SHORT COMMUNICATIONS

Hydrogen Sulphide Ameliorates the Toxic Effect of Clotrimazole Against *Trichophyton rubrum*

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ABSTRACT

Introduction: Reactive sulphur species (RSS) have been recently discovered to be a part of a major endogenous antioxidant system. Nonetheless, the RSS was implicated as an underlying mechanism in reducing the efficacy of several antibiotics, such as penicillin and carbapenem. The emergence of drug resistance has been a global concern. In fact, several incidences on the azole resistant *Trichophyton* sp. isolates in the clinical setting, such as clotrimazole, have also been reported. Clotrimazole is a broad-spectrum antifungal commonly used in dermatophytosis management caused by the Trichophyton, Epidermophyton, and Microsporum genera. This study investigated the role of RSS, as a potential element underlying the resistance against clotrimazole. **Methods:** The *Trichophyton rubrum* growth in sulphide-rich environment was initially established by exposing towards several concentrations of sodium hydrosulphide (NaHS). Then, to investigate the potential interaction between the RSS and clotrimazole, the *T. rubrum* were treated with 1000 µM of clotrimazole, with or without co-treatment several concentrations of NaHS. Broth microdilution method was performed to observe the growth of the fungus. **Results:** As expected, clotrimazole exhibited cytotoxic effect towards *T. rubrum* at high concentration. Interestingly, the growth of *T. rubrum* was significantly recovered ($p<0.01$) in samples co-treated with clotrimazole (1000 µM) and NaHS in a dose-dependent manner. Co-treatment with the highest concentration of NaHS (1000 µM), the *T. rubrum* growth was recorded approximately 90%, despite being exposed to 1000 µM of clotrimazole. **Conclusion:** In conclusion, RSS potentially interferes with the efficacy of clotrimazole. The exact mechanism underlying such activity warrants further investigation.

Keywords: Dermatophytes; *Trichophyton rubrum*; Sodium hydrosulphide (NaHS); Clotrimazole; Reactive sulphur species (RSS)

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INTRODUCTION

Dermatophytosis, commonly known as tinea, affect 20-25% of the global population (1). The prevalence of dermatophytosis is continuously rising and has gained recent attention due to significant public health concern in recent decades. Alarmingly, with an inclination to simmer, the problem of epidemic of antifungal therapeutic failure is on the rise has been reported in South Asian countries (2), Europe (3), and India (4,5) are undeniable. High relapse incidence, recurrent cases, and chronic persistent of dermatophytosis impede the pharmacotherapeutic management of superficial cutaneous mycoses and raise the issue of the effectiveness of antifungal medications on the market. However, the underlying causes of these phenomena are unknown, and the "azole menace" which cause the emergence of multi-azoles resistant (also known as 'superbugs'), led to virulent fungal pathogens and newly acquired resistance mechanisms as a result of widespread and prolonged use of azoles and/ or repeated clinical exposure to suboptimal concentrations of antifungal drugs, particularly clotrimazole and itraconazole (6). In addition to overcome these challenges, various strategies have been proposed by researchers, such as introducing alternative treatment derived from natural products (7), discovering a novel class of antifungal agent that works by a new mode of action (8), or formulating novel topical drug delivery systems, i.e., vesicular carrier systems (9), nanoparticles (10,11), and film-forming systems (12). However, to date, none of these strategies successfully acknowledge the main core problems that are facing the global community.

Reactive sulphur species (RSS) are an important signalling mechanism. It has been linked to several physiological mechanisms, including the detoxification process in organisms (13,14). RSS includes several

sulphur-containing endogenous molecules, including hydrogen sulphide (H_2S), cysteine hydropersulphide (CysSSH), and glutathione persulphide (GSSH). In fact, most proteins in the biological system are heavily polysulphurated in the cysteine residue. The sulphur atom (S) is a chalcogen with six valence electrons and over 30 allotropes, making it accessible for a broad range of oxidation. RSS is undoubtedly highly flexible, as it may receive or donate electrons and exists in a wide oxidation state range from -2 to +6 (15). Additionally, the RSS, such as persulphides or polysulphides, contain more than a single sulphur atom and are known to be highly nucleophilic (16). Hence, considering major drug metabolites are electrophiles, it is hypothesised that endogenous RSS might influence the efficacy of drugs and that fungi can use RSS to evade the cytotoxic effects of antifungal agents.

Recent studies have demonstrated the involvement of RSS in the emergence of antimicrobial resistance through the facilitation of intrinsic resistance via the RSS-mediated composition of certain beta-lactam rings. An illustration of this can be seen when *Escherichia coli* utilises the RSS-mediated mechanism to modify certain beta-lactam antibiotics (17). Also, Akaike et. al., found that RSS produced by Salmonella suppresses anti-bacterial autophagy by lowering the cellular 8-nitro-cGMP level. The findings indicate that bacteria-derived RSS have an immunomodulatory effect as they can enhance bacterial intracellular survival by inhibiting autophagy (18). Nonetheless, such observations in fungi, which are a more complex biological system than bacteria are still loosely documented. Possibly, fungi may also be able to utilize the RSS to gain survivability and adaptability against a harsh environment, leading to antifungal resistance. Taking this into account, this study was done to investigate the potential interactions between the RSS and clotrimazole in *T. rubrum*. The information from this study can contribute to a new perspective from the clinical and toxicology perspectives for healthcare providers in strategizing the use of future antifungal drugs. This study will also emphasize on the involvement of redox biology in the emergence of the antifungal resistance, which can be utilized as a strategy for improving drug discovery and pharmacotherapeutic management of dermatophytosis.

MATERIALS AND METHODS

Materials

The dermatophyte fungus, *T. rubrum* (ATCC 28188) was obtained from the American Type Culture Collection. Sodium hydrosulphide (NaHS) was purchased from Saint Louis, USA. The antifungal drug, clotrimazole, Sabouraud Dextrose Agar (SDA), and RPMI-1640 media were purchased from Sigma-Aldrich, Merck Millipore (Darmstadt, Germany). All chemicals purchased are at the highest grade available.

Inoculum preparation

T. rubrum is cultured on Sabouraud Dextrose Agar (SDA) at 28°C for 7 to 14 days (12,19). The inoculum of the dermatophyte was prepared according to the Clinical and Laboratory Standard Institute (CLSI) M38-A2 (2008) protocol. The *T. rubrum* colonies that grow in the SDA plate were covered with 10 ml of sterilised distilled water. The tip of the transfer pipette was used to rub the colonies gently prior to transferring the suspension to a sterile 15-ml centrifuge tube and letting it settle for 15 minutes. The sterile saline was added to adjust the cell density to match the turbidity of 0.5 McFarland's standard (1.5×10^8 CFU/ml) at 625 nm wavelength. The suspension was further diluted (1:50) in RPMI 1640 that buffered with 0.165 M of morpholine-propanesulfunic acid (MOPS) at pH 7.0 (modified RPMI 1640 media) to get a cell count in the range of 2-4 X 10^6 CFU/ml.

Determining *T. rubrum* growth in sulphide-rich environment

The broth microdilution method according to CLSI M38-A2 (2008) protocols that were performed on 96-well microtiter plates was used to determine the *T. rubrum* growth that was subjected to various concentrations (1-1000 µM) of NaHS and clotrimazole (positive control). This method involves two-fold serial dilutions of the tested compounds in a modified RPMI-1640 medium by dispensing 100 µl of liquid medium in the sterile 96-well microplate, followed by 100 µl of 2000 µM of NaHS in the first three rows and 2000 µM of Clotrimazole in the next three rows. Starting from the first row, two-fold serial dilution was conducted up to the 12th column. Subsequently, 100 µl of prepared inoculum was dispensed in the treated wells. The seventh and eighth rows were for growth control (negative control) and sterility control, respectively. Then, the 96-well microplate was incubated at 35 °C for 72 h (20). The percentage of dermatophyte growth was obtained using the microplate reader (Infinite M1000, Switzerland) at 625 nm.

Identification potential interaction of sulphide donour and antifungal agents

The broth microdilution method was performed in NaHS cotreated samples of clotrimazole. Briefly, the sterile 96-well microplates were filled with various concentrations (up to 1000 µM) of NaHS and 1000 µM clotrimazole in a one-to-one ratio in modified RPMI-1640 media. Following that, 100 µl of prepared inoculum was dispensed into the treated wells. Non-NaHS co-treated samples served as a negative control.

Statistical analysis

The data experiments were expressed as the mean of three consistent replicates. The percentage of *T. rubrum* growth was calculated based on the given formula equation:

$$\frac{(x-y)}{(z-y)} \times 100\%$$

where x represents the average absorbance of the strain treated with the tested compounds. The y is the medium control average absorbance, and the z is the strain control average absorbance. The data was analysed statistically using the t-test, where *P* values of < 0.05 were considered as statistically significant.

RESULTS

T. rubrum growth in sulphide-rich environment

To determine whether *T. rubrum* can survive in sulphide-rich environment, the dermatophyte was treated with NaHS at several concentrations (0.5 µM, 1.0 µM, 2.0 µM, 3.9 µM, 7.8 µM, 15.6 µM, 31.3 µM, 62.5 µM, 125 µM, 250 µM, 500 µM, and 1000 µM) by using the broth microdilution study. Figure 1 shows the percentage growth of *T. rubrum* at different concentrations of NaHS and clotrimazole. The data obtained shows that *T. rubrum* can significantly ($p < 0.01$) survive up to 45.1% of the population at the highest NaHS concentration, 1000 µM. There was no growth (< 10%) of *T. rubrum* that was treated with clotrimazole in the range of 2 – 1000 µM.

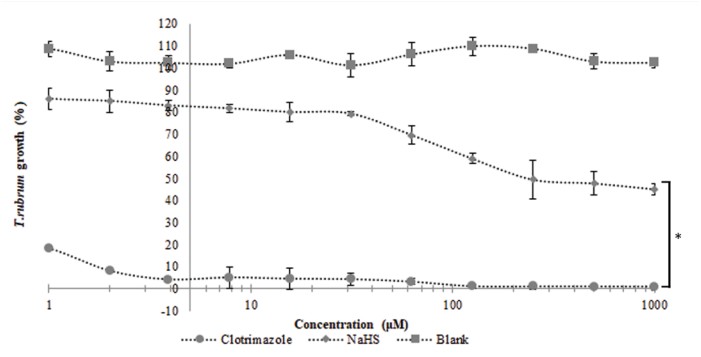

Figure 1 : Percentage of *T. rubrum* growth in different concentrations of NaHS and clotrimazole. *$p < 0.05$.

Identification of a potential interaction between sulphide donour and antifungal agent

To determine whether NaHS can directly modulate antifungal activities, the antifungal agent was co-treated with NaHS using the broth microdilution method. Figure 2 shows the co-treatment results of treating *T. rubrum* with NaHS and clotrimazole as well as clotrimazole alone. The data obtained show that co-treated *T. rubrum* with NaHS and clotrimazole significantly ($p < 0.01$) caused inactivation of the antifungal agent as the *T. rubrum* grew with the highest percentage of 93% at a NaHS concentration of 1000 µM. Untreated cells were used as controls.

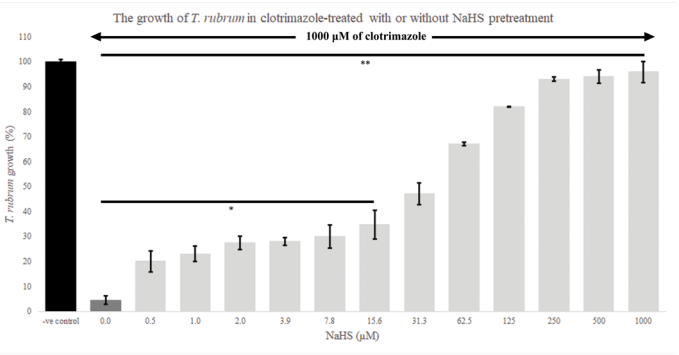

Figure 2 : Percentage of T. rubrum growth co-treated with various concentration of NaHS and 1000 µM of clotrimazole. *$p < 0.05$, **$p < 0.01$.

DISCUSSION

Limited antifungal options have been a concern particularly in the issue of treatment failure and antifungal resistance. Inadequate efficiency in the treatment of antifungal potentially can lead further spreading of the infection and perhaps develop resistance towards the treatment. It is undeniably true that the prevalence of antifungal resistance is a growing concern that demands numerous investigations and efforts in the search for new drugs. For instance, in India, the resistant incidence has recently ranged from 16% (Southern India) to 75-77% (West, North and East India) (22). In the present study, it was shown that clotrimazole, an antifungal drug, exhibits high fungicidal activity against *T. rubrum* by fundamentally reducing the permeability barrier that is present in the cytoplasmic membrane of fungal cells (23,24) By preventing the 14-alpha-demethylation of lanosterol, clotrimazole is able to inhibit the biosynthesis of ergosterol in a manner that is proportional to the concentration of the compound (24,25). Ergosterol is a crucial component of the membranes of fungal cells that controls the fluidity, permeability, and activity of membrane-associated proteins. It is a complex biosynthesis process that consumes a high energy pathway which involves the presence of various enzymes (26). In the present study, NaHS partially inhibits the growth of pathogens at the highest concentration of 1000 µM. Brenna and David (2020) revealed that upon infection, there is a rise in hydrogen sulphide and reactive sulphur, which will impede bacterial survivability in the myriad of infected cells or animals (27). The presence of hydrogen sulphide disrupted various enzymes in *Cryptococcus neoformans* (28), *Aspergillus fumigatus* (29), and *Candida spp.* (29), which theoretically may disrupt the dermatophyte enzyme responsible for the production of ergosterol. Thus, if the synthesis of ergosterol is stopped, the

cell will be unable to construct a cell membrane that is both intact and functional. To date, Jordá and Puig (2020) recently discovered that defects in sterol production generate pleiotropic defects that limit cellular proliferation and tolerance to stress (26). Apart from that, ergosterol also directly promotes the growth of fungal cells in a hormone-like manner; therefore, the rapid onset of the above events leads to a dose-dependent inhibition of fungal growth (24).

The highest concentration of clotrimazole (1000 µM) has been noted to exert maximum efficacy against *T. rubrum* (Figure 1), which can subsequently provide a stronger baseline for investigating the impact of NaHS on the potency of this antifungal drug at its optimal efficacy. As a result, the treatment of 1000 µM of clotrimazole with various concentrations of NaHS reduces the efficacy of the antifungal drug against fungal pathogens in a dose-dependent manner. This result embark that NaHS attenuates the antifungal activity of clotrimazole. The fungicidal activity of clotrimazole was significantly ($p < 0.01$) diminished (fungal growth >80%) due to the presence of NaHS at a concentration of more than 125 µM. Here, we showed that the presence of NaHS increased the cell survivability (Figure 2), it is possible to note that the addition of sulphide potentially leads to accumulation of endogenous persulphide or polysulphides that can somewhat cause nucleophilic attack on clotrimazole structure which consequently can disrupt its pharmacological properties (30). This study is relatable to the previous study reported by Ono *et al.* (2021) showed that ß-lactam antibiotics such as penicillin were inactivated when exposed to RSS. They discovered that the ring of ß-lactam antibiotics was vulnerable to attack by thiol-containing nucleophiles of RSS, which further led to the formation of ß-lactam ring-opened products (17). Clotrimazole (Figure 3) is found to possess negative, neutral, and electron-deficient regions so that they may be subject to electrophilic, lyophilic, and nucleophilic attacks. Prior studies indicated that xenobiotics or their metabolites are kinetically unstable and abundant in electron-deficient regions on the molecular surface. Both of these tend to make cells toxic due to glutathione depletion and damage DNA by oxidising its nucleobases (31).

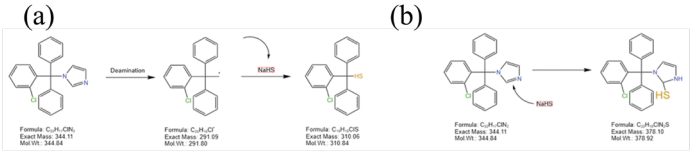

Figure 3 : Proposed pathway of NaHS reduces the clotrimazole efficacy. (a) neutralising electrophilic clotrimazole metabolite (b) induce nucleophilic attack on the azole ring.

It is further hypothesized that there are two possible different ways in which NaHS could interfere with clotrimazole. First, it may be due to the activity of NaHS, that neutralizes the reactive by-product of clotrimazole after it is metabolised by cytochrome p450 specifically the CYP3A enzyme. The deamination of clotrimazole will lead to the formation of a reactive metabolite to which it will be later sulphydrated and neutralised as predicted in Figure 3(a). Secondly, it is also hypothesised that NaHS possibly impinged nucleophilic attack on the pentene ring, and structurally change the efficacy and pharmacological properties of clotrimazole as depicted in figure 3(b). This hypothesis is based on the findings reported by Ono et al. (2021), that RSS (i.e., CysSSH) initially reacted with penicillin G to form a penicillin G-CysSSH intermediate, followed by cleavage of the disulfide bond to form the ß-lactam ring-opened penicillin G carbothioic S-acid (PG-COSH). In parallel, it is also believed that if the experiment uses compounds that exert more nucleophilicity compared to NaHS, such as persulphides and polysulphides, we may expect that such RSS can greatly affect the metabolism and potentially efficiently disrupt the action of clotrimazole.

Even so, additional research is required to corroborate these findings and ideas. Making use of nuclear magnetic resonance (NMR) spectroscopy, reverse phase high-performance liquid chromatography (RP-HPLC), and metabolomic analysis, for instance, can verify the extent to which the structure of antifungal changed when interacting with RSS.

CONCLUSION

In conclusion, the data obtained suggest that RSS had the possibility of having a potential interaction with resistance to antifungal activity. Last but not least, due to the increasing number of infections that are caused by antifungal-resistant, this study may serve as a stepping stone to a new perspective that can help to improve the treatment efficacy and strategize the development of novel antifungal treatments. Overall, it is hoped that this literature could potentially give a solid foundation for more investigation and discussion in the future.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge research grants from UniversitiTeknologi MARA (UiTM) in the form of Geran Penyelidikan Khas 600-RMC/GPK 5/3 (170/2020) and (245/2020).

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