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In vivo antifungal activity of dipyrithione against *Trichophyton rubrum* on guinea pig dermatophytosis models

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ABSTRACT

The treatment of dermatophytosis has improved considerably over the past several decades following the introduction of the oral antifungals such as azoles and amphotericin B. However, these drugs have had limited success because the treated fungi often develop drug resistance, resulting in recurrence when applied in various topical formulations. Thus, there are constant needs for new topical agents that are effective against dermatophytosis. Dipyrithione is an attractive candidate to become an antifungal agent due to its broad spectrum of antimicrobial activities. In this study, we determined that dipyrithione could potently inhibit the growth of *Trichophyton rubrum*, which is the most common cause of dermatophytosis. The MIC_{50} value of dipyrithione against *T. rubrum* was measured as 6.03 μM, as compared with miconazole (MIC_{50}: 1.38 μM). Additionally, the compound caused morphological changes in the fungi, which was examined using the morphological interference assay. The *in vivo* experiment further revealed that dipyrithione had a healing effect on the skin of guinea pigs infected with *T. rubrum*. Our studies have demonstrated that dipyrithione had a potent antifungal activity *in vitro* and *in vivo*, suggesting that it could be formulated as a potential antifungal lead compound in search for novel therapeutic agents against dermatophytosis.

1. Introduction

Dermatophytosis (also named as tinea pedis, athlete's foot and Hong Kong foot) is one of the most frequent fungal infections worldwide, and it is caused by fungi known as dermatophytes, such as *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis*, *M. gypseum* and *Epidermophyton floccosum* [1]. The fungi grow best in moist skin areas like between the toes [2], and lead to signs and symptoms such as itching, scaling, and redness in the affected area [3]. The disease is typically transmitted on the warm and damp surfaces around pools, public showers and bathrooms [2]. The incidence of this infection has increased over recent years, with an estimated worldwide prevalence of 20–25% [4]. The fungal infection is particularly common and widespread in immunocompromised patients [5,6]. Although dermatophytes tend to affect the feet, it can infect or spread to the other parts of the body, such as the groin, and the name of the condition changes according to the infected location, such as tinea corporis on the body [7].

There is a long felt need for a practical and efficacious topical treatment of dermatophytosis that can be prescribed over the counter. The topical antifungal agents (allylamines and azoles), including the form of spray, ointment, powder, cream, aerosol solution or gel, have been developed and used for external treatment of dermatophytosis. However, relapse or treatment failure is very common, and the currently used antifungals are often associated with side effects and variable bioavailability [8–10]. Moreover, allylamines are much more expensive than other treatments such as azoles. Thus, despite there are the topical anti-fungal drugs available on the market, new antifungals are continuously being sought and developed for better treatment of invasive fungal infections. *In vivo* studies using animal models are pivotal to develop a lead compound as an antifungal drug candidate. Different dermatophyte species including *Trichophytons* and *Microsporum canis* have successfully been used in animal models since these species quickly cause acute inflammatory infections that can be analyzed by clinical assessment [11,12]. The use of guinea pigs as an animal model for dermatophytosis is based on the predisposition of this animal species to dermal fungal infections with clinical features comparable to those seen in humans [13,14]. Guinea pig models infected with *T.*

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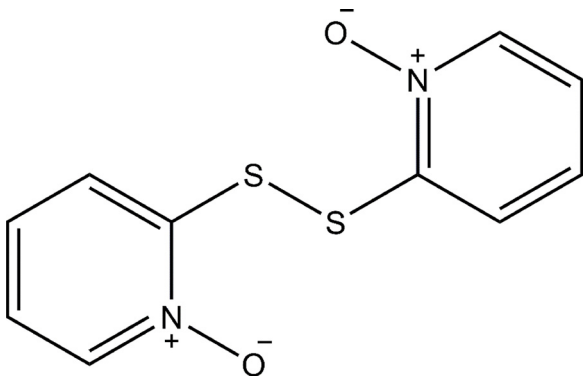

Fig. 1. The chemical structure of dipyrithione.

rubrum are commonly used to evaluate the efficacy of antifungal agents and for studying host defense mechanisms [15].

Dipyrithione (2, 2′-dithiobispyridine-1,1′-dioxide), a pyrithione derivate (Fig. 1), is usually used as an antimicrobial drug [16,17]. It has previously been isolated from the genus *Marsypopetalum*, basidiomycete (mushroom) and *Allium stipitatum* [16]. Due to its broad antibacterial and antifungal spectrum, it is widely added into cosmetics and shampoo. However, the compound has not been studied for its antifungal efficacy in animal models against *T. rubrum*. The purpose of the current study is to determine the antifungal effects of dipyrithione against *T. rubrum* both *in vitro* and *in vivo*. A guinea pig dermatophytosis model was used to investigate the therapeutic efficacy of topical application of dipyrithione.

2. Materials and methods

2.1. Dermatophyte isolates

T. rubrum ATCC-MYA4438 isolate was cultured on potato dextrose agar (PDA; sigma, St. Louis, USA) plates and incubated at 30 °C.

2.2. Animal study

Female guinea pigs (mean body weight, 300 g) were obtained from Guangdong Medical Laboratory Animal Center (Guangdong, China). All animal experiments in this study were approved by the laboratory animal ethics committee of Shenzhen University [Approval number: SYXK (Yue) 2014-0140].

2.3. Antifungal agents

Dipyrithione was purchased from J&K Chemical LTD (Beijing, China). Miconazole was purchased from Dalian Meilun (Dalian, China).

2.4. Broth microdilution antifungal assay

To evaluate the antifungal activity, *T. rubrum* was cultured at 30 °C on PDA for 2 weeks to produce an adequate amount of conidia. A mixed suspension of conidia and hyphae fragments was obtained by covering the fungal colonies with sterile saline (0.85%) and gently rubbing the colonies with an inoculation loop. The suspension was then filtered through four layers of sterile lens paper to remove the hyphae, and the filtrate was centrifuged at 1000 × *g* for 10 min to separate the conidia. These conidia were then washed twice with sterile saline and finally suspended in RPMI 1640 medium with L-glutamine buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) and 2% glucose (m/v). The conidia concentration was adjusted with the medium to 1×10^4 cells/mL using hemocytometer counts. The antifungal susceptibility test was performed as outlined in document M38-A2 with minor revisions [18]. The conidia suspension was seeded into 96-well plates that contained a serial two-fold dilution of dipyrithione starting the concentration of 20 μg/mL (190 μL of cell suspension and 10 μL of the test agent in each well). Each treatment was run in triplicates. Miconazole (20 μg/mL) was served as a positive control and the well with conidia and DMSO only was acted as a negative control (Ac) in the assay. After 7 days of incubation in a plate shaker at 30 °C, the optical density at 510 nm (OD) of each well was measured using a microplate reader (Bio-Rad, USA) and the fungal growth inhibition was determined using the below equation:

$$\text{Growth Inhibition}\,\% = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

Ac was the average OD value of the negative controls, and At was the average of OD value of the triplicated treatment wells. The median inhibitory concentration (MIC_{50}) was calculated by Graphpad prism 5. The minimal inhibitory concentration (MIC) is the antifungal concentration at which at least 80% decrease in absorbance is detected compared with the control in the absence of drug.

2.5. Microscopic examination of fungal morphology

The morphological alternations of *T. rubrum* after the drug treatment was examined. The fungi were treated with 10 μg/mL of diphyrithione or miconazole in RPMI medium and incubated at 30 °C for three days before the examination. DMSO treated fungi were used as a negative control. After incubation, hyphae were fixed with lacto-phenol–cotton blue stain and observed under light microscopy at 400× to examine morphological alterations. The microscopic structural alterations in the test were recorded and compared with the normal growth in the negative control groups.

2.6. Cytotoxicity assay

Cytotoxicity of dipyrithione on 293 T cell line was examined using the sulforhodamine (SRB) assay [19]. Briefly, 293 T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Camarillo, USA). Serial dilutions of dipyrithione were prepared using 10% DMSO. 190 μL of the cell suspension was added into the 96-well plate to achieve a final cell density of 5000 cells/well. One plate was fixed immediately *in situ* with cold trichloric acetic acid (TCA) to represent a no growth control at the time of drug addition (day 0). 10 μL of 10% DMSO was used as a negative control group. After 72 h incubation, the cells were fixed by adding cold TCA to each well. The wells were washed with running water and air-dried, and SRB solution was added into the well and stood for 30 min. Afterwards, the wells were washed with 1% acetic acid, and the cell-associated SRB was solubilized with 200 μL of 10 mM unbuffered Tris base (pH 10). The OD of each well was measured at 515 nm using a microplate reader after shaking the plate for 5 min or until the dye was completely solubilized. The percentage of the cell growth inhibition was calculated as follow.

$$\%\ \text{Cytotoxicity} = \left(1 - \frac{\text{OD (cells + tested agents)} - \text{OD (day 0 only cells)}}{\text{OD (cells + 10\% DMSO)} - \text{OD (day 0 only cells)}}\right) \times 100$$

IC_{50} is defined as the drug concentration causing a 50% decrease of absorbance over that of negative control.

2.7. Guinea pig dermatophytosis model and antifungal treatment

A guinea pig infection model was established based on the research by Ghamnoum et al. [13]. *T. rubrum* isolate was subcultured on PDA plates and incubated at 30 °C for 14 days. The conidia was prepared at 1×10^8 conidia/mL using the same conidia preparation procedure as above, which were used to inoculate the animals.