Journal of Pharmaceutical Research International

33(31A): 198-207, 2021; Article no.JPRI.69458
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

In vivo and *In vitro* Antifungal Activity of 2,3-Dimethylquinoxline

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AA, HAA and ASA designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors HMA and MWA observed the performance of the experiments and edited the manuscript. Authors HYA and HAA managed the literature searches and performed the experiments. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i31A31684
Editor(s):
(1) Dr. Mohamed Fawzy Ramadan Hassanien, Zagazig University, Egypt.
Reviewers:
(1) Mohamad Aris Mohd Moklas, Universiti Putra Malaysia, Malaysia.
(2) Mubashir Zafar, University of Hail, Saudi Arabia.
Complete Peer review History: http://www.sdiarticle4.com/review-history/69458

Original Research Article

Received 04 April 2021
Accepted 10 June 2021
Published 11 June 2021

ABSTRACT

Aims: To explore the antifungal activity of 2,3-dimethylquinoxaline.
Study Design: A preclinical study of a compound against 10 fungal species.
Backgrounds: Severe fungal infections cause significant clinical problem and need more effort to search for new antifungals.
Methodology: We evaluated the susceptibility of 2,3-dimethylquinoxaline in vitro against a wide range of pathogenic fungi, including six *Candida* species, two *Aspergillus* species, one *Cryptococcus* species, and one *Trichophyton* species. Also, we evaluated the susceptibility of 2,3-dimethylquinoxaline in vivo against oral candidiasis using a mice model.

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Results: The highest score of the minimum inhibitory concentration was 9 µg/ml against Cryptococcus neoformans. While, the lowest score was 1125 µg/ml against Candida tropicalis. The oral candidiasis in a mouse model was resolved using 2,3-dimethylquinoxaline 1% gel.
Conclusion: The 2,3-Dimethyquinoxaline has interesting antifungal activity. Quinoxalines in general need to be further developed as a promising antifungal candidate.

Keywords: Antifungal; aspergillus; candida; cryptococcus; 2,3-dimethylquinoxaline; trichophyton.

1. INTRODUCTION

Fungal infections significantly affect human health, with disease severity ranging from mild unpleasant superficial infections to severe life-threatening invasive infections [1,2]. Severe infections are rising in parallel with an expanding population at high-risk, including transplantation, cancer, immunodeficiency, and critically ill patients [1].

Fungal infection includes a wide range of diseases and requires prolong treatment [2]. Aspergillus, Candida, and Cryptococcus are the most common life-threatening infection in humans [2]. Invasive aspergillosis is a progressive disease, often fatal in transplant recipients and critically ill patients [3]. Candidemia is a common bloodstream infection associated with a high mortality rate [4]. Cryptococcosis caused thousands of deaths annually among the papulation of immunocompromised [5].

Treatment options for severe fungal infections are limited to only a few classes, including polyenes, azoles, and echinocandins (Table 1) [6]. Polyenes have a broad-spectrum antifungal activity but with significant toxicity [6]. Squibb isolated and introduced amphotericin B in the 50s [7]. It has become and still the standard treatment for severe fungal infection [7]. However, the dose-limiting adverse effects and nephrotoxicity have prompted a further search for alternatives that are equally effective but less toxic [7].

Azoles are known to cover a broad spectrum of antifungal activity with relatively low toxicity but with a high degree of drug interactions [6]. In the 40s, Woolley reported benzimidazole activity, the first parent compound to azole [8]. In the 60s, Bayer introduced clotrimazole, and Janssen introduced miconazole [9]. In the 80s, Janssen introduced ketoconazole as the only oral agent available to treat systemic fungal infections [10]. However, unacceptable side effects have limited the use of all imidazoles for topical use only. Pfizer introduced fluconazole in 1988 as a broad-spectrum triazole antifungal that can be given intravenously and orally [11]. It has excellent and predictable pharmacokinetics with a wide distribution in tissues and significantly less toxicity risk [12]. It shortly becomes one of the most widely prescribed antifungal agents [10]. However, the lack of activity and intrinsic resistance among some fungal species created a need for an alternative [10]. Four newer broad-spectrum triazoles were introduced between 1992 and 2015, leading to significant improvement in the management of invasive fungal infections Table 1 [13].

Table 1. Timeline of antifungal drugs development

Drug Class	Discovery date	Approval date
	1949	1957 (Amphotericin B)
		1989 (Amphotericin B lipid formulations)
Azoles	1944	1981-2013 (Ketoconazole)
		1990 (Fluconazole)
		1992 (Itraconazole)
		2002 (Voriconazole)
		2006 (Posaconazole)
		2015 (Isavuconazole)
Echinocandins	1970	2001 (Caspofungin)
		2005 (Micafungin)
		2006 (Anidulafungin)
5-Fluorocytosine	1957	1971 (Flucytosine)

**Approval date for invasive fungal infections*

Fig. 1. Chemical Structure of 2,3-dimethylquinoxaline on the left (Molecular weight =158.2 g/mol) versus quinoxaline on the right (Molecular weight =130.2 g/mol)

Echinocandins have relatively excellent safety profiles but with a limited spectrum of activity [6]. In the 70s, Nyfeler reported the first parent compound of an echinocandin [14]. Merck introduced caspofungin in 2001, Astellas introduced micafungin in 2005, and Pfizer introduced anidulafungin in 2006 to treat invasive fungal infections with a favorable safety profile [15]. However, echinocandins still lack activity against Cryptococcus species, Fusarium species, Absidia species, Mucor species, and Trichosporon species, which often develop breakthrough infections [15].

Less popular antifungal drugs include flucytosine, a pyrimidine analog introduced in the 70s, and griseofulvin, a mitotic inhibitor introduced in the 50s by Oxford [16]. Their use is limited by toxicity and the emergence of drug resistance [16].

The emergence of resistance to the antifungals is a clinical problem and causes failure in the therapy of severe life-threatening infections [17]. Candida auris, among other fungal species, showed resistance to most antifungal drug classes [18,19]. Candida auris has been highlighted as critical pathogens and urgent threats by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), with a priority for searching and developing new drugs [20,21].

The discovery and development of new antifungal drugs are needed to improve the current situation [22,23]. Small molecule screening was and is still considered a valuable resource of new drugs [24].

We aim to explore the antifungal activity of small molecules representing quinoxalines' simplest chemical structure (Fig. 1). In vitro, we screened 2,3-dimethylquinoxaline against several fungal species. In vivo, we tested 2,3-dimethylquinoxaline efficacy against oral candidiasis in a mouse model.

2. MATERIALS AND METHODS

2.1 Fungal Species

Seventy-three clinical isolates were used for in vitro studies with a wide diversity of pathogenic fungi, including yeasts: Candida albicans, Candida auris, Candida krusei, Candida glabrata, Candida tropicalis, Candida parapsilosis, Cryptococcus neoformans, and molds: Aspergillus niger, Aspergillus fimugatius, and Trichophyton mentagrophyte. A reference American Type Culture Collection strain Candida albicans ATCC 10231 was used for both in vitro and *In vivo* studies.

2.2 The 2,3-Dimethylquinoxaline Formulation

The 2,3-dimethylquinoxline was purchased from Sigma (Aldrich-D184977, Taufkirchen, Germany). The structure of 2,3-dimethylquinoxaline is shown in Fig 1. The stock solution of 2,3-dimethylquinoxaline was prepared in 100% dimethyl sulfoxide (DMSO; Sigma Aldrich-D8418, Taufkirchen, Germany). The working solution of 2,3-dimethylquinoxaline was diluted in RPMI-1640 (Gibco, Maryland, United States of America) medium to a final DMSO concentration of less than 5%.

To make 1% gel of 2,3-dimethylquinoxaline, two gram of 3% hydroxypropyl methylcellulose (HPMC; Sigma Aldrich-H7509, Taufkirchen, Germany) was added to 50 ml of hot distilled water and allowed to soak then, 10 ml of glycerol (Sigma Aldrich-G5516, Taufkirchen, Germany) was added and allowed further soaking. Then, one gram of 2,3-dimethylquinoxaline was dissolved in five milliliters of 99% alcohol and 35 ml of distilled water, then added with stirring to the HPMC-Glycerol, to form a homogenous gel.

The gel was protected from light and stored at a four-degree centigrade.

2.3 Validation of the Gel Formulation

To test the efficacy of gel formulation of 2,3-dimethylquinoxaline against Candida albicans ATCC 10231, well diffusion assay was used following the Clinical Laboratory Standard Institute (CLSI) guideline [25]. Two wells, four millimeters in diameter, cut out of the Sabouraud dextrose agar (Himedia-ME063, Mumbai, India). 20 µl of 5 mg/ml solution of 2,3-dimethylquinoxiline was placed into the first well. 20 µl of 1% gel of 2,3-dimethylquinoxiline was placed into the second well. The plates were incubated at 33˚C for 48 hours. Clear zones of inhibition were measured. This test was done in duplicate on the same day and done in triplicate over three days.

2.4 Susceptibility of 2,3-Dimethylquinoxaline toward the Fungal Species

The antifungal activity of 2,3-dimethylquinoxaline was tested in vitro against seventy-three clinical isolates and one reference strain. The minimum inhibitory concentration (MIC) was determined following the CLSI guideline [26]. The inoculum of 5×103 CFU/ml was prepared from a 36-hour culture on sabouraud dextrose agar incubated at 35°C. RPMI-1640 medium was distributed at 100 µl per well in a 96-well microtiter plate. 2,3-dimethylquinoxaline at 3 mg/mL was added at 200 µl per well to the second column of the plate, followed by a two-fold serial dilution along with all subsequent wells. The concentrations of 2,3-dimethyquinoxaline ranged from 2.9 to 1500 µg/mL. The inoculum was added at 100 µl/well to the second column and subsequent column until the last column. The last column represents the positive control that consisted of wells containing inoculum only. The first column represents the negative control that consisted of wells containing RPMI-1640 medium only. The plates were incubated at 33ºC for 48 hours. This test was done in duplicate on the same day and done in triplicate over two weeks.

2.6 Fungicidal Activity of 2,3-Dimethylquinoxaline

The inoculum of 5×103 CFU/ml was prepared using the molds from a 36-hour culture on sabouraud dextrose agar. Melted Bacto-Casitone (Gibco-225930, Maryland, United States of America) medium was inoculated with Aspergillus niger, Aspergillus fimugatius, and Trichophyton mentagrophyte at a ratio of 20 ml of the medium to 1 ml of the inoculum and 1 ml of the tested compound. The concentrations of 2,3-Dimethyquinoxaline ranged from 0.156 to 2500 µg/mL. Bottles were incubated at 35˚C for fourteen days. Growth was observed and documented daily. This test was done in triplicate for two weeks.

2.7 Activity of 2,3-Dimethylquinoxaline Against Oral Candidiasis

The mouse model of oral candidiasis was used to evaluate in vivo activity of 2,3-dimethylquinoxaline as described previously in the literature [27,28]. BALB/c mice, 10 Male, 6–8 weeks old, were used to develop oral candidiasis. Mice were immunosuppressed with prednisolone (Sigma Aldrich-P6004, Taufkirchen, Germany) at a dose of 100 mg/kg subcutaneous one day before and three days after the infection with Candida albucans ATCC 10231. Tetracycline hydrochloride (Sigma Aldrich-T7660, Taufkirchen, Germany) in drinking water at a concentration of 0.9 mg/ml was administered orally to mice beginning one day before the infection. Mice were anesthetized by 100 µg chlorpromazine hydrochloride (Sigma) intramuscular in each femur. The entire oral cavity was swabbed with sterile cotton pads soaked in a cell suspension (2x108 CFU/ml) of Candida albicans ATCC 10231 to produce oral infection. The infection severity was evaluated daily by the severity of whitish and curd-like patches on the tongue surface.

2.8 In Silico Drug-likeness, ADME and Toxicity Prediction

The 2,3-Dimethyquinoxaline compound was assessed for drug-likelihood properties and compliance with Lipinski's Rule of Five using the SwissADME tool (Swiss Institute of Bioinformatics, Lausanne, Switzerland) [29]. The pharmacokinetics and toxicity of 2,3-dimethylquinoxaline were assessed in silico using the Discover Studio 4.0 tool (BIOVIA, San Diego, United States of America) and the Toxtree 3.1.0 tool (Ideaconsult, Sofia, Bulgaria).

3. RESULTS AND DISCUSSION

The 2,3-dimethyquinoxaline exhibited a broad spectrum of antifungal activity against all the species tested with MIC in the range from 9 to 1125 µg/ml (Table 2). The compound showed fungicidal activity as no growth was observed in all bottles containing 2,3-dimethyquinoxaline after fourteen days of incubation compared to the control, Fig. 2. Almost equal clear zones of inhibition against Candida albicans were observed for both 5 mg/ml solution and 1% gel of 2,3-dimethylquinoxaline (Fig. 2).

It was observed that at day three post-infection, there was an apparent reduction of the infection severity in mice treated with 2,3-dimethylquinoxaline 1% gel compared to the control group. No Candida CFUs were detected in the oral cavities of the 2,3-dimethylquinoxaline treated mice. Dorsal tongue surfaces of the 2,3-dimethylquinoxaline treated mice were glossy and regular on day five (Fig. 3).

Table 2. Susceptibility of 2,3-dimethylquinoxaline on pathogenic fungal species

Fungal species	MIC (µg/ml)	
	24 hr	48 hr
Candida species		
Candida albicans ATCC 10231 (*n*=1)	190	370
Candida albicans (*n*=32)	854	935
Candida auris (*n*=3)	280	370
Candida glabrata (*n*=6)	470	560
Candida krusei (*n*=3)	370	560
Candida parapsilosis (*n*=7)	560	750
Candida tropicalis (*n*=13)	935	1125
Aspergillus species		
Aspergillus fimugatius (*n*=2)	370	370
Aspergillus niger (*n*=2)	750	750
Cryptococcus species		
Cryptococcus neoformans (*n*=3)	9	9
Trichophyton species		
Trichophyton mentagrophyte (*n*=2)	750	750

ATCC=American Type Culture Collection. MIC=minimum inhibitory concentration. n=number of isolate

Fig. 2. Effect of 2,3-Dimethylquinoxaline on *Aspergillus niger* (first panel), *Aspergillus fumigatus* (second panel), *Trichophyton mentagrophyte* (third panel) and *Candida albicans* (fourth panel)

The 2,3-Dimethylquinoxaline produced a complete and sustainable fungal growth inhibition at concentration been tested. Also, it produced a clear inhibition zone in a well diffusion assay against Candida albicans for both 5 mg/ml solution (well A) and 1% gel (well B)

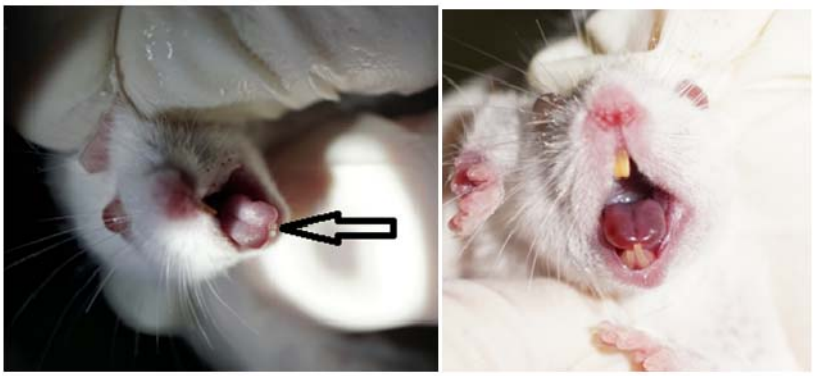

Fig. 3. Effect of 2,3-dimethylquinoxaline on a mouse model of oral candidiasis
A thick lesions of oral thrust was observed in untreated mice (left panel). 2,3-Dimethylquinoxaline-treated mice displayed near to healthy tongue surface (right panel)

Table 3. Properties of 2,3-dimethylquinoxaline using Swiss-ADME

Physiochemical		**Pharmacokinetics**	
Formula	$C_{10}H_{10}N_2$	Skin permeation	-6 cm/s
Molecular weight	158.2	Gastrointestinal absorption	High
Molar refractivity	49.47	BBB permeant	Yes
Synthetic accessibility	1.54	Bioavailability score	0.55
Hydrogen bond		CYP1A2 substrate	Yes
Acceptors	2		
Donors	0	CYP2C9, 2C19, 2D6, 3A4, P-glycoprotein substrate	No
Rotatable bonds	0		
Polar surface area	25.78 $Å^2$	Lipophilicity	2.09 ± 0.7
Lipinski rule of five	zero violation	Solubility	-2.53

The 2,3-Dimethylquinoxaline compound did not violate the drug-likeness rules. It showed good physicochemical and pharmacokinetic properties (Table 3). This compound has good gastrointestinal absorption and can cross the blood-brain barrier. This compound is not a substrate to P-glycoprotein that cannot be flushed out and less susceptible to microbial resistance by such a mechanism.

The 2,3-Dimethylquinoxaline structure has no alert for the potential mutagenicity or any safety concern. Its structure has no skin sensitization reactivity alerts. Its structure contains no enhanced toxicity functional groups.

In the past two decades, quinoxalines have emerged as a bright spot for drug discovery and development against pathogenic microorganisms [30]. There are currently only three drug classes used to treat the life-threatening fungal infection, azoles, echinocandins, and polyenes. There appears to be a time lag between discovery and license for using these drug classes (Table 1). Such gab happened to polyenes and also repeated to azoles and echinocandins. It seems that this may happen with quinoxalines.

Quinoxaline has been used for more than 55 years as an antimicrobial to enhance animal growth and improve animal husbandry [31]. Carta and his colleagues back in 2002 were the first to report quinoxaline activity against Candida species [32]. In the same year, Waring and his colleagues reported excellent antifungal activity against Fusarium oxysporum of synthetic quinoxalines bearing substitution at positions 2 and 3 of the ring [33].

The inhibition of topoisomerase (Topo) is one among other explanation for the mode of action of quinoxaline against eukaryotic organisms [30]. Fungal topoisomerase is a good target as an antifungal with a sufficiently distinct form of the human enzyme [34,35].

Minimal reports addressed 2,3-dimethylquinoxaline in the published literature. The current application of 2,3-dimethylquinoxaline is in the laboratory as a reagent to determine the level of specific chemicals in body samples, foods, or beverages [36].

Few reports indicate that 2,3-dimethylquinoxaline is a competitive enzyme inducer toward the hepatic P-450 [37,38]. These results are consistent with our in silico prediction pharmacokinetic results. In silico prediction, considered our compound as a substrate for the CYP1A2 enzyme. The CYP1A2 enzyme is known to be induced by smoking, rifampin, oral contraceptive steroids, and barbiturate [39]. The CYP1A2 is also known to be inhibited by cimetidine and ciprofloxacin [39]. Further studies are needed to define the effect of CYP1A2 genetic variations on the response to our tested compound.

Mutagenicity potential of 2,3-dimethylquinoxaline was examined by Hashimoto T et al., among other 33 quinoxaline and quinoline compounds using Salmonella/microsome assay. These results are consistent with our in silico prediction showing our tested compound has no risk for mutagenicity [40].

Besides the quinoxalines' safety profile, one of their most prominent characteristics is their ability to reach target tissues at an appropriate concentration [41]. In contrast, amphotericin B has minimal or no value for deep in vivo infection, although it has excellent in vitro activity, explained by the limited drug distribution into the infected tissues [42]. These results are supported and consistent with our in silico pharmacokinetic prediction results.

4. CONCLUSION

The results obtained from this study revealed a promising activity of 2,3-dimethylquinoxaline against common pathogenic fungal species and merited further optimization. Drugs available to treat fungal infections are minimal. The balance between harm and interest may drive the acceptance of quinoxalines as a candidate antifungal drug class. The antifungal effects probably rely on a new mechanism of action. Quinoxalines serve as platforms and show good affinity to bind to multiple targets. Our future study will explore the quinoxalines drug targets as an antifungal as well as their toxicity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable (The Biomedical Ethics Committee approved the experimental protocol at King Abdulaziz University, KAU and the National Committee of Bioethics, NCBE, Registration No. HA-02-J-008). All experiments have been examined and approved by the appropriate ethics committee. Animal handling was performed in strict compliance with the ethical guidelines for treating animals as defined by KAU and NCBE.

ACKNOWLEDGEMENTS

The study was supported by grant G-1256-140-1440 from the Deanship of Scientific Research (DSR) at King Abdul-Aziz University (KAU). We are grateful to the King Fahd Medical Research Center (KFMRC) for their valuable collaboration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/69458