INTERACTION BETWEEN KETOCONAZOLE, AMPHOTERICIN B AND TERBINAFIN AND THREE DIAZENUMDIOLATES IN CONCOMITANT USES AGAINST SOME FUGAL SPECIES

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ABSTRACT

A checkerboard broth microdilution method was performed to investigate the in vitro antifungal activities of three diazeniumdiolates derivatives (DETA/NO, DPTA/NO, DEA/NO) alone and in combination with ketoconazole, amphotricin B or terbinafine against five *Candida* species, *Cryptococcus neoformance* and four *dermatophyte* strains. MICs and MLCs were recorded, and synergy was calculated by using fractional inhibitory and fractional lethal concentration index. DETA/NO with a half-life of 57h at 25°C showed antifungal activity against all tested *dermatophyte* species (MIC 0.150 to 2.5mg/ml), DPTA/NO with a half life of 3h at 37°C showed antifungal activity against five species of *Candida* and *Cryptococcus neoformans*, and DEA/NO with a half life of 2 min at 37°C and 16 min at 25°C did not show antifungal activity against tested strains. Combinations of DPTA-NO with either ketoconazole or amphotericin B were either synergistic or indifferent for all tested strain of *Candida* and *Cryptococcus neoformance*. DETA/NO was unable to enhance the antifungal activity of terbinafine against *dermatophyte* strains. Even where no synergistic activity was achieved, there was still a decrease in the MIC of one or both drugs which were used in combination. Antagonism was observed between terbinafine and DETA-NO against *Trichophyton rubrum*. Our result suggests that DETA/NO and DPTA/NO may be useful for development of new therapeutic strategies for treatment of *dermatophyte* and *Candida* infections. Clinical studies are warranted to elucidate the potential utility of these combination therapies.

Keywords: NO, Diazeniumdiolates, Antifungal activities, Terbinafine, Amphotericin B, Ketoconazole

INTRODUCTION

The patient populations at risk for serious fungal infections have increased dramatically in recent years. These populations include patients with AIDS (1, 2), those receiving cancer chemotherapy (3) or organ transplantation (4) and others receiving immunosuppressive medications (5). In addition, the need for prolonged and repeated therapy has led to the emergence of fluconazole-resistant isolates of *Candida albicans*, as well as the appearance of other, more resistant species, such as *Candida glabrata*, *C. tropicalis*, and *C. krusei* (6, 7, 8).Thus, any combination therapy that enhance antifungal activity should be actively pursued. Combination therapy might be a promising approach in such circumstances (9). The use of antifungal combinations may increase the rates of microbial killing, shorten the durations of therapy, reduces the emergence of drug resistance, and expand the spectrum of activity (10, 11). Since combination therapy carries a much higher cost and can increase the potential for drug interactions and toxicities, it is important to evaluate their effects carefully.

Diazeniumdiolates are capable of releasing NO in a biologically usable form. (12) The candidacidal activity of diazeniumdiolates, alone and in combination with ketoconazole, fluconazole, and miconazole has been reported (8). The reported data suggest that DETA-NO or compounds with similar properties may be useful in development of new therapeutic strategies for treatment of *Candida* infections.

In this study, in vitro activities of three diazeniumdiolates, DPTA-NO, DETA-NO and DEA-NO alone and in combination with ketoconazole, amphotricin B and terbinafine, against *C.albicans*, *C.glabrata*, *C.tropicalis*, *C.parapsilosis* and four *dermatophyte* species including *Trichophyton mentagrophytes*, *T.rubrum*, *Microsporum canis*, *M.gypseum* and *Cryptococcus neoformans* were investigated.

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MATERIALS AND METHODS

Fungal strains

Candida strains included PTCC (Persian Type Culture Collection)5027 *Candida albicans* and *dermatophyte* strains included *Microsporum gypseum*, PTCC 5070 *Microsporum canis* PTCC 5060 and *Trichophyton rubrum* PTCC 5143 which were obtained from Iranian Scientific and Industrial Institute. *Candida parapsilosis*, *C. tropicalis*, two type of *C. glabrata* (resistance and susceptible to ketoconazole), *Cryptococcus neoformance* and *Trichophyton mentagrophyte* were isolated from patients at Mycology department of Iran Medical Science University.

All *Candid*a strains were evaluated on SC agar (Sabouraud's dextrose agar + chloramphenicol) for presumptive identification, and identity was verified by API kit (api 20 C AUX, boimerieux sa). Strains were stored at –70°C in 20% glycerol. Prior to initiation of the study, the strains were subcultured on antimicrobial agent-free medium to ensure viability and purity. (13)

After presumptive identification, *Cryptococcus neoformance* was subcultured on BHI agar (Brain Heart Infusion Agar). *Dermatophyte* strains' identity were confirmed by slide culture and urease test (14). All strains were maintained in 20% glycerul-10% lactose in liquid nitrogen.

Antifungal agents

Ketoconazole and amphotricin B were obtained from Sigma chemical Co. Terbinafine was a gift from Tehran Chemistry Co. NO releasing compounds including DEA/NO; sodium (Z)-1-(N, N-diethlamineo), DETA/NO; (Z)-1-[N-(2-amino-ethyle)– N- (2-aminoethyle) amino] diazen-1-ium-1,2-Diolate and DPTA/NO (3,3'-(Hydroxynitroso-hydrazino)bis-1-propanamine) were obtained from Alexis Chemical Co.

Stock solutions of ketoconazole, amphotricin B and terbinafine were prepared in dimethyl sulfoxide (Sigma). Stock solutions of diazenium-diolates were prepared in sterile RPMI-1640. Further dilutions of all drugs were prepared in the test medium.

Broth dilution assay

Drug activity was assessed by a checkerboard method derived from the standardized procedure established by the National Committee for Clinical Laboratory Standards (NCCLS) for broth microdilution antifungal susceptibility testing. Briefly, testing was performed in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Gibco Laboratories) buffer. One hundreds microliters of a two fold concentration of each drug (or 50µl of a four fold concentration for the studying of two drugs in combination) were dispensed in the wells of 96-well microtiter plates (Falcon 3072; Becton Dickinson). The range of concentrations of the antifungal agents after addition of inocula were 0.125-64 µg/ml for ketoconazole, 0.007-1 µg/ml for terbinafine, 0.001-64 µg/ml for amphotricin B and 0.01-5 mg/ml for NO-generation compounds. Fungi inocula (100µl), were prepared spectrophotometrically and diluted further in order to obtain concentrations ranging from 10^4 CFU/ml of 18h old *Candida* cells or 48h old *Cryptococcus neoformans* cells and 10^5 CFU/ml containing 4-7 days old *dermatophytes* (2x inoculum), when added to each well of the microtitre plates to a final volume of 200µl. Plates were incubated for 24 -48 h at 37ºC for yeast species and 4-7 days at 28ºC for *dermatophytes* (13, 15). The minimum inhibitory concentration (MIC) of agents was determined visually, as the lowest concentration at which no growth was observed. The minimum lethal concentrations (MLC) of each drug, alone or in combination, were determined by plating 100µl sample from wells showing no observable growth onto sabouraud dextrose agar. MLCs were defined as the lowest concentration of the agent that at which no colonies were observed after 24h for *Candida* spp. and 4 days for *dermatophytes*. The reported final data are the average of three independent experiments.

In synergy studies, the fractional inhibitory concentrations (FIC) of each drug used in combination were calculated and added to obtain the FIC indices.

FIC index is equal to: (MIC of drug A in combination/MIC of drug A alone)+(MIC of drug B in combination/MIC of drug B alone).

Drugs interactions were judged on the basis of the following criteria: synergistic effect (≥0.5) indifferent effect (0.5 - ≤4) and antagonistic effect (>4) (16). The fractional lethal concentration (FLC) index was calculated from a formula similar to that for the FIC index but by substitution of MLC for MIC.

RESULTS

Antifungal effects of DPTA/NO

The results of susceptibility to DPTA-NO alone and in combination with ketoconazole and amphotricin B by the broth dilution assay are presented in Table 1. When DPTA/NO was used alone, the MICs ranged from 156 to 2500 µg/ml and when DPTA-NO was combined with ketoconazole, there were significant reductions in its MIC values ranging from 39 to 625 µg/ml. The most and the least sensitive *Candida* strains against DPTA-NO alone were *C. parapsilosis* and *C. glabrata*(R). MICs of ketoconazole alone

Table 1. Inhibitory effects of DPTA/NO on proliferation of selected fungal species in the presence or absence of antifungal drugs.

Strain	MIC (µg/mL)			MIC (µg/mL)			
	Ketoconazole	Ketoconazole with DPTA-NO	FIC	DPTA-NO	DPTA-NO with azole	FIC	FIC Index
C. albicans	1	0.5	0.5	625	312	0.5	0.99
C. parapsilosis	0.5	0.125	0.25	156	78	0.5	0.75
C. glaborata NR	4	2	0.5	1250	625	0.5	1
C. glaborata R	32	4	0.125	2500	312	0.124	0.24
C. tropicalis	16	0.5	0.031	1250	39	0.03	0.06

MICs were determined visually as the concentration that gave 100% inhibition. The MIC values represent the average of 3 independent experiments.
NR: Susceptible to ketoconazole, R:Resistance to ketoconazole

Table 2. Inhibitory effects of DPTA/NO on proliferation of selected fungal species in the presence or absence of amphotricin B.

	Amphotricin B	Amphotricin B with DPTA-NO		DPTA-NO	DPTA-NO with Amphotricin B		
Cryptococcus neoformans	1	0.003	0.003	19	0.1	0.005	0.008

MICs were determined visually as the concentration that gave 100% inhibition. The MIC values represent the average of 3 independent experiments.

Table 3. Fungicidal effects of DPTA/NO on selected fungal species in the presence or absence of antifungal drugs.

Strain	MLC (µg/mL)			MLC (µg/mL)			
	Ketoconazole	Ketoconazole with DPTA-NO	FLC	DPTA-NO	DPTA-NO with azole	FIC	FLC Index
C. .albicans	4	2	0.5	2500	1250	0.5	1
C. parapsilosis	4	2	0.5	5000	1250	0.25	0.75
C. glaborata NR	32	16	0.5	5000	1250	0.25	0.75
C. glaborata R	64	32	0.5	5000	2500	0.5	1
C. tropicalis	32	16	0.5	2500	1250	0.5	1

MLC was determined as the lowest concentration of the agent at which no colonies were observed after 24 h for yeasts and 4 days for *dermatophytes*. The MLC values represent the average of 3 independent experiments.
NR: Susceptible to ketoconazole, R:Resistance to ketoconazole

Table 4. Fungicidal effects of DPTA/NO on selected fungal species in the presence or absence of amphotricin B.

	Amphotricin B	Amphotricin B with DPTA-NO		DPTA-NO	DPTA-NO with Amphotricin B		
Cryptococcus neoformans	2	0.001	0.0005	39	0.5	0.01	0.0125

MLC was determined as the lowest concentration of the agent at which no colonies were seen after 24 h for yeasts and 4 days for *dermatophytes*. The MLC values represent the average of 3 independent experiments

Table 5. Inhibitory effects of DETA/NO on proliferation of selected fungal species in the presence or absence of antifungal drugs.

Strain	MIC (µg/mL)			MIC (µg/mL)			
	Terbinafine	Terbinafine with DETA-NO	FIC	DETA-NO	DETA-NO with Terbinafine	FIC	FIC Index
Microsporum gypseum	0.03	0.03	1	1250	312	0.24	1.24
Microsporum canis	0.003	0.007	2.33	156	19	0.12	2.45
Trichophyton rubrum	0.01	0.06	6	2500	625	0.25	6.25
Trichophyton mentagrophytes	0.03	0.03	1	625	312	0.49	1.49

MICs were determined visually as the concentration that gave 100% inhibition. The MIC values represent the average of 3 independent experiments.

ranged from 0.5 to 30 µg/ml, while in combination with DPTA-NO ranged from 0.125 to 4 µg/ml. *C. parapsilosis* was the most susceptible strain against ketoconazole alone and in combination. The FIC index showed synergy between DPTA-NO and ketoconazole against *C. glabrata*(R) and *C. tropicalis* and indifferent interaction against other tested *Candida* species The MLCs values of DPTA-N0 alone, ranged from 2.5 to 5 mg/ml and in combination with ketoconazole, MLC values of both agents were reduced significantly. The FLC indices confirmed the synergistic effect inferred from the FIC values (Table2).

DPTA/NO showed the greatest antifungal activity against *Cryptococcus neoformans*. When DPTA/NO was combined with amphotricin B, there was an almost 200 fads decrease in the MIC value for DPTA/NO against *Cryptococcus neoformans* . In the presence of DPTA/NO a similar decrease in the MIC of amphotricin B against *Cryptococcus neoformans* (almost 300 fold) was observed. The MLC value of DPTA-NO in combination with amphotricin B was at least 80-fold lower than that for DPTA-NO alone. Higher decrease (2000-fold) was shown for MLC of ketoconazole in combination with DPTA-NO against *Cryptococcus neoformans*. Data are presented in table 2.

Antifungal effects of DETA/NO

The MIC values for the anti fungal effects of DETA/NO, alone and in combination against selected strains of *Tricophytone* and *Microsporum* are presented in Table (3). Presence of DETA/NO didn't produce a great change in the MIC value of terbinafine against these organisms and the interaction was indifferent. However, the presence of terbinafine reduced the MIC value of DETA/NO against *M. canis* and *M. gypseum* significantly. The results also showed that there was an antagonism between DETA-NO and terbinafin against *T.rubrum*

Antifungal activity of DEA/NO

DEA/NO did not show antifungal activity against any of the tested fungi including: *Candida* spp., *Cryptococcus neoformans* and *dermatophyte* strains.

DISCUSION

The emergence of antifungal resistant strain of various fungi such as *Candida*, *dermatophyte* and *Cryptococcus neoformans* has prompted research for development of new strategies for treatment of fungal infections (7, 17, 18). The identification of NO as an antifungal agent (19), which is active against a number of pathogenic fungi, has offered new opportunities for development of such strategies (20, 21, 22, 23, 24), while at the same time has presented new challenges due to the wide physiological effects of NO which increases likelihood of unacceptable adverse effects (6). In addition, instability of NO under physiological conditions makes delivery of the agent to the site of infections difficult (25) and the main problem with the use of No has been reported to be its short half life and its limited solubility (26).

Diazeniumdiolates with the general structure of XN (O) N=O (where X is a nucleophile residue) are capable of releasing NO in an aqueous environment (27, 28, 29). These compounds do not require activation through a redox reaction or electron transfer as do glyceryl trinitrate and sodium nitroprusside (30). Diazeniumdiolate are stable and capable of delivering NO in a biologically useable form at a predictable rate. Three of such compounds are DPTA/NO, DETA/NO and DEA/NO. Work by other researchers showed in vitro synergy or additive effect between DETA-NO and azole against some candida species (12) has been reported. In the present study a checkerboard broth microdilution method was performed to investigate the in vitro antifungal activities of DPTA-NO, DETA-NO and DEA-NO alone and in combination with three currently used drugs: ketoconazole, amphotericin B and terbinafine against five *Candida* species, *Cryptococcus neoformance* and four *dermato-phytes* strains. The results obtained in this work, suggest that the rate of NO release is an important factor in the antifungal effects of these compounds, as DEA/NO with a half life about 2 minutes didn't show any antifungal activity.

The data obtained in this work demonstrated an effective interaction between DPTA-NO and ketoconazole against five *Candida* species especially clinical isolates of *C. glabrata*. The FIC index calculated for the combined effects of ketoconazole and DPTA-NO, on various *Candida* species suggests that the detoconazole and DPTA-No have synergistic effect against C.*tropicalis* and C.*glabarata* R. *Candida glabrata* R has recently emerged as a significant pathogen involved in voth superficial and deep-seated infections (16,19). The prominence of C.*glabrata* R as a pathogen is of particular clinical concern because it is innately less susceptible to fluconazole and amphotericin B than most other spesies of *Candida* (19), and infections due to C.*glabrata* are characterized by a high mortality rate.

Combination of, DPTA-NO and ketokonazole had indifferent effect, against C.*albicans*, C.*para-psilosis* and C.*galaborata* NR. Also, the different effect values of FIC index obtained for different species of the *Candida* genus, suggest that the effects of combination of two drugs, depends not

only on the drugs themselves, but also on the microorganism that the combination acts on.

Results of this study also demonstrated that there is a notable synergistic interactions between DPTA-NO and amphotericin B against *Cryptococcus neoformans*. The encapsulated fungus *Cryptococcus neoformans* is a significant cause of morbidity and mortality in patients with impaired cell-mediated immunity, especially those with AIDS (It is an opportunistic fungal pathogen that causes life-threatening meningoencephalitis in 5 to 10% of AIDS patients) (32, 33).

All three fungicidal compounds tested, change fungal cell membrane permeability via different mechanisms. Ketoconazole, like other azoles, affects ergosterol biosynthesis (34,35), amphotericin B binds to ergosterol (36), and terbinafine binds squalene epoxidases leading to ergosterol deficiency (37). However, the effects of NO donors, when combined with these fungicidal agents were not similar. DPTA-NO exhibited synergistic effect with ketoconazole and amphotericin B, while DETA-NO showed little effect when combined with terbinafine. The effects of DPTA-NO when used in combination with ketoconazole and amphotericin B may be due to enhanced entry of the NO donor into the cell due to membrane damage which is caused by the fungicidal agents. However, enhanced entry of the NO donor into the cell is not the only factor involved. Since when terbinafine and DETA-NO were used in combination, there were no enhancement of the fungicidal effects of the former. This could be due to the long half-life of DETA-NO (57h) which would mean a much slower release of NO compared to DPTA-NO (half life = 2.5h). Therefore, although in the presence of terbinafine, DETA-NO enters the fungal cell more easily (as suggested by the decrease in the MIC of DETA-NO in the presence of terbinafine), the rate of generation of NO by DETA-NO is not fast enough to generate effective fungicidal levels of NO.

CONCLUSION

DPTA-NO appears to be the most suitable NO donor which was tested in this work. Our data suggest that DPTA-NO may be effective when it is used in combination with ketoconazole and amphotericin B for the treatment of *Candida* especially *C. glabrata* resistant to ketoconazole and *Cryptococcus neoformans* infections.

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