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Demonstration of Synergy with Fluconazole and Either Ibuprofen, Sodium Salicylate, or Propylparaben against *Candida albicans* In Vitro

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The combination of fluconazole with either ibuprofen, sodium salicylate, or propylparaben resulted in synergistic activity (fractional inhibitory index, <0.5) against *Candida albicans* NCYC 610 in a microdilution checkerboard assay. Synergism between miconazole and ibuprofen was also demonstrated. In three of four clinical isolates of *C. albicans* from AIDS patients, the combination of fluconazole and ibuprofen was synergistic. Preparation of the inoculum and the growth conditions used were those recommended by the National Committee for Clinical Laboratory Standards for susceptibility testing. A visual estimation of total inhibition of growth and determination of an 80% reduction in the optical density at 492 nm compared with those for the control were taken as endpoints for the calculation of synergy, and a good correlation between both estimates was demonstrated.

A popular reason for using a combination of antimicrobial agents is to obtain a synergistic effect whereby the antimicrobial outcome exceeds that predicted on the basis of the addition of individual effects. For a number of reasons, synergistic combinations for the treatment of fungal infections such as candidiasis would be beneficial. First, there is an increase in the incidence of fungal infections. For example, *Candida* species are now the fourth most common organism recovered from the blood of hospitalized patients (13). Notwithstanding the increasing need for effective therapy, the range of antifungal agents available is limited, and some of the most effective agents are also toxic. In addition, while the azoles have been used successfully for the treatment of *Candida* infections, numerous reports of treatment failures are now appearing in the literature (6). The main aim of the study described here was to investigate ways of enhancing the antifungal activities of the azoles. We have focused on combinations of other agents with fluconazole, because fluconazole is one of the most popular drugs for treating candidiasis, especially in immunocompromised patients.

Previous reports of synergistic combinations of antifungal compounds have not been extensive or encouraging. In a review of combination therapy in systemic mycosis, Polak (8) discussed the combination of amphotericin B with flucytosine as one of the better-established synergistic combinations of antifungal agents that has been used clinically to treat candidiasis. The combination of an azole with a polyene resulted in conflicting outcomes, depending on the species and the strain tested, and specific antagonism was observed with *Candida albicans* (8). In the present investigation, we decided to include compounds for which claims of antifungal activity were limited but which were also known to have low toxicity. Propylparaben is an ester of *p*-hydroxybenzoic acid with antifungal activity (11) and is widely used as a preservative in pharmaceuticals, cosmetics, and food. Salicylic acid is a constituent of some topical antifungal preparations. Ibuprofen, a nonsteroidal anti-inflammatory drug widely used for its analgesic, antipyretic, and anti-inflammatory activities, was recently shown to have limited activity against *C. albicans* (10). Combinations of traditional agents such as miconazole and nystatin with fluconazole were also tested.

Considerable effort has recently been directed at standardization of in vitro testing of antifungal agents against *C. albicans* in order to eliminate the poorly controlled sources of test variation, and a National Committee for Clinical Laboratory Standards standard for susceptibility testing has been proposed (5). Microdilution susceptibility testing methods based on these recommended procedures have also been reported (7, 9). We have applied this microtiter methodology in a checkerboard assay to investigate synergy among antifungal agents and other possible potentiating compounds.

MATERIALS AND METHODS

***C. albicans* strains.** The NCYC 610 strain of *C. albicans* (Robin) Berkhout was mainly used in the investigation. Four clinical isolates of *C. albicans* obtained from patients with AIDS were also used. These isolates were kindly supplied by the Public Health Laboratory Service, Royal Sussex County Hospital, Brighton, United Kingdom. All isolates were maintained on Sabouraud dextrose agar (SDA; Oxoid) slants at 4°C.

Antifungal agents and other compounds. The compounds tested were fluconazole (kindly supplied by Pfizer Ltd.), miconazole (Sigma Chemical Co. Ltd. U.K.), nystatin (Sigma Chemical Co. Ltd. U.K.), ibuprofen (Boots Pharmaceuticals), sodium salicylate (BDH Ltd.), and propylparaben (propyl-*p*-hydroxybenzoate; Sigma Chemical Co. Ltd. U.K.). Fluconazole and sodium salicylate solutions were prepared in water. Solutions of the other agents were prepared by dissolving the individual compounds in 10% dimethyl sulfoxide (DMSO) and diluting the solution with medium so that the final concentration of the solvent was 0.5%.

Checkerboard broth microdilution method for synergy testing. Eight doubling dilutions of the two agents being tested were prepared in the susceptibility testing medium RPMI 1640 (Sigma Chemical Co. Ltd. U.K.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma Chemical Co. Ltd. U.K.). The range of concentrations of drugs for use in the checkerboard assay was based on an initial determination of the MICs of individual agents. Each dilution (50 μl) of each agent was placed in wells of a microtiter plate to provide 64 drug combinations. Additional rows were used to determine the MIC of each agent alone (inoculated with 100 μl of individual agent), for the growth

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TABLE 1. Influence of day and solvent on control growth of *C. albicans* NCYC 610 in RPMI 1640 medium on the basis of OD measurements

Day[a]	Solvent (DMSO)[b]	No. of wells measured	Mean (SD) OD at 492 nm
1	Yes	4	1.41 (0.011)
1	No	8	1.39 (0.033)
2	Yes	8	1.28 (0.061)
2	No	8	1.24 (0.072)
3	Yes	16	1.38 (0.031)
3	No	8	1.41 (0.046)
4	Yes	8	1.25 (0.302)
4	No	16	1.37 (0.067)
5	Yes	16	1.47 (0.040)
5	No	8	1.52 (0.036)
6	Yes	16	1.46 (0.039)
6	No	8	1.47 (0.045)

[a] Significant difference ($P < 0.001$; analysis of variance) with day.
[b] No significant difference ($P > 0.9$; analysis of variance) with the solvent, DMSO (used to solubilize some of the compounds in synergy tests), present or absent.

of the organism alone, with and without DMSO, and the sterility of the growth medium. The same predefined well-filling pattern was used in all experiments.

The yeast cell inoculum was prepared according to the proposed standard (5) except that the final suspension was adjusted to an optical density (OD) at 530 nm equivalent to 2×10^6 to 10×10^6 CFU/ml on the basis of a linear regression calibration graph of viable count versus OD ($r^2 = 0.974$). The final inoculum suspension was diluted 1:100; this was followed by a 1:20 dilution in RPMI 1640 to give the test inoculum of 1×10^3 to 5×10^3 CFU/ml. The viable count of this inoculum was determined by serial dilution in 0.1% peptone water and a spread plating method on SDA. The final inoculum (100 μl) was added to each well of the microtiter plate, which was incubated at 35°C for 24 h without shaking.

Plates were then scored visually by using the following numerical scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity compared with control growth (7). The MIC-0, MIC-1, MIC-2, etc., were the minimum concentrations resulting in the corresponding reduction in turbidity. MICs were also determined for each component of the combination by using the same criteria, and the fractional inhibitory concentrations (FICs) were calculated, where FIC($drug_1$) = MIC of $drug_1$ in the combination/MIC of $drug_1$ on its own and FIC($drug_2$) = MIC of $drug_2$ in the combination/MIC of $drug_2$ on its own. The fractional inhibitory index (FIX) (1) is the sum of FIC($drug_1$) and FIC($drug_2$) and was interpreted as follows: ≤0.5, synergy; >0.5 to <4, indifference or additivity; ≥4, antagonism. The OD at 492 nm of each well was also read on a microplate reader (Titretex Plus MS 212) so that MICs based on a reduction in absorbance could be calculated.

RESULTS

Preparation of inoculum for microtiter plates. The OD at 492 nm of control growth of *C. albicans* NCYC 610 after 24 h of incubation in microtiter plates with and without DMSO was measured on 6 separate days. The results were analyzed by using a two-factor analysis of variance (Table 1) to determine if growth was significantly affected by the day on which the experiment was carried out or the presence in the growth medium of the solvent required to solubilize some of the antifungal compounds. There was a significant difference in control growth on different days ($P < 0.001$), but growth was not significantly affected by the presence of DMSO ($P > 0.9$). However, the range (1.24 to 1.52) of OD measurements for control growth was, in practical terms, quite small when the full range of the OD scale is considered. Also, there was little variance in the within-day results. From an analysis of the control growth of the culture collection strain and the four clinical isolates, the isolate was shown to significantly influence growth in microtiter plates (Table 2).

TABLE 2. Influence of isolate of *C. albicans* on control growth at 24 h obtained in microtiter plates in RPMI 1640 medium on the basis of OD measurements

Isolate[a]	No. of wells measured	Mean (SD) OD at 492 nm
NCYC 610	24	1.46 (0.040)
A1	32	0.78 (0.064)
A2	8	1.29 (0.043)
A3	8	1.17 (0.068)
A4	8	1.45 (0.063)

[a] Significant difference ($P < 0.001$; analysis of variance) with isolate. A1 to A4 are clinical isolates.

Correlation of visual scale and OD measurements. The results of the visual scores for five microtiter plates was regressed against the corresponding mean OD at 492 nm measurement (Fig. 1) and indicated that the visual scores correlated with the OD ($r^2 = 0.994$). The mean OD of all scores of 2 was 0.9, which was approximately 60% of the mean OD of all scores of 4. The variance in scores of 2 and 3 was greatest, indicating that these were the most difficult to interpret consistently.

Checkerboard investigations for synergy. The results from the visual scoring of microtiter plates containing combinations of agents in the checkerboard assay are provided in Table 3 for the MIC-0. Problems of solubility limited the upper concentration that could be used. Consequently, some compounds used on their own were not assigned an MIC-0. A further problem was in assigning an MIC-2 in the same concentration range that made it possible to determine the MIC-0. For example, no concentration of fluconazole achieved total inhibition of growth. However, within the range of 8 doubling dilutions (usually 128 to 1), the MIC-2 of fluconazole was less than the lowest concentration used in all experiments, and thus, it was not possible to estimate the FIX and the possibility of a

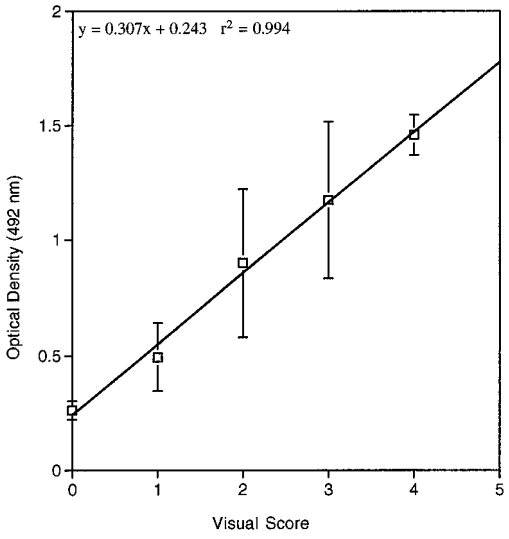

FIG. 1. Correlation of visual score with mean OD at 492 nm measurements for *C. albicans* NCYC 610. Visual score interpretations (and the number of individual scores contributing to the mean OD value) were as follows: 0, optically clear ($n = 79$); 1, slightly hazy ($n = 115$); 2, prominent decrease in turbidity ($n = 89$); 3, slight reduction in turbidity ($n = 38$); and 4, no reduction in turbidity compared with control growth ($n = 114$). Error bars are standard deviations.

TABLE 3. Checkerboard assay of the effect of drug combinations on *C. albicans* NCYC 610 on the basis of a visual endpoint of total inhibition of growth (MIC-0)

Combination	Agents in combination	MIC-0 (μg/ml) of each agent		FIC (μg/ml)[a]	FIX[b]	Outcome
		In the combination	On its own			
1.	Fluconazole	1	>128[c]	0.004	1.004	Indifference
	Miconazole	16	16	1.0		
2.	Fluconazole	1	>128[c]	0.004	2.004	Indifference
	Nystatin	4	2	2.0		
3.	Fluconazole	8	>128[c]	0.031	0.156	Synergy
	Ibuprofen	128	1,024	0.125		
4.	Fluconazole	32	>128[c]	0.125	0.375	Synergy
	Sodium salicylate	256	>512[c]	0.25		
5.	Fluconazole	2	>128[c]	0.008	0.258	Synergy
	Propylparaben	64	256	0.25		
6.	Miconazole	2	16	0.125	0.375	Synergy
	Ibuprofen	256	1,024	0.25		

[a] The ratio of the MIC-0 of the agent in the combination to the MIC-0 of the agent on its own is the FIC.
[b] The FIX is the sum of the FICs of the agents in the combination.
[c] When the MIC-0 of the agent on its own was higher than the maximum concentration used, it was assigned the next higher concentration for calculation of the FIC. This may result in the underestimation of synergy.

synergistic interaction with drug combinations by using the MIC-2 criterion. Most of the visual scores for fluconazole were 1.

One of the difficulties in the visual scoring scheme is the subjective interpretation of a prominent decrease in turbidity. In an attempt to remove this source of error, the MIC-80% was determined from the scanned OD readings as follows. The average OD for control growth and the background OD for medium only was calculated, and the difference was taken as the true increase in OD due to control growth. Twenty percent of this value was calculated, and the value obtained was added to the background OD to provide the limits for determination of the MIC-80%, i.e., the concentration giving an 80% reduction in the OD compared with the OD for the control. The results are presented in Table 4.

The combination of fluconazole and ibuprofen was tested against four clinical isolates of *C. albicans*. The MIC-0 results assessed after 48-h incubations are presented in Table 5 and indicate that the combination had synergistic activity against three of four isolates. The MIC-80% determined for OD values produced similar results and interpretations.

TABLE 4. Checkerboard assay of the effect of drug combinations on *C. albicans* NCYC 610 on the basis of an 80% reduction in OD at 492 nm compared with control growth (MIC-80%)

Combination	Agents in combination	MIC-80% (μg/ml) of each agent		FIC (μg/ml)[a]	FIX[b]	Outcome
		In the combination	On its own			
1.	Fluconazole	1	>128[c]	0.004	0.504	Indifference
	Miconazole	8	16	0.5		
2.	Fluconazole	1	>128[c]	0.004	1.004	Indifference
	Nystatin	2	2	1.0		
3.	Fluconazole	1	>128[c]	0.004	0.066	Synergy
	Ibuprofen	32	>256[c]	0.062		
4.	Fluconazole	8	64	0.125	0.25	Synergy
	Sodium salicylate	64	>256[c]	0.125		
5.	Fluconazole	1	128	0.008	0.133	Synergy
	Propylparaben	64	>256[c]	0.125		
6.	Miconazole	0.25	4	0.062	0.070	Synergy
	Ibuprofen	4	512	0.008		

[a] The ratio of the MIC-0 of the agent in the combination to the MIC-0 of the agent on its own is the FIC.
[b] The FIX is the sum of the FICs of the agents in the combination.
[c] When the MIC-80% of the agent on its own was higher than the maximum concentration used, it was assigned the next higher concentration for calculation of FIC. This may result in the underestimation of synergy.

TABLE 5. Checkerboard assay of the effect of the combination of fluconazole and ibuprofen on *C. albicans* isolates on the basis of a visual endpoint of total inhibition of growth (MIC-0)

Clinical isolate	Agents in combination	MIC-0 (μg/ml) of each agent		FIC (μg/ml)[a]	FIX[b]	Outcome
		In the combination	On its own			
1.	Fluconazole	8	256	0.031	0.047	Synergy
	Ibuprofen	32	>1,024[c]	0.016		
2.	Fluconazole	4	>256[c]	0.008	0.258	Synergy
	Ibuprofen	512	>1,024[c]	0.25		
3.	Fluconazole	8	>256[c]	0.016	0.266	Synergy
	Ibuprofen	512	>1,024[c]	0.25		
4.	Fluconazole	>128	256	NC[d]	NC	NC
	Ibuprofen	>512	>1,024[c]			

[a] The ratio of the MIC-0 of the agent in the combination to the MIC-0 of the agent on its own is the FIC.
[b] The FIX is the sum of the FICs of the agents in the combination.
[c] When the MIC-0 of the agent on its own was higher than the maximum concentration used, it was assigned the next higher concentration for calculation of FIC. This may result in the underestimation of synergy.
[d] NC, not calculable.

DISCUSSION

The microdilution method with an inoculum prepared in a manner similar to that used for the National Committee for Clinical Laboratory Standards standard (5) proved satisfactory for synergy testing. Control growth in the test medium was rapid and reproducible, reaching an OD at 492 nm of approximately 1.2 to 1.5 within 24 h. This is equivalent to a viable count of at least 3×10^7 CFU/ml and represents a 4-log increase in the 24-h period. Visual and OD endpoints could be read clearly after 24 h of incubation. OD readings do not increase linearly with cell mass in the higher absorbance range, and therefore, a reading at 48 h offers no advantage. The exception to this was with clinical isolates, which were often slower growing and required longer incubation periods to obtain clear endpoints. While the correlation between visual and OD readings was good, the advantage of the latter method is that it avoids interoperator bias in endpoint assessment. Nevertheless, there was good agreement between the outcomes predicted in synergy tests with both visual and OD endpoints. In order to take account of the partial inhibition of growth that is a common feature of the inhibitory activity of azoles, the MIC-2 concept can be used whereby the MIC is the concentration that gives a prominent decrease in turbidity (7). However, in the present investigation, visual endpoints based on the total inhibition of growth were mainly attained, especially with drug combinations, and the MIC-0 was used to calculate FIXs and make interpretations of synergy. A variety of methods for determining endpoints from OD readings of microtiter plates have been described, including 70% reduction in isolate growth compared with control growth (2) and 25% or less growth than that of the control (9). We selected an 80% reduction in growth relative to control growth, which related to the guidelines of the National Committee for Clinical Laboratory Standards for macrodilution methods (5).

The results of the microtiter checkerboard assay for combinations of the traditional antifungal agents (azoles and polyenes) used in the study indicated that their activity was not synergistic. Additivity or indifference was the outcome obtained with a nystatin and fluconazole combination. Also, the additive effect observed with the combination of fluconazole, a triazole, and miconazole, an imidazole, was anticipated because both drugs share a similar mode of action and are unlikely to enhance the activity of each other. The most interesting outcome was in the synergism displayed with combinations of fluconazole and either ibuprofen, sodium salicylate, or propylparaben. Synergism between miconazole and ibuprofen was also demonstrated. Against three of the four clinical isolates of *C. albicans* from AIDS patients, the combination of fluconazole and ibuprofen was synergistic. A recent investigation of the antifungal activity of ibuprofen claimed that it has good activity against dermatophytes but poor activity against *C. albicans* (10), although no mechanism of action was proposed by those investigators. However, ibuprofen is a propionic acid derivative, and the similarity of its structure to those of the salicylates may be a contributing factor. Sodium salicylate, the salt of salicylic acid, was recently shown to potentiate the inhibitory action of aminoglycoside antibiotics against *Klebsiella pneumoniae* (3), which the investigators suggested might occur by facilitation of transport through the cytoplasmic membrane. Propylparaben is an ester of *p*-hydroxybenzoic acid with antifungal activity (11) and is considered to act as an uncoupling agent preventing the uptake of substrates which depend on a proton motive force for their entry into the cell and which also inhibit electron transport (4). It is possible that all three compounds have some membrane activity either in facilitating the uptake of the azole or in enhancing the membrane damage associated with the mode of action of the azole.

The checkerboard method aims to provide a simple estimate of the interaction between two compounds whereby the lower the FIX value that is obtained, the greater the synergistic activity. The low FIX values observed with fluconazole or miconazole in combination with ibuprofen in the present study are particularly noteworthy. In a decimal assay for additivity, we have also demonstrated in vitro synergism between econazole and ibuprofen against *C. albicans* (12). Further investigations of azole-ibuprofen combinations are merited, especially for fungicidal activity, in which it is known that azoles on their own have limited activity.

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