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Article

Is the efflux pump inhibitor Verapamil a potential booster for isoniazid against *Mycobacterium tuberculosis*?

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The membrane-based efflux pump systems are recognized to have an important role in pathogenicity and drug resistance in *Mycobacterium tuberculosis* by the extrusion of toxic substrates and drugs from the inner bacillus. This study aimed to investigate the *in vitro* interaction of Verapamil (VP), an efflux pump inhibitor, with the classical first-line anti-tuberculosis drug isoniazid (INH) in resistant and susceptible *M. tuberculosis* clinical isolates. Seven multidrug-resistant (MDR), three INH monoresistant and four susceptible *M. tuberculosis* clinical isolates were tested for the INH and VP combination by modified Resazurin Microtiter Assay Plate (REMA). Fractional Inhibitory Concentration (FIC) and Modulation Factor (MF) were determined. The INH plus VP combination showed no significant change in the Minimum inhibitory concentration (MIC) values of INH (FIC≥ 0.5; MF=1 or 2).The use of VP in tuberculosis therapy should be managed carefully, considering the resistance caused by specific mutation in *katG* and *inhA* genes, in which the use of these EPIs may have no success. The use of EPIs as an adjunctive drug in the anti-tuberculosis therapy should be further investigated on a larger number of *M. tuberculosis* clinical isolates with different resistant profile.

Keywords: Tuberculosis. Multidrug-resistance. Efflux pumps. Efflux pumps inhibitors. Isoniazid.

INTRODUCTION

Drug resistance is a matter of great concern for tuberculosis (TB) control programs worldwide. Patients who harbor resistant TB must have alternative treatment and, in some cases, the drugs used are more expensive and toxic, and less effective (WHO, 2018).

The multidrug-resistant (MDR) *Mycobacterium tuberculosis* phenotype is caused by sequential mutations in specific chromosomal genes, which are related to the mechanism of rifampicin (RIF) and isoniazid (INH) actions (Zhang, Yew, 2009). However, the genetic basis of these two and other anti-TB drugs resistance are not fully known. In some resistant bacilli, the classical mutations related to resistance to specific drugs are not present, suggesting other resistance mechanisms

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are responsible for the resistant phenotype (Ghajavand *et al*., 2019).

At present, the membrane-based efflux pump systems (EPs) are recognized to have an important role in the bacterial pathogenicity and antimicrobial resistance, including in *M. tuberculosis*, by the extrusion of toxic substrates and drugs from the inner cell. Nonetheless, not only have EPs important biological role in drug extrusion, but also in the uptake of substrates and nutrients necessary to the bacillus survival (Gupta *et al*., 2010a; Ghajavand *et al*., 2019; Rodrigues *et al*., 2012; Chen *et al*., 2018).

These transporters can be classified as primary active transporters, which use ATP direct energy to promote the transport of molecules across the bacterial membrane, against their concentration gradient. The secondary active transporters are membrane proteins that use an electrochemical gradient of cations, the proton motive force, across the membrane coupled to drug extrusion against its concentration gradient (Sharma, Gupta, Pathania, 2019).

Verapamil (VP), a calcium channel blocker, is a drug commonly used to treat hypertension and has shown to have activity as EPs inhibitors (EPI) in *M. tuberculosis* (Machado *et al*., 2012). More recently, the *M. tuberculosis*'s membrane pharmacologic target mechanistic basis for the role of verapamil's potentiation of TB drugs was recognized as having the ability to directly impact on membrane energetic (Chen *et al*., 2018).

In the search for new treatments in cases of resistant TB, in which classical anti-TB drugs are not effective, the combinatory use of efflux pumps inhibitors (EPI), such as VP, has become the subject of some studies (Gupta *et al*., 2010a; Rodrigues *et al*., 2012; Cloete *et al*., 2018; Sharma *et al*., 2010). However, the experimental procedures for understanding EPI activity in *M. tuberculosis* are mostly limited to laboratory strains and few have been performed with clinical isolates (Machado *et al*., 2012), and divergence in results have been observed. Our study aimed to know how the combination of VP with the classical first-line anti-TB drug INH has activity, *in vitro*, against susceptible and resistant (including MDR) *M. tuberculosis* clinical isolates.

MATERIAL AND METHODS

Clinical isolates

A total of 14 resistant and susceptible *M. tuberculosis* clinical isolates (seven MDR, three INH monoresistant and four susceptible to the anti-TB first-line drugs), previously determined by proportion method in Löwenstein-Jensen and genotyped by Mycobacterial Interspersed Repetitive Unit - Variable Number Tandem Repeat (MIRU-VNTR) (Supply *et al*., 2001) and Spoligotyping (Molhuizem*et al*.,1998), were selected for the study (Table I). *M. tuberculosis* wild type 37: subscribed (ATCC 27294) strain was used in all assays as control. Sequencing reactions, to determine mutation in *inhA* regulatory and *inhA* and *katG* structural genes, were conducted previously (Cardoso *et al*., 2004).

Minimum inhibitory concentration (MIC)

The MIC of INH and VP (Sigma-Aldrich, St. Louis, MO, USA) were determined, three times on different days for *M. tuberculosis* $H_{37}Rv$ and all clinical isolates by REMA (Resazurin Microtiter Assay Plate), as previously described (Palomino *et al*., 2002). Carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), a strong protonophore, which specifically dissipates the transmembrane proton gradient component of the proton motive force, was used as EPI reference drug (Chen *et al*., 2018). The drugs concentrations ranged from 0.0009 to 50.0 μg/mL, 3.90 to 500.0 μg/mL and 0.39 to 100.0 μg/mL for INH, VP and CCCP, respectively. Growth and sterility controls for *M. tuberculosis* $H_{37}Rv$ and all clinical isolates were carried out in all assays. MIC was defined as the lowest drug concentration that prevented a color change of resazurin from blue to pink. Isolates with MIC ≥ 0.25 μg/mL were considered INH resistant (Palomino *et al*., 2002).

Drugs Combination assay

The combination of VP and CCCP with INH were performed using VP and CCCP at constant concentration (½ x MIC previously determined by REMA). For the assay, 100 μL of OADC-supplemented Middlebrook 7H9 (Difco Laboratories, Detroit, MI, USA) were added to each well in the microtiter plates and two-fold serial dilutions of INH were carried out. Mycobacterial inoculums were standardized with 1 McFarland scale and diluted (1:20) in OADC-supplemented Middlebrook

TABLE I - Mycobacterial Interspersed Repetitive Unit (MIRU) and Spoligotyping patterns, drugs susceptibility profiles, *katG* and *inhA* mutations in *Mycobacterium tuberculosis* $H_{37}Rv$ and clinical isolates

Strain/Isolates	MIRU	Spoligotyping	Susceptibility pattern	Mutation	
				katG	*inhA*
$H_{37}Rv$	-	-	S	-	-
9S	-	-	S	-	-
46	224326132323	777777774020771	S	-	-
47	123323132321	777777777760771	S	-	-
25252	-	000000007760771	S	-	-
34R	122222153321	777777777760771	INH^R	Ser 315Thr	NM
91R	-	677737607760771	INH^R	Ser 315Thr	NM
4250	-	777777777760710	INH^R	Ser315Ile	NM
64A	124325163322	677737607760771	INH^R/RIF^R	NM	c to t at -15; Ile21Thr
73A	224225123321	677737607760771	$INH^R/RIF^R/PZA^R$	Ser315Thr	NM
109	224326153325	776177607760771	INH^R/RIF^R	Ala109Val	NM
18	224326153324	776177607760771	$INH^R/RIF^R/EMB^R$	Ser315Thr	NM
19	224327153324	776177607760771	$INH^R/RIF^R/EMB^R$	Ser315Thr	NM
71A	225313153323	777777777720771	$INH^R/RIF^R/EMB^R$	Ser315Thr	NM
3614	224225163321	677737607760771	INHR/RIFR/EMBR ETH^R, STR^R	NM	c to t at -15; Ile21Thr

S, susceptible; ^{R}, resistant; INH, isoniazid; RIF, rifampicin; EMB, ethambutol; PZA, Pyrazinamide; STR: streptomycin; ETH: ethionamide *katG*, gene that encode Catalase peroxidase enzyme; *inhA,* gene that encode the enoyl ACP reductase NADH dependent enzyme; -, not performed; NM, no mutation detected.

7H9 (BBL/Becton Dickinson, Sparks, MD, USA) added of VP or CCCP to achieve ½ x MIC final concentration in all microplates wells. The final concentration of INH in the microplates wells ranged from 0.0009 to 50 µg/mL. Growth and sterility controls for *M. tuberculosis* $H_{37}Rv$ and all clinical isolates were carried out in all assays. Incubation conditions and reading were carried out according to REMA (Palomino *et al*., 2002).

The effect of VP and CCCP on the activity of INH were determined by the fractional inhibitory concentration (FIC) according to Pillai *et al*. (2005), where FIC=MIC drugs combination/MIC INH. The results were interpreted according to Coelho *et al*. (2012), considering FIC ≤ 0.25 as synergism, FIC > 0.25 <2 as indifferent, and FIC ≥ 2 as antagonism. To quantify the effect of each inhibitor (VP and CCCP) in reducing the INH MIC values, the modulation factor (MF) was applied by dividing the MIC value determined for INH by the MIC obtained with the INH plus EPI combination (MF=MIC INH/MIC INH+EPI). A four-fold reduction on the MIC value of the INH combined with VP or CCCP (MF value ≥ 4) was considered significant for INH in having better action against the bacillus.

RESULTS AND DISCUSSION

Table II shows MIC values of INH, VP, CCCP and INH combined with the two EPIs at constant concentration for *M. tuberculosis* $H_{37}Rv$ and all studied isolates. All isolates were genetically differentiated and the presence of mutation in specific genes related with resistance to INH was known. The resistant isolates that had Ser315Thr or Ser315Ile mutations in the Catalase enzyme (*katG*) showed MIC range from 3.125 to 12.5 µg/mL. The isolate with Ser 109Val mutation in *katG* had lower INH MIC, 1.56 µg/mL. These MIC values, obtained in our study, corroborate with the literature inferring resistance to INH in *M. tuberculosis*. The cumulative mutations in the regulatory region and in the open reading frame of *inhA* certainly could induce the MIC value observed for the two INH resistant isolates 64A and 3614 (Walker *et al*., 2015).

Analyzing the MIC of VP and CCCP, in susceptible and in resistant isolates, lower values were detected for CCCP than VP. This assay was previously carried out for all isolates to determine the ½ x MIC concentration to perform the drugs combination assays.

As far as we have knowledge, both VP and CCCP are inhibitors of secondary active transporters. This kind of transporters act extruding different compounds by using an electrochemical gradient of cations, the proton motive force, across the cell membrane inducing extrusion against its concentration gradient. VP can modify the proton-motive force of the cell membrane, which is an essential requirement for the function of specifics EPs. Then, VP blocks the activity of EPs and the concentration of antimicrobial agents inside the cells will be higher and may cause bacterial death (van Bambeke, Balzi, Tulkens, 2000).

The effects of VP and other EPIs on reducing the drug resistance in *M. tuberculosis*, have been previously reported (Gupta *et al*., 2010b; Rodrigues *et al*., 2012; Cloete *et al*., 2018; Machado *et al*., 2012; Sharma *et al*., 2010). However, we must emphasize that the *M. tuberculosis* isolates in the above studies were laboratory-induced resistant mutants, which showed EPs (over)expression under drug pressure status, such as prior bacillus exposure to sub-inhibitory concentrations of drugs (Rodrigues *et al*., 2012; Sharma *et al*., 2010).

Like other studies (Coelho *et al*., 2015; Machado *et al*., 2016), the combination of VP with INH in our work showed no significant change in the MIC values of INH (FIC ≥ 0.5; MF = 1 or 2) in the studied clinical isolates (Table II). Coelho *et al*. (2015), working with clinical isolates, observed a MF = 4 only for the pan susceptible reference strain $H_{37}Rv$, which had an initial MIC 0.1 µg/mL for INH and, after the addition of VP at ¼ MIC concentration, the MIC value for INH decreased to 0.025 µg/mL. In our study, the used $H_{37}Rv$ reference strain showed a lower initial MIC 0.06 µg/mL, and no decrease in this value after the addition of VP at ½ MIC concentration was observed. Machado *et al*. (2016) in the same field of study, observed excellent action of VP in decreasing the MIC of INH in only one resistant isolate, which had a MF = 20. No interference of VP, in this sense, was observed in the susceptible $H_{37}Rv$ and in the INH resistant $H_{37}Rv$ mutant. Of course, we could not fail to see, in the above study, a little decrease in the INH MICs values in the other isolates, which account for a MF = 3 in three resistant isolates.

Despite the limitation of using a low number of clinical isolates in our study, the addition of VP at ½ x MIC concentration did not enhance the INH activity in all *M. tuberculosis* (FIC ≥ 0.5; MF = 1 and 2) studied. The result of no significant reduction of the INH MIC by the combination with VP might be due to the studied isolates, which had specific mutation in the *katG* or *inhA* genes. Our data corroborates with

TABLE II - Minimum inhibitory concentration (MIC) of Isoniazid and efflux pump inhibitors alone and in combination with ½ x MIC of efflux pump inhibitors, Fractional Inhibitory Concentration (FIC) and Modulation Factor (MF) for *Mycobacterium tuberculosis* H_{37}Rv and clinical isolates

Strain/ Isolates	REMA MIC (µg/mL)			REMA MIC (µg/mL)		FIC		MF	
	CCCP	VP	INH	INH+CCCP	INH+VP	INH+CCCP	INH+VP	INH+CCCP	INH+VP
H_{37}Rv	3.125	125	0.06	0.03	0.03	0.5	0.5	2	2
9S	1.56	62.5	0.06	0.03	0.03	0.5	0.5	2	2
46	1.56	125	0.03	0.03	0.03	1	1	1	1
47	3.125	250	0.06	0.06	0.03	1	0.5	1	2
25252	1.56	62.5	0.125	0.06	0.125	0.48	1	2	1
34R	3.125	125	6.25	3.125	6.125	0.5	1	2	1
91R	1.56	62.5	12.5	12.5	12.5	1	1	1	1
4250	1.56	125	6.25	6.25	3.125	1	0.5	1	2
64A	1.56	125	12.5	6.25	6.25	0.5	0.5	2	2
73A	3.125	125	6.25	3.125	3.125	0.5	0.5	2	2
109	1.56	125	1.56	1.56	1.56	1	1	1	1
18	0.78	125	3.125	3.125	3.125	1	1	1	1
19	1.56	62.5	3.125	1.56	3.125	0.5	1	2	1
71A	3.125	125	12.5	6.25	6.25	0.5	0.5	2	2
3614	3.125	62.5	6.25	6.25	3.125	1	0.5	1	2

INH, isoniazid; VP, verapamil; CCCP, Carbonyl cyanide *m*-chlorophenyl-hydrazone. REMA: Resazurin Microtiter Assay Plate.

Machado *et al.* (2016), who found a limited effect of VP and other EPIs in isolates harboring mutation in *inhA* and little or no effect in isolates with mutation in *katG*, in which those mutations can induce low and high level resistance, respectively.

Although our and other *in vitro* studies have shown low effectiveness of the INH and VP combination in some *M. tuberculosis* isolates, studies in animal model, combining VP at a dosage of 6.25 mg/kg restored the susceptibility to this classical first-line anti-TB drug in mice infected with MDR *M. tuberculosis* (Louw *et al*., 2011). Additionally, an *in vitro* study with *M. tuberculosis* infected macrophages (Machado *et al*., 2016) showed EPIs cause alterations in the transport of cations in eukaryotic vacuolar efflux pumps and enhancement of the macrophages mediated the killing of the bacillus. This effect in the macrophage associated with the proposed direct interference of the EPIs, as VP,

on the bacterial cell energy (Black *et al*., 2014, Chen *et al*., 2018) could improve the TB therapy.

In the opinion of these authors, there is no doubt on EPs role as an alternative mechanism for anti-TB drug resistance in some *M. tuberculosis* clinical isolates, or even influence to acquire other resistance mechanisms. However, a consideration on the lack of influence of VP on restoring INH susceptibility in some MDR *M. tuberculosis* isolates is clear in our study. The resistance in these isolates is consequence of mutations in the *katG* and *inhA* genes and, even VP having the ability to directly impact bacillus membrane energetics, no synergism with INH plus VP could be observed.

As no effect in decreasing the MIC of INH by the addition of VP, including with our EPI control, CCCP, was observed in our study with susceptible and resistant clinical isolates, it is clear that the activity of VP in the acquisition, as well as in the maintenance of INH resistance in the *M. tuberculosis* clinical isolates studied is only one of the involved mechanisms, as reported by Song and Wu (2016). Then, the consideration of efflux activity and the use of EPI in therapy of TB still need further studies on a larger number of MDR *M. tuberculosis* isolates with different resistant genetic profiles. The use of VP in the therapy of TB should be managed carefully, considering the resistance caused by specific mutations in *katG* and *inhA* genes or other genes, which may lead to the failure of the use of these EPIs. The use of EPIs as adjunctive anti-TB therapy should be more investigated and the issue of their activity in infected macrophage and in reducing anti-TB drugs side effects considered.

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Competing interest

The authors declare that they have no competing interest.

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