free DNase. Verso cDNA synthesis kit (Thermo Scientific, Lithuania, USA) was used to reverse transcribe to total RNA cDNA using the commercially available kit (. The real-time PCR reactions were performed using Thermoscientific ABI-7500 one-step reverse transcription (RT)–PCR in a 20 µl total reaction volume of consisting of 10 pmol of each primer and SYBR green. Protocol: 55°C for 10 min, followed by PCR activation at 95°C for 30 sec and 45 cycles of amplifications at room temperature (RT). The experiment was performed in triplicates and the housekeeping gene 16s rRNA was used as the internal control.

Cloning and over-expression of Rv1218c

Mycobacterial transformation and expression of recombinant protein: The expression of Rv1218c was carried out as described earlier[24]. Briefly, Rv1218c was overexpressed in the *E. coli* DH5α strain using the pMV261 vector. The recombinant strains were screened by plating on LB agar plates with 35 µg/ml of Kanamycin (Merck-25389-94-0). Rv1218c-pMV261 plasmid was isolated from the recombinant strains using a Qiagen miniprep kit and 200 to 400 ng of the same was transformed into *M. smegmatis mc*^{2} 155 (MSM) competent cells through electroporation. The transformation was confirmed through PCR using Rv1218c gene-specific primers. The recombinant *M. smegmatis mc*^{2} 155 (rMSM) cultures were incubated for 48 h at 37°C until it reaches an optical density of 0.6 at 600 nm (OD_{600}) for further studies.

Determination of MIC against recombinant *M. smegmatis* mc^2 155 (rMSM) and MDR-TB clinical isolates: MICs of the lead molecules (100-1000 µg/ml) in comparison with RMP were determined in triplicates against rMSM and two MDR-TB clinical isolates (RMP MIC >128 and 32 µg/ml), both individually and in combinations. Dilutions were prepared as described previously[24]. In brief, 100 µl of drug solution (RMP and other efflux inhibitors) was added to each first well of the 96 well plate and serially diluted to achieve 2× concentration of the desirable test range. Then an equal volume of culture suspension of rMSM and MDR-TB clinical isolates was added to all the wells to achieve 1× concentration of the desirable test range. This culture suspension was previously prepared from the cultures

grown in 7H9 at 37°C until it reaches an OD_{600} of 0.1 and then diluted 1000 and 10 times, respectively, in 7H9 broth[25]. The cells were exposed to a range of drug concentrations for two days (rMSM) and seven days (MDR-TB clinical isolate) at 37°C. The wells with bacterial turbidity (for rMSM) and cord formation (for MDR-TB) were considered positive, whereas negative wells were denoted by clear broth.

Checkerboard titration assay: This broth dilution assay was carried out in triplicates as described earlier[26] to determine the interaction between these shortlisted molecules and RMP. One in hundred diluted untreated bacterial culture was used as culture control. Different combinations of efflux inhibitors with rifampicin starting from its respective MIC was prepared at two-fold dilutions using 7H9 broth. One million cells (rMSM and MDR-TB clinical isolates 1 and 2) of all three isolates were treated with the selected molecules (range 10-1000 µg/ml) in the presence of RMP at varying concentration ranges (0.0078-128 µg/ml for MDR-TB clinical isolate 1,0.0078-32 µg/ml for MDR-TB clinical isolate 2 and 0.015-8 µg/ml for rMSM). Piperine, a known EPI, ranging from 1.56-25 µg/ml was tested in combination with RMP as a control. The plates were incubated for five days at 37°C and observed under a microscope for cord formation.

Ethidium bromide (EtBr) accumulation assay: This was carried out on rMSM on a real-time basis as described earlier[27] to assess the Rv1218c EP inhibition by the selected molecules. The rMSM was grown in 7H9 until 0.8 OD_{600} was centrifuged at 3000xg 15 min and the pellet was re-suspended in phosphate buffered saline (*p*H 7.4). The suspended cells at 0.1 OD_{600} were added to each well of 96-well black plates and EtBr at the final concentration of 1 µg/ml was added, followed by the addition of 10 µl of each test lead molecules at its half-MICs. Spectra Max multimode reader (Spinco Biotech) was used to measure fluorescence (excitation ~530 nm; emission ~590 nm for 30 min at 5 min intervals).

Cytotoxicity testing of the shortlisted efflux pump inhibitors: The cytotoxicity of these lead molecules was tested on haemocytes and epithelial cells using two *in vitro* and *ex vivo*-based approaches.

Haemolytic activity testing: Haemolytic activity of identified EP inhibitors at the concentration of 2×, 1×, and 0.5× of its respective MIC was tested in triplicates through a checkerboard assay as described previously using 1/10th diluted complement-free human red blood cells (RBCs) in phosphate-buffered saline (PBS *p*H 7.0)[28]. After 2 h of exposure, the absorbance was measured at 541 nm and the percentage of hemolysis was calculated by comparing the values with that of positive control (distilled water).

Cytotoxicity testing on cell lines: The cytotoxicity of the shortlisted molecules at the concentration of 1×, 2×, 4×, 8× and 10× of their MIC was initially tested on commercially purchased peripheral blood mononuclear cells (PBMCs) (HiMedia) using tryphan blue viability testing[29] and shortlisted efflux inhibitors were further tested on Vero cells, HepG2 cells, and THP1 using resazurin assay as described previously[30]. Positive (Triton-X 100) and negative controls (un-treated) were included. The percentage of viability was calculated by comparing the values of the test with the untreated controls. The 10^4 cells were treated with the respective EPIs after 24 h of seeding.

Time-kill assay: The efficacy of shortlisted efflux inhibitors was further evaluated against MDR-TB clinical isolate (MIC >128 µg/ml) by time-kill kinetics assay as described elsewhere[31]. In brief, the culture was treated with RMP (128, 64, 32, 0.03, 0.015 and 0.0078 µg/ml) in the presence of efflux inhibitors at specified concentration. Based on its MIC, the known EP inhibitor Piperine was used as control.

Results

In silico screening for efflux pump inhibitors against Rv1218c was carried out. The compounds from Duke's database were prepared for docking and induced fit docking was performed in the search for active EP inhibitors against Rv1218c. The top ten compounds were selected based on the Lipinski's rule of five.

The compounds raffinose pentahydrate (RP), palmitic acid (PA), tetra decanoic acid (TD), magnesium stearate (MS), 3, 4, 5-trihyhydroxy benzoic acid (TH), dodecanoic acid (DA), flavone (FL), rhamnose monohydrate (RM), Ol and stearic acids (SA) were selected for *in vitro* validation based on parameters including the glide score, glide energy, ADMET (absorption, distribution, metabolism, elimination and toxicity) properties and interactions with the target protein (Table I). The 2D and 3D interactions of the identified EPs with the protein (Rv1218c) are given in Figure 1.

A significant differential expression of Rv1218c was observed only in the MDR-TB clinical isolates when exposed to sub-inhibitory levels of RMP

Table I. Hydrogen bond interaction observed in compounds with Rv1218c

Compounds	Binding energy (Kcal/mol)	Interaction residues	Molecular weight (g/mol) (<500)	Lipophilicity (MLog *P*) (<5)	Hydrogen bond donors (<5)	Hydrogen bond acceptors (<10)
RP	−7.68	Thr102, Tyr86, Asp79, Ala149, Ser152	594	−2.91	6	5
PA	−7.53	Arg110, Ser152	256	−1.52	4	2
TD	−7.50	Tyr86, Ile84	228	−1.535	5	7
MS	−6.90	Arg110, Leu106	591	−1.418	4	0
TH	−6.85	Tyr86, Thr92, Thr103	170	−0.607	3	5
DA	−6.76	Ile84, Ser152	200	−0.7	4	6
FL	−6.69	Thr102, Tyr86	222	−2.443	5	6
RM	−6.25	Tyr86, Ser152, Asp79	182	−0.149	6	5
OL	−6.15	Ala 85	282	4.57	1	2
SA	−5.4	Ser152	284	4.67	1	2

RM, rhamnose monohydrate; RP, raffinose pentahydrate; PA, palmitic acid; TD, tetradecanoic acid; MS, magnesium stearate; TH, 3,4,5-trihyhydroxy benzoic acid; DA, dodecanoic acid; FL, flavone; OL, oleic; SA, stearic acids

(¼ MIC). However, such a difference was not observed in the RMP-sensitive MTB H37Rv (both RMP treated and untreated) (Fig. 2). Hence, increased expression of Rv1218c can be considered as one of the RMP-resistant mechanisms of the strains used in the study.

The MICs of RMP against MDR-TB and rMSM were determined in the range of 128 to 0.003 µg/ml. Similarly, the MICs for the shortlisted molecules were also determined. The MIC of RMP for the MDR-TB clinical isolates (n=2) and rMSM was found to be >128 µg/ml, 32 µg/ml, and 8 µg/ml, respectively. The compounds OI and SA showed minimal inhibition even at higher concentrations with MDR-TB clinical isolates. MIC of TD, MS and RP was 1000 µg/ml each. MICs of the molecules RM, PA, TH and FL were 700, 100, 100 and 100 µg/ml, respectively, while the DA was highly potent even at a lower concentration of 10 µg/ml. The MIC of the molecules was comparatively higher in rMSM (Table II).

The RMP-MIC lowering the potential of the selected molecules was determined using the checkerboard method in microtitre plates. As OI and SA exhibited poor inhibition activity (MIC >1500 µg/ml against MDR-TB clinical isolates and rMSM), except for these two, the remaining 8 molecules were included in the checkerboard assay. The MIC of RMP was reduced by more than 4-8 fold in the presence of identified active compounds (Table II).

New EP inhibitors counteracted Rv1218c and retained intracellular EtBr accumulation: The EP inhibitory efficiency of the identified putative EP inhibitors RP, PA, TD, MS, TH, DA, FL and RM was tested against rMSM in the presence of EtBr. Bacterial cells unaccompanied by identified active compounds and the known efflux inhibitor piperine were considered as controls. The compound MS, RP, TH, RM, PA and DA showed increased efflux inhibition as compared to piperine (P<0.001). Even though the molecules FL and TA reduced the MIC of RMP, it showed minimal accumulation of EtBr (Fig. 3). The expansion of the fluorescence intensity of EtBr in the presence of most of the inhibitors used confirms that the mechanism of inhibition through the EP inhibition in MDR clinical isolates.

Dodecanoic acid and palmitic acid are cytologically safe: The lysis of RBC was more than 80 per cent at the 1× concentration for TA and MS whereas < 5 per cent RBC lysis was seen in the presence of RM, TH and PA while the integrity of RBCs was completely secured in the presence of DA and FL even at 2× MIC concentration. The molecules RM and RP had normal ranges of 2.7 and 2.9 per cent, respectively, at a 2× MIC. However, due to their increased individual MIC with MDR clinical isolates, these compounds were not pursued further (Fig. 4). Although MS showed a high efflux inhibition, it indeed showed higher haemolytic activity in a concentration-dependent manner. The toxicity of molecules TH, DA, PA and FL was further tested by MTT assay using PBMCs. DA and PA showed the least toxicity even at higher concentrations

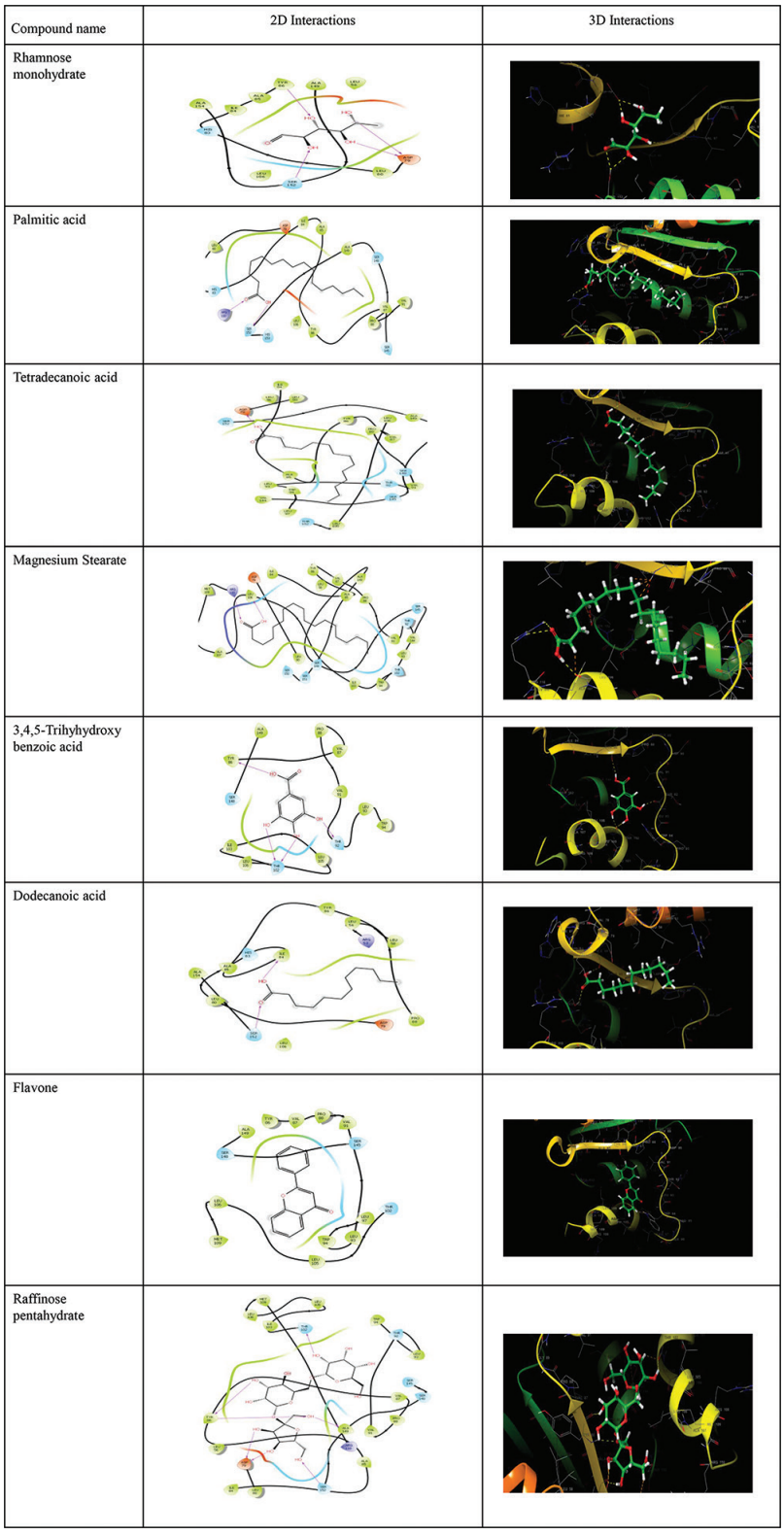

Fig. 1. Docking pose of identified efflux pump inhibitors interacting with RV1218C visualized using Maestro v9.1 for two-dimensional and Pymol viewer version 1.3 for three-dimensional images.

(8× MIC) (Fig. 5). Both FL and TH showed only <20 per cent of cell viability even at 1× MIC.

The cytotoxicity of short-listed EPIs along with Piperine was further tested on Vero (kidney),

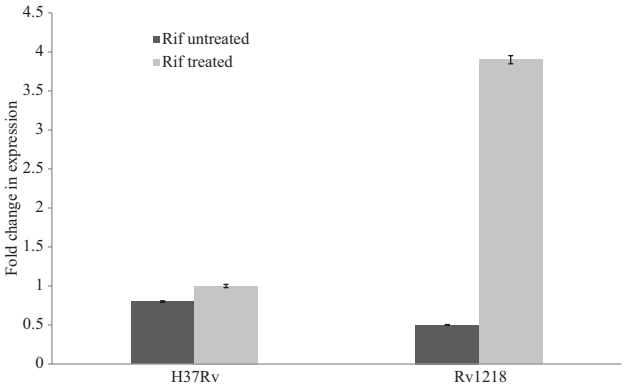

Fig. 2. Analysis of mRNA transcriptional levels of Rv1218c in MDR-TB clinical isolate and H37Rv. Rv1218c: RMP Untreated versus treated P=0.035, H37Rv: RMP Untreated *vs*. treated P=0.287. MDR-TB, multi-drug resistance-tuberculosis; RMP, rifampicin.

THP-1 monocyte (macrophage) and HepG2 liver cells belonging to different cell origins. As predicted, EPIs which showed less toxicity in PBMCs (PA and DA) showed promising results with >85 per cent cell viability in all three cell lines. In THP-1, both DA and PA showed 80 per cent of cell viability in 1× of MIC. In Vero and HepG2 cells, both EPIs (DA and PA) showed 85 and 90 per cent of cell viability, respectively (Fig. 6). The *in vitro* and *ex vivo* study outcomes align to each other supporting that these molecules DA and PA as a possibly reliable EPIs.

Time kill kinetics: The potentiality of the identified molecules on the time-dependent killing activity of RMP against the MDR clinical isolate (MIC >128 µg/ml) was assessed. There was a significant five $\log_{10}$ reduction

Table II. *In vitro* rifampicin and efflux inhibitors combinational studies

Clinical isolates used	Compound name	Concentration of shortlisted compound with RMP (µg/ml)	MIC of RMP	
			Without shortlisted inhibitors (µg/ml)	With shortlisted inhibitors (µg/ml)
MDR-clinical isolate NIRT-001	Piperine	25	>100	64
	RM	700	>128	4
	PA	100		0.0078
	TD	1000		8
	MS	1000		1
	TH	100		0.5
	DA	10		0.25
	FL	100		0.25
	RP	1000		0.0078
MDR-clinical isolate NIRT-002	RM	700	32	8
	PA	100		0.5
	TD	1000		0.062
	MS	1000		4
	TH	100		0.062
	DA	10		0.062
	FL	100		0.062
	RP	1000		1
rMSM	RM	700	8	2
	PA	800		0.5
	TD	1000		4
	MS	1000		2
	TH	600		4
	DA	320		2
	FL	800		2
	RP	1000		4

NIRT, National Institute for Research in Tuberculosis; MDR, multi-drug resistance; rMSM, recombinant *Mycobacterium smegmatis* mc^2 155; RMP, rifampicin; MIC, minimum inhibitory concentration; RM, rhamnose monohydrate; RP, raffinose pentahydrate; PA, palmitic acid; TD, tetradecanoic acid; MS, magnesium stearate; TH, 3,4,5-trihyhydroxy benzoic acid; DA, dodecanoic acid; FL, flavone

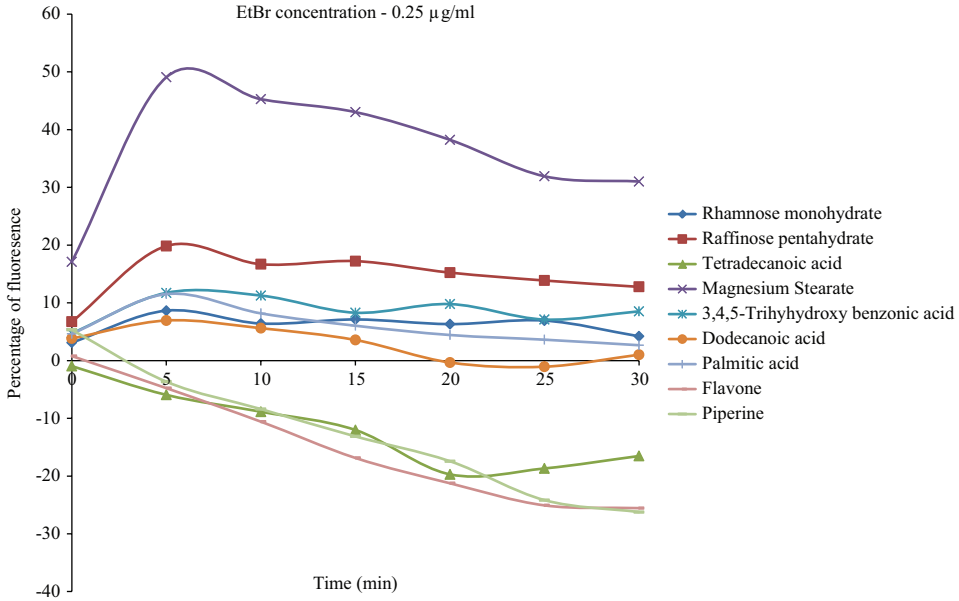

Fig. 3. Effect of shortlisted efflux pump inhibitors on the accumulation of ethidium bromide. The efflux inhibitors were used at ½ MIC and ethidium bromide at 0.5 µg/ml concentration. The rMSM without efflux pump inhibitor serves as a control. MIC, minimum inhibitory concentration; rMSM, recombinant *Mycobacterium smegmatis* mc^2 155.

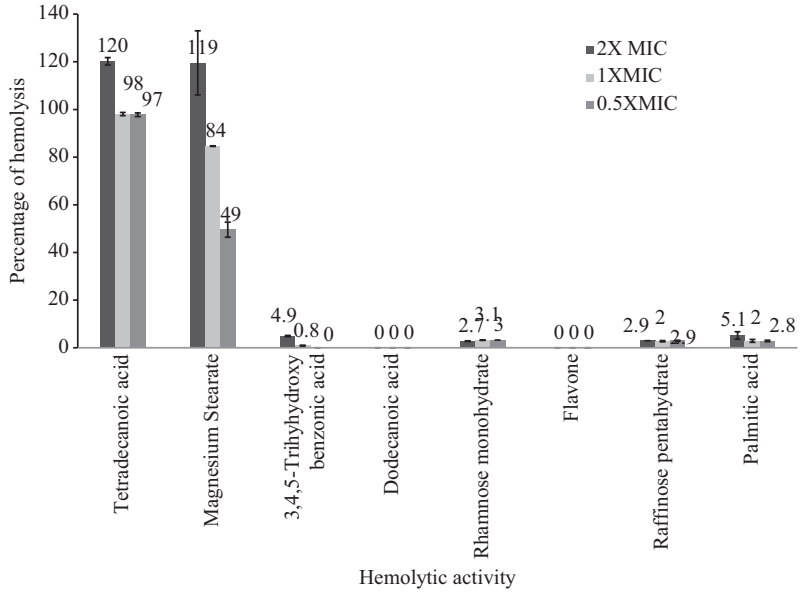

Fig. 4. Haemolytic activity of shortlisted active compounds. Dodecanoic acid, Flavone caused no haemolysis at its 2× MIC. RBCs treated with distilled water and PBS was considered as positive and negative control, respectively. PBS, phosphate buffered saline; RBCs, red blood cells.

in the CFU (P<0.001) for all identified EP inhibitors (data shown for only PA and DA), at the earliest of 48 h in combination with RMP, when compared with Piperine (Fig. 7).

Discussion

RMP and INH are known to be two drugs which are more efficient in terms of efficacy and toxicity to treat drug-sensitive TB infections[32]. However, MDR-TB creates an unenviable dispute in treating and controlling TB. Since 1980, when the influence of EPs on drug resistance was reported, EPs are being viewed as a scope for managing bacterial drug resistance possibly with the first-line anti-TB drugs themselves[33,34]. The expression of EPs increases in the presence of anti-TB drugs. The clinical TB isolates (RMP MIC >128 µg/ml) used in

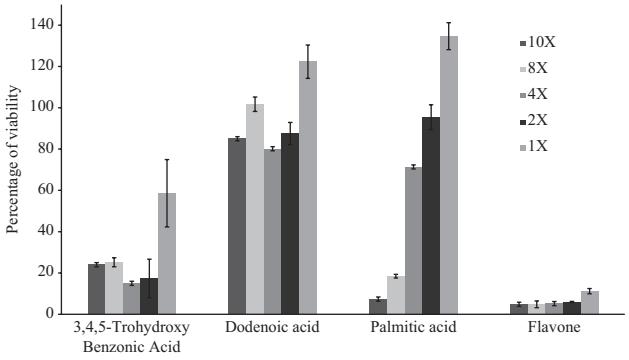

Fig. 5. Cytotoxicity of shortlisted efflux pump inhibitors on the PBMCs. Palmitic acid and Dodenoic acid showed more than 90 per cent cell viability at 2× MIC. Untreated PBMCs were used as a control.

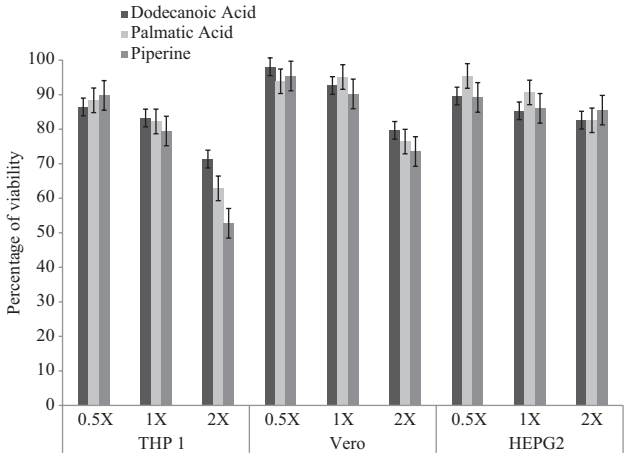

Fig. 6. *Ex vivo* cytotoxicity of dodecanoic acid, palmitic acid and Piperine on different cell lines.

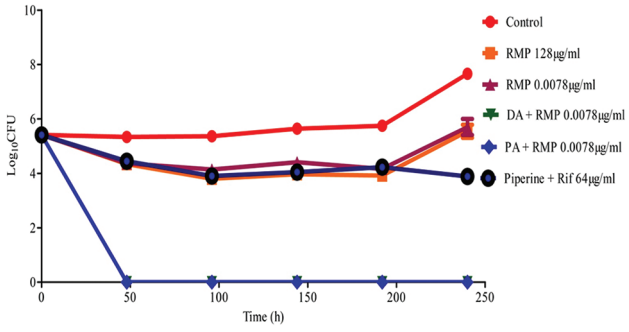

Fig. 7. Representative graph showing time-dependent killing by RMP and reduction in viable MTB MDR-TB clinical strain in the presence of DA and PA. The actual RMP MIC of 128 µg/ml was drastically reduced to 0.0078 µg/ml and 0.25 µg/ml, respectively by PA and DA, which efficiently killed MTB MDR strain within 48 h of RMP exposure (P<0.001).

this study were also found to overexpress Rv1218c, an EP of the ABC transporter family in the presence of RMP (Fig. 2). This portrays the significance of these EPs in the pumping-out of the drug molecules[19] and as potential targets to handle the drug resistance menace. Hence, there has been a pursuit of natural molecules with EPI activity in the recent past[18]. Using *in silico* methods, we selected ten molecules from the pool of 25000 plant-based molecules. Based on the outcome of *in vitro* experiments for anti-TB activity and RMP-MIC lowering activity, eight molecules were chosen for toxicity testing based on which these were further narrowed down to two molecules, namely DA and PA. DA and PA potentially decreased the MIC of RMP by 8 to 1000 folds in the MDR-TB clinical isolates notably a drop in MIC from >128 µg/ml to 0.0078 µg/ml in one of the studied isolates (Table II). The time-kill kinetic study showed that both DA and PA can reduce the time to kill these MDR isolates of RMP by 48 h which otherwise could not be achieved even after 240 h in the presence of RMP alone (Fig. 7). DA showed more than 80 per cent cell viability even at 10-fold higher concentrations of its MIC. PA was found to be non-toxic at 2× of its MIC while being toxic at a higher concentration of 1 mg/ml (Fig. 5). Though the other molecules like TH and FL showed significant RMP MIC lowering activity and EP inhibition, these were more toxic to PBMCs. The non-toxic characteristics of DA and PA were found to be consistent across different cell lines belonging to different organ sources (Figs 5-6). However, both TH and EL were non-lytic to RBCs (Figs 4 and 5). Interestingly, even though TA and FL efficiently minimized the MIC of RMP and showed significant binding with Rv1218c (*in silico*), these did not cause accumulation of EtBr, unlike DA and PA. This could be due to a combined inhibitory activity mediated by efflux inhibition but by targeting other EPs that could be present in the respective clinical strains. Since the EtBr accumulation assay was carried out in recombinant *M. smegmatis* transformed with the Rv1218c, only the Rv1218c EP inhibitors showed the accumulation. Interestingly, a time-dependent fluorescence quenching was noted which could be due to EtBr decay[35]. Hence, to summarize, both DA and PA were able to reduce the MIC of RMP by specifically binding with Rv1218c in the limited evidence provided. This emphasizes the need for further in-depth studies (Fig. 3).

PA is a saturated fatty acid that is abundant in the human physiological system and can be consumed as a part of a regular diet. Formerly, PA was putatively contemplated as a detrimental molecule, but later

scientific insights contradicted this view[36]. PA is also plentiful in the natural food products such as milk, coconut oil and palm oil[37]. PA by itself was found to inhibit MTB in this study. Previously, PA was reported to bind with the Rv3249c and Rv1816 transmembrane proteins of MTB that further hindered the MTB Mmpl protein expression[38]. DA, otherwise called lauric acid, is present abundantly in coconut oil along with PA[39]. DA has been shown to possess anti-microbial properties against various microbial pathogens against a broad range of bacteria, fungi and viruses. Besides a previous report that showed the anti-TB activity of DA[40], this study has demonstrated the RMP-MIC lowering capacity of DA to the scientific community. Coherently, we have also previously reported the anti-TB activity of a herbal plant, *Lantana camara* that was known to contain DA according to the Duke's Database[41]. DA exhibits rapid non-ionic passive membrane transport across lipid bilayer[42]. This quality could be advantageous as MTB is an intracellular pathogen. However, DA gets quickly cleared from the bloodstream and easily digested and metabolized in the liver when administered through intravenous and oral routes, respectively[39]. This may be surmounted by using an efficient and targeted drug delivery system. Interestingly, PA in combination with stearic acid itself could be used for the construction of such solid-lipid nanodelivery systems[43]. We also reported the drug efflux-inhibitory anti-TB activity of other molecules such as FL and TH that was dropped due to toxicity. Using advanced chemical technologies, non-toxic potent analogues of these compounds can be generated.

Several plant-based compounds, including the secondary metabolites, were reported previously to encompass the EPI activity against various pathogens[18,43]. However, authors believe that this is the first report to indicate that both DA and PA were able to bring down the MICs of RMP in more than one MDR-TB isolate, while both were found to putatively bind with RV1218C. Even though this supports the high likelihood of re-establishing RMP into the MDR regimen, these compounds should be validated in the supplementary number of drug-resistant clinical isolates of MTB, including bedaquiline resistance. Furthermore, the specificity of DA and PA by gene knock-out studies also need confirmation. The smaller number of strains included in this study is, however, the major limitation. Further validations to understand other potential targets of these EPIs, their chemical as well as functional interaction with other anti-TB drugs, *in vivo* safety and *in vivo* efficacy testing need to be undertaken before these molecules could be considered for therapeutic purposes.

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