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RESEARCH ARTICLE

Antimycobacterial activity of usnic acid against resistant and susceptible strains of *Mycobacterium tuberculosis* and non-tuberculous mycobacteria

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Abstract

Tuberculosis remains a serious public health problem, with nine million cases being reported annually. Treatment with antibiotics is the most effective mechanism to control this disease, although the increase in cases with resistant strains, co-infection with HIV, and the long duration of treatment has established the need to develop new drugs. Here we show the activity of usnic acid against susceptible and resistant Mycobacterium tuberculosis strains and against nontuberculous mycobacteria. Further, we did not identify any contribution of efflux in innate resistance to usnic acid.

Keywords: *Usnic acid;* Mycobacterium tuberculosis*; nontuberculous mycobacteria; resistance; efflux*

Introduction

Antimicrobial treatment is the most suitable method for tuberculosis (TB) control; however, the rise in the number of cases with resistant strains, HIV coinfection, length of the treatment, and persistent strains reduce the therapy efficacy, establishing the need to develop new drugs (WHO, 2008). In recent years, the number of opportunistic infections caused by saprophyte mycobacterial species, whose treatment may be even more difficult than that observed for TB, has increased. This strengthens the need to develop new drugs for the treatment of mycobacteriosis (Reddy et al., 2008; Hsieh et al., 2008; Garcia-Navarro et al., 2008). Among the different strategies for the search of new drugs, the screening of crude extracts from natural resources is an important tool. In fact, it has recently been shown that several compounds derived from marine, micro- and macroorganisms, plants, lichens, fungi, bacteria, etc. have antimycobacterial activity (Mata et al., 2004; Stavri et al., 2003; Okunade et al., 2004; Pauli et al., 2005; Rojas et al., 2006; Azevedo et al., 2008).

Usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3 (2H, 9bH)-dibenzo-furandione] is one of the most intensively studied secondary metabolites regarding its biological activities. Several species of lichens, such as those belonging to families Cladoniaceae, Usneaceae, Lecanoraceae, Ramalinaceae, and Parmeliaceae, produce usnic acid (Ingólfsdóttir, 2002).

Usnic acid gives lichens a protection against microbial infections, UV radiation, and drying (Cocchietto et al., 2002). In addition to antibacterial activities, different studies have confirmed antiproliferative, antiviral, anti-inflammatory, and antiprotozoan activities of this molecule (Vijayakumar et al., 2000; De Carvalho et al., 2005; Fazio et al., 2007; Bazin et al. 2008).

In vitro studies have shown the antimicrobial activity of usnic acid against clinical isolates of *Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus, Streptococcus mutans,* and *Escherichia coli* (Ingólfsdóttir, 2002). It has also been reported that extracts and substances obtained from some lichen species show antimycobacterial activity, particularly against *Mycobacterium aurum, Mycobacterium*

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avium, and *Mycobacterium smegmatis* (Ingólfsdóttir et al., 1998; Ingólfsdóttir, 2002; Copp, 2003). In this study, we evaluated the antimicrobial activity of usnic acid against susceptible and resistant clinical isolates of *M. tuberculosis,* and against four species of non-tuberculous mycobacteria (NTM).

Material and methods

Strains

Antimicrobial activity was evaluated against *M. tuberculosis* H37Rv (ATCC 27294); isoniazid (INH) resistant *M. tuberculosis* (INHr); rifampicin resistant *M. tuberculosis* (RMPr); streptomycin resistant *M. tuberculosis* (SMRr) with mutation in the *loci* KatG S315T, RpoB S531L, RpsL K43R, respectively; *M. fortuitum* (ATCC 35931); *M. chelonae* (ATCC 946); and *M. kansasii* (ATCC 12478).

Antimycobacterial activity evaluation

Resazurin microtiter assay (REMA) was used to determine antimycobacterial activity (Palomino et al., 2002). Briefly, bacterial suspensions were prepared in sterile water containing 3-mm glass bead, homogenized by vortex, and the turbidity was adjusted with a tube of McFarland scale (3.2×10^6 cfu/mL). The inoculum was prepared by diluting the bacterial suspension into the 1:20 ratio in 7H9 broth medium (4.7 g of Middlebrook 7H9 broth base (Difco -Becton Dickinson resazurin (Sigma) CCCP 5 µM and verapamil 100 Mm (Sigma)), 2 mL of glycerol (Vetec) in 900 mL water) enriched with 10% oleic acid, albumin, dextrose, and catalase (OADC-BBL). The assay was carried out in a 96-well microplate using resazurin as a cellular-viability indicator. Ten percent OADC-enriched 7H9 medium was used. Usnic acid (Sigma) (Figure 1) was dissolved in DMSO and minimal inhibitory concentration (MIC) was determined starting from 100 µg/mL in 1:2 serial dilutions.

Relation between efflux mechanism and usnic acid

The possible existence of efflux mechanisms involved in the innate resistance to usnic acid was evaluated. The REMA was used for MIC determination with the presence of classic efflux inhibitors (CCCP 5 µM and verapamil 100 µM) in 7H9-OADC medium.

Results and discussion

The earliest description of the antimycobacterial activity of usnic acid was recorded over 60 years ago (Shibata et al., 1948; Stoll et al., 1950). Usnic acid activity against *M. aurum*, a saprophyte species whose susceptibility profile has been considered similar to that of *M. tuberculosis* was described (Ingólfsdóttir et al., 1998). Recently, antimicrobial activity of usnic acid against *M. tuberculosis* (Copp, 2003) was shown; however, to our knowledge there is no report about the antimicrobial activity of usnic acid to clinical isolates which are susceptible and resistant to the current drugs used for TB treatment.

In our study, usnic acid showed activity against both resistant and susceptible strains (Table 1), permitting us to infer that there is no cross resistance with INH, RMP, and SMR when the molecular basis to resistance is mutation of the *loci* KatG S315T, RpoB S531L, and RpsL K43R. This is a pertinent point because these drugs are the basis of the current therapy for TB, and the molecular alteration observed in these strains molecular alteration responsible to resistance these strains studied.

Contrasting with an earlier study, we observed a MIC which was five times lower in *M. tuberculosis* than *M. aurum* (Ingólfsdóttir et al., 1998). This discrepancy could be based on the difference of the methodologies used to determine the susceptibility and/or variation of intrinsic resistance between the species that were studied.

Interestingly, the MIC of usnic acid to INHr strain was five times lower than the susceptible strain (Table 1). This INHr strain has a mutation of S315T in *kat*G, which is observed in most *M. tuberculosis* strains

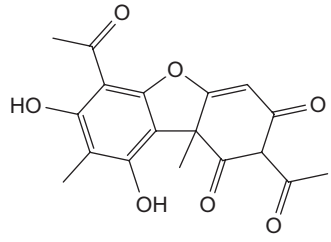

Figure 1. Structure of usnic acid.

Table 1. Usnic acid antimicrobial activity.

	Usnic acid	CCCP	Verapamil
H37Rv	12.25 µg/mL	12.5 µg/mL	12.5 µg/mL
INHr	1.56 µg/mL	NR	NR
SMRr	6.25 µg/mL	NR	NR
RMPr	12.5 µg/mL	NR	NR
M. kansasii	12.5 µg/mL	12.5 µg/mL	6.5 µg/mL
M. avium	100 µg/mL	100 µg/mL	100 µg/mL
M. fortuitum	50 µg/mL	25 µg/mL	25 µg/mL
M. chelonae	25µg/mL	50 µg/mL	25 µg/mL

MIC values of usnic acid against isoniazid (INH), streptomycin (SMR) and rifampicin (RMP) resistant and susceptible strains *M. tuberculosis,* and NMT with and without inhibitor efflux.

of INHr. This molecular alteration is related with the production of catalase-peroxidase with decreased intracellular detoxification capacity. Recently, it was shown that among the several actions of usnic acid in hepatocytes, the most important is the hydrogen peroxide production, increasing the oxidative stress (Han et al., 2004). These results when taken together allowed one to infer a possible link between oxidative stress produced by usnic acid and high activity against the INHr strain with deficient activity catalase-peroxidase. INH is one of the main drugs for treatment of TB; however, in the last few years there has been an increase of TB cases with INHr strains, so the possibility of finding drugs which act against INHr strains is an important method to control TB.

Usnic acid was also active to all NTM that were evaluated in our study with MIC of 12.5-100 μg/mL (Table 1). These four mycobacterial species have gained prominence as infectious agents in the last few years. The slow-growing species, *M. avium* and *M. kansasii,* are most often associated with disseminated and chronic pulmonary infections among immunosuppressed and elderly patients, while the rapid growing *M. fortuitum* and *M. chelonae* are mainly related with soft tissue and skeletal infections. Treatment of NTM infection is difficult, and requires long courses of multidrug therapy with or without adjunctive surgical intervention. In fact, most NTM species pose a particular therapeutic challenge because of a lack of effective and well-tolerated antimycobacterials (Jarzembowski & Young, 2008). So, the development of new drugs to treat these infectious diseases is an important goal.

Drug efflux is an important mechanism of intrinsic and acquired antimicrobial resistance, decreasing the activity of several natural or synthetic antibiotics. To evaluate the relationship between efflux mechanism and usnic acid resistance in these mycobacterial species studied, the MIC was determined in presence of CCCP, an ionophore, and change to verapamil, a calcium channel blocker, both classical efflux inhibitor mechanisms. The results observed show that there is no link between efflux mechanism and usnic acid resistance in any of the species studied. This is an important observation because the new antimicrobial must overcome the intrinsic resistance mechanism as a means to be more effective in its activity.

Even though the mechanism of the action of usnic acid is unknown, it presents a good activity against mycobacterial species, and it is one of the few cases where an isolated substance from a natural source shows good antimicrobial activity. Other studies with usnic acid derivatives could permit to achieve a more potent substance.

Declaration of interest

This work was supported by grants from CNPq and CAPES-Brazil. The authors report no conflict of interests.

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