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Miconazole and terbinafine induced reactive oxygen species accumulation and topical toxicity in human keratinocytes

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There are an estimated 1 billion cases of superficial fungal infection globally. Fungal pathogens form biofilms within wounds and delay the wound healing process. Miconazole and terbinafine are commonly used to treat fungal infections. They induce the accumulation of reactive oxygen species (ROS) in fungi, resulting in the death of fungal cells. ROS are highly reactive molecules, such as oxygen (O_2), superoxide anion (O_2•−), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH). Although ROS generation is useful for killing pathogenic fungi, it is cytotoxic to human keratinocytes. To the best of our knowledge, the effect of miconazole and terbinafine on HaCaT cells has not been studied with respect to intracellular ROS stimulation. We hypothesized that miconazole and terbinafine have anti-wound healing effects on skin cells when used in antifungal treatment because they generate ROS in fungal cells. We used sulforhodamine B protein staining to investigate cytotoxicity and 2´,7´-dichlorofluorescein diacetate to determine ROS accumulation at the 50% inhibitory concentrations of miconazole and terbinafine in HaCaT cells. Our preliminary results showed that topical treatment with miconazole and terbinafine induced cytotoxic responses, with miconazole showing higher cytotoxicity than terbinafine. Both the treatments stimulated ROS in keratinocytes, which may induce oxidative stress and cell death. This suggests a negative correlation between intracellular ROS accumulation in keratinocytes treated with miconazole or terbinafine and the healing of fungi-infected skin wounds.

Keywords: antifungal; HaCaT cells; miconazole; reactive oxygen species; terbinafine.

Introduction

According to recent global estimates, there are approximately 1 billion cases of superficial (skin, hair, and nail) fungal infections (Bongomin et al. 2017). Superficial fungal infections affect approximately 1.7 billion people and 10–20% of people in the United States (Lam et al. 2018). Superficial fungal infections are commonly caused by

dermatophytes (Hay 2017), a group of fungi that infect keratinized tissues such as skin, hair, and nails (Khurana et al. 2019). These fungal pathogens from biofilms within wounds and contribute to delayed healing (Kalan et al. 2016). The antimycotic drugs miconazole and terbinafine are used to treat fungal infections, including otomycosis (Zarei Mahmoudabadi et al. 2015) and vaginal candidiasis (Daniel et al. 2018). Miconazole belongs to the imidazole family of antifungal drugs that have a broad spectrum of activity. Its effect is based on decreasing the ability of fungi to produce ergosterol, an important part of their cell membranes, via the inhibition of lanosterol-14α-demethylase, a cytochrome P450 dependent enzyme (Ramírez-Villalva et al. 2015, Campoy and Adrio 2017). This dependent enzyme has an iron protoporphyrin unit at its active site. Miconazole binds to the iron of the porphyrin and disrupts the biosynthesis of fungal ergosterol, resulting in the accumulation of 14α-methylated sterols (Campoy and Adrio 2017). The depletion of ergosterol and accumulation of 14α-methylated sterols lead to the leakage of intracellular contents, thereby inhibiting fungal growth and replication (Ramírez-Villalva et al. 2015). Terbinafine is an allylamine antifungal agent (Tolomeotti et al. 2015, Campoy and Adrio 2017). It disrupts the synthesis of ergosterol via the inhibition of fungal squalene epoxidase, an enzyme that catalyzes the conversion of squalene into squalene-2,3-epoxide, which is then converted into lanosterol and ergosterol (Campoy and Adrio 2017). Recent studies have shown that both miconazole and terbinafine inhibit fungal growth by inducing the production of reactive oxygen species (ROS). Miconazole triggers the generation of ROS via the activation of cyanide-insensitive NADH oxidase and avoids the enzyme-mediated breakdown of H2O2 (mediated by cytochrome peroxidase and catalase), which result in toxic levels of H2O2 in yeasts (Francois 2007). It also acts on the organization of the actin cytoskeleton in yeasts and leads to actin clumping prior to the induction of an increase in endogenous

ROS levels. The production of ROS subsequently causes fungal cell death (Thevissen et al. 2007). Terbinafine also induces the intracellular production of ROS in yeasts. The accumulation of ROS subsequently causes mitochondrial lipid peroxidation, thus inhibiting fungal growth (Shekhova et al. 2017).

Microbial infection is one of the most common causes of chronic skin wounds (Mangoni et al. 2016). Antifungal drugs, such as miconazole and terbinafine, are commonly used for the topical treatment of fungal skin infections. Previous studies have shown that miconazole and terbinafine exert their antifungal actions via the stimulation of intracellular ROS production in fungal cells (Francois 2007, Thevissen et al. 2007, Shekhova et al. 2017). ROS may be deleterious and cause oxidative stress, possibly leading to cell damage and cell death (Ghosh et al. 2018). However, ROS accumulation is not only detrimental to fungal cells but also to skin cells. Skin, rich in unsaturated fatty acids, is vulnerable to damage caused by ROS. ROS, especially the hydroxyl radical (•OH), induce oxidative damage to biological macromolecules, such as DNA, carbohydrates, lipids, and proteins. Among the most susceptible targets are polyunsaturated fatty acids, as ROS induces the initiation of lipid peroxidation. Oxidative damage to the lipids that constitute the cell membrane affects cellular components, which ultimately leads to cell death (Pillai et al. 2005). Furthermore, cytotoxicity to human skin HFF-1 cells (Ghaderi-Shekhi Abadi et al. 2018) and HaCaT cells (Ali et al. 2018) is proportional to intracellular ROS production.

This raises a question on the impact of ROS-inducing antimycotic drugs (i.e., miconazole and terbinafine) on skin wound healing, considering that the generation of ROS can cause topical toxicity. We hypothesized that miconazole and terbinafine have anti-wound healing effects on skin cells when used in antifungal treatment because they have been recently demonstrated to have a strong ability to generate ROS. Therefore, they

are believed to inhibit fungal growth via the inhibition of ergosterol synthesis, and owing to the ability of miconazole and terbinafine to produce ROS in fungi, we suspect that when used topically, they may also stimulate ROS generation in skin cells. Of note, keratinocytes are the predominant cell type in the epidermis (Jang et al. 2017, Bhushan et al. 2019) and are fundamental to the structural and barrier functions of the epidermis (Kim et al. 2018, Choi et al. 2019). To the best of our knowledge, the effect of miconazole and terbinafine on HaCaT cells (an immortalized human keratinocyte line) has not been studied previously with respect to intracellular ROS stimulation. Thus, in this study, we aimed to investigate whether miconazole and terbinafine exert anti-wound healing effects on HaCaT cells.

Materials and Methods

In vitro cytotoxicity

HaCaT cells (Cell Lines Service, Eppelheim, Germany) were removed from cell culture flasks and washed with phosphate-buffered saline. After centrifugation, they were re-suspended in complete cell culture medium (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum), and the number of cells was adjusted to approximately 1×10^5 cells/mL. Cells were seeded in 96-well microtiter plates for 24 h prior to the evaluation of each antifungal drug (miconazole and terbinafine, both from Sigma-Aldrich, St. Louis, MO). These drugs were incubated with keratinocytes (1×10^5 cells/mL) for 24 h at the concentration gradients 50, 25, 12.5, 6.25, 3.125, and 1.56 μg/mL. Dimethyl sulfoxide (DMSO) (1%) and doxorubicin added in decreasing order (10, 5, 2, 1, and 0.5 μg/mL) were used as vehicle and positive controls, respectively. To evaluate the cytotoxicity to HaCaT cells, the sulforhodamine B protein (SRB) staining assay was conducted. Briefly, cells were fixed with trichloroacetic acid, washed with distilled water, stained with SRB

at room temperature for 30 min, and washed with acetic acid. Optical images of treated cells were captured. Finally, cells were dissolved in Tris buffer, and optical absorbance was measured using a microplate reader. Each experiment (miconazole, terbinafine, and doxorubicin) was conducted in triplicate. The 50% inhibitory concentrations (IC_{50}) of miconazole, terbinafine, and doxorubicin were calculated from these experimental results (Lam et al. 2016a and 2016b).

Determination of ROS

HaCaT cells were seeded at a concentration of approximately 1×10^5 cells/mL and counted manually using a haematocytometer under an inverted microscope. After 24 hours, the culture medium was changed and incubated with the antifungal drugs (miconazole and terbinafine) and doxorubicin at their IC_{50} values on HaCaT cells. DMSO at 1% was used as a vehicle control. After a further incubation of 24 hours, H_2O_2 at 100 μM was added as the positive reference. All the culture media were changed after 2 h and cells were incubated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probe) for a further of 30 min. Skin keratinocytes were washed and collected. Cells were subsequently lysed and total cellular protein was collected while debris was discarded. Protein content was determined by using the Bradford reagent. The relative level of ROS from each of 20 μg of total protein sample was determined by measuring its fluorescence units at 515 nm after an excitation at 485 nm using a microplate reader (Chui et al., 2009). Experiments were performed in triplicate.

Statistical analysis

A Student’s t-test was conducted, and the p-value was used to determine the statistical significance of the results.

Results

In vitro cytotoxicity to human keratinocytes

The SRB assay was used to determine the IC_{50} of miconazole and terbinafine on HaCaT cells. Figure 1 shows the possible cytotoxic responses of miconazole and terbinafine at various concentrations. High degrees of cell rounding, cellular shrinkage, and condensation were observed in cells treated with miconazole at 50 and 25 µg/mL (Figures 1c and 1d), terbinafine at 50 µg/mL (Figure 1g), and doxorubicin at 2 µg/mL (Figure 1j). The IC_{50} values of miconazole and terbinafine on HaCaT cells were 6.25–12.5 µg/mL and 12.5–25 µg/mL, respectively. Moderate degrees of cell rounding and cell shrinkage were shown with the IC_{50} values of miconazole (Figures 1e and 1f) and terbinafine (Figures 1h and 1i) compared to the untreated control (Figure 1a) and vehicle control (1% DMSO) (Figure 1b), which displayed a high integrity of cellular morphology. As the positive control, doxorubicin achieved an IC_{50} of 0.5–1 µg/mL (Figures 1k and 1l).

ROS generation in human keratinocytes

The effect of miconazole and terbinafine on intracellular ROS production in HaCaT cells, evaluated using the DCFH-DA assay, is shown in Figure 2. The fluorescence intensity in HaCaT cells treated with 12.5 µg/mL miconazole and 25 µg/mL terbinafine was significantly higher ($p < 0.05$) that that in the untreated control. The fluorescence intensity of DCFH-DA, a fluorimetric probe for the detection of oxidative stress, was proportional to the ROS generation in HaCaT cells. The intracellular ROS level induced by the IC_{50} of miconazole (12.5 µg/mL) in HaCaT cells was approximately three-fold higher than that of the untreated control and approximately two-fold higher than that induced by the IC_{50} of terbinafine (25 µg/mL) (Figure 1). The treatment with doxorubicin (at the IC_{50} of 1

μg/mL) caused a significant increase in fluorescence intensity, which was comparable with that of the positive control (100 μM of H_2O_2) with respect to the untreated control.

Discussion

Superficial fungal infections affect over 1 billion people worldwide (Bongomin et al. 2017, Lam et al. 2018). Traditionally, miconazole and terbinafine exert their antifungal activity via interference with ergosterol biosynthesis in the fungal membrane (Ramírez-Villalva et al. 2015, Campoy and Adrio 2017). In addition to the interference with ergosterol biosynthesis, these agents lead to the accumulation of ROS in fungi, which results in the death of fungal cells (Francois 2007, Thevissen et al. 2007, Shekhova et al. 2017). As many open wound injuries are prone to fungal infection and as miconazole and terbinafine are commonly used to treat open wounds, we hypothesized that these antifungal drugs may induce excessive ROS generation in keratinocytes. As hypothesized, both miconazole and terbinafine stimulated excessive ROS production at their IC_{50} values of cellular cytotoxicity, at which the level of ROS production of miconazole was higher than that of terbinafine. This suggested that considerable cell death occurred.

The overproduction of ROS generates many highly reactive molecules, such as O_2, $O_2{\bullet}^-$, H_2O_2, and •OH. These species attack cellular macromolecules, which include DNA, carbohydrates, lipids, and proteins. These chemical reactions result in the formation of oxidized products and lead to deleterious effects, such as blockage of cell cycle progression, induction of apoptosis, and necrosis in a cell type- or dose-dependent manner (Lambert and Yang 2003, Collin 2019). Owing to these properties, ROS-inducing antimycotic agents, such as miconazole and terbinafine, are commonly used to treat superficial fungal infections. This presents a question: as miconazole and terbinafine exert

their antifungal activities via ROS generation in fungal cells, can these antimycotic agents also prompt ROS generation in keratinocytes when applied topically? This is therapeutically relevant because fungal infections are usually associated with chronic wounds, such as ulcers (Morton and Phillips 2016). Therefore, on the one hand, keratinocytes play an important role in cutaneous wound healing; thus, any substance that is cytotoxic to keratinocytes may impair wound healing (Bhushan et al. 2019). On the other hand, from the therapeutic point of view, fungi-infected wounds may require topical application of antimycotic drugs. However, miconazole and terbinafine may affect the healing of infected skin wounds, as the induction of ROS not only inhibits and kills pathogenic fungi but also inhibits the growth of keratinocytes, which may impair wound healing. Skin wounds usually involve microbial invasion into the tissue, which elicits a host response, resulting in impaired wound healing (Hurlow et al. 2018). As open wounds with microbial infection (including fungal infection) are very common, special attention should be given to the application of antimycotic drugs during the treatment process. Our preliminary results suggest that there is a negative correlation between miconazole- and terbinafine-induced intracellular ROS accumulation in keratinocytes and the healing of fungi-infected skin wounds. In conclusion, our results support our hypothesis that the cytotoxic effects of these ROS-inducing drugs cause the death of keratinocytes, which may delay the repair of fungi-infected wounds (Figure 3). However, all experiments were conducted *in vitro*; thus, simulation of the microenvironment *in vivo* may fail. Therefore, future research using *in vivo* experiments is necessary to verify this hypothesis.

Conflict of interests

The authors have no conflicts of interest to declare.

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References

Ali D, Tripathi A, Al Ali H, Shahi Y, Mishra KK, Alarifi S, Alkahtane AA, Manohardas S. (2018). ROS-dependent Bax/Bcl2 and caspase 3 pathway-mediated apoptosis induced by zineb in human keratinocyte cells. Onco Targets Ther 11:489–497.

Bhushan P, Umasankar Y, Hutcheson JD, Bhansali S. (2019). Toxicity assessment of wearable wound sensor constituents on keratinocytes. Toxicol In Vitro 58:170–177.

Bongomin F, Gago S, Oladele RO, Denning DW. (2017). Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision. J Fungi (Basel) 3:57.

Campoy S, Adrio JL. (2017). Antifungals. Biochem Pharmacol 133:86–96.

Choi M, Park M, Lee S, Lee JW, Choi WJ, Lee C. (2019). Establishment of Nrf2-deficient HaCaT and immortalized primary human foreskin keratinocytes and characterization of their responses to ROS-induced cytotoxicity. Toxicol In Vitro 61:104602.

Chui CH, Wong RSM, Gambari R, Cheng GYM, Yuen MCW, Chan KW, Tong SW, Lau FY, Lai PB, Lam KH, Ho CL, Kan CW, Leung KS, Wong WY. (2009). Antitumor

activity of diethynylfluorene derivatives of gold(I). Bioorg Med Chem 17:7872–7877.

Collin F. (2019). Chemical Basis of Reactive Oxygen Species Reactivity and Involvement in Neurodegenerative Diseases. Int J Mol Sci 20:pii:E2407.

Daniel S, Rotem R, Koren G, Lunenfeld E, Levy A. (2018). Vaginal antimycotics and the risk for spontaneous abortions. Am J Obstet Gynecol 218:601.e1-601.e7.

Francois I. (2007). Investigating the role of reactive oxygen species in the mechanism of action of miconazole and effects on the treatment of diaper dermatitis. J Am Acad Dermatol 56:AB77.

Ghaderi-Shekhi Abadi P, Shirazi FH, Joshaghani M, Moghimi HR. (2018). Influence of formulation of ZnO nanoblokes containing metallic ions dopants on their cytotoxicity and protective factors: An in vitro study on human skin cells exposed to UVA radiation. Toxicol Rep 5:468–479.

Ghosh N, Das A, Chaffee S, Roy S, Sen CK. (2018). Chapter 4 – Reactive oxygen species, oxidative damage and cell death, in: Chatterjee S, Jungraithmayr W, Bagchi D (Eds.), Immunity and Inflammation in Health and Disease Emerging Roles of Nutraceuticals and Functional Foods in Immune Support. Academic Press, United Kingdom, pp. 45–55.

Hay R. (2017). Superficial fungal infections. Medicine 45:707–710.

Hurlow JJ, Humphreys GJ, Bowling FL, McBain AJ. (2018). Diabetic foot infection: A critical complication. Int Wound J 15:814–821.

Jang Y, Lee AY, Chang SH, Jeong SH, Park KH, Paik MK, Cho NJ, Kim JE, Cho MH. (2017). Trifloxystrobin induces tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in HaCaT, human keratinocyte cells. Drug Chem Toxicol 40:67–73.

Kalan L, Loesche M, Hodkinson BP, Heilmann K, Ruthel G, Gardner SE, Grice EA. (2016). Redefining the chronic-wound microbiome: fungal communities are prevalent, dynamic, and associated with delayed healing. mBio 7:e01058–16.

Khurana A, Sardana K, Chowdhary A. (2019). Antifungal resistance in dermatophytes: Recent trends and therapeutic implications. Fungal Genet Biol 132:103255.

Kim HJ, Lee E, Lee M, Ahn S, Kim J, Liu J, Jin SH, Ha J, Bae IH, Lee TR, Noh M. (2018). Phosphodiesterase 4B plays a role in benzophenone-3-induced phototoxicity in normal human keratinocytes. Toxicol Appl Pharmacol 338:174–181.

Lam PL, Lee KKH, Kok SHL, Gambari R, Lam KH, Ho CL, Ma X, Lo YH, Wong WY, Dong QC, Bian ZX, Chui CH. (2016a). Antifungal study of substituted 4-pyridylmethylene-40-aniline Schiff bases. RSC Adv 6:104575–104581.

Lam PL, Lu GL, Choi KH, Lin Z, Kok SHL, Lee KKH, Lam KH, Li H, Gambari R, Bian ZX, Wong WY, Chui CH. (2016b). Antimicrobial and toxicological evaluations of binuclear mercury(II)bis(alkynyl) complexes containing oligothiophenes and bithiazoles. RSC Adv 6:16736–16744.

Lam PL, Lee KKH, Wong RSM, Cheng GYM, Bian ZX, Chui CH, Gambari R. (2018). Recent advances on topical antimicrobials for skin and soft tissue infections and their safety concerns. Crit Rev Microbiol 44:40–78.

Lambert JD, Yang CS. (2003). Mechanisms of cancer prevention by tea constituents. J Nutr 133:3262S-3267S.

Mangoni ML, McDermott AM, Zasloff M. (2016). Antimicrobial peptides and wound healing: biological and therapeutic considerations. Exp Dermatol 25:167–173.

Morton LM, Phillips TJ. (2016). Wound healing and treating wounds: Differential diagnosis and evaluation of chronic wounds. J Am Acad Dermatol 74:589–605.

Pillai S, Oresajo C, (2005). Hayward J. Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation - a review. Int J Cosmet Sci 27:17–34.

Ramírez-Villalva A, González-Calderón D, González-Romero C, Morales-Rodríguez M, Jauregui-Rodríguez B, Cuevas-Yáñez E, Fuentes-Benítes A. (2015). A facile synthesis of novel miconazole analogues and the evaluation of their antifungal activity. Eur J Med Chem 97:275–279.

Shekhova E, Kniemeyer O, Brakhage AA. (2017). Induction of Mitochondrial Reactive Oxygen Species Production by Itraconazole, Terbinafine, and Amphotericin B as a Mode of Action against Aspergillus fumigatus. Antimicrob Agents Chemother 61:e00978–17.

Thevissen K, Ayscough KR, Aerts AM, Du W, De Brucker K, Meert EM, Ausma J, Borgers M, Cammue BP, François IE. (2007). Miconazole induces changes in actin cytoskeleton prior to reactive oxygen species induction in yeast. J Biol Chem 282:21592–21597.

Tolomeotti D, de Castro-Prado MA, de Sant'Anna JR, Martins AB, Della-Rosa VA. (2015). Genotoxic evaluation of terbinafine in human lymphocytes in vitro. Drug Chem Toxicol 38:306–311.

Zarei Mahmoudabadi A, Seifi Z, Gharaghani M. (2015). Lamisil, a potent alternative antifungal drug for otomycosis. Curr Med Mycol 1:18–21.

Figure captions

Figure 1. Representative results of cytotoxicity caused by miconazole and terbinafine in HaCaT cells: (a) untreated control, (b) vehicle control 1% DMSO, (c) 50 µg/mL miconazole, (d) 25 µg/mL miconazole, (e) 12.5 µg/mL miconazole, (f) 6.25 µg/mL miconazole, (g) 50 µg/mL terbinafine, (h) 25 µg/mL terbinafine, (i) 12.5 µg/mL terbinafine, (j) 2 µg/mL doxorubicin, (k) 1 µg/mL doxorubicin, and (l) 0.5 µg/mL doxorubicin. DMSO, dimethyl sulfoxide.

Figure 2. Effects of oxidative stress induced by IC_{50} of miconazole and terbinafine in HaCaT cells. $*p < 0.05$ when compared with untreated control. Experiments were performed in triplicate. DMSO, dimethyl sulfoxide; IC_{50}, 50% inhibitory concentration.

Figure 3. Delayed repair of fungi-infected wound due to miconazole- or terbinafine-induced ROS accumulation in keratinocytes. ROS, reactive oxygen species.