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Antifungal relative inhibition factors: BAY l-9139, bifonazole, butoconazole, isoconazole, itraconazole (R 51211), oxiconazole, Ro 14-4767/002, sulconazole, terconazole and vibunazole (BAY n-7133) compared *in vitro* with nine established antifungal agents

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Nine new antifungal agents were tested for their activity *in vitro* in terms of relative inhibition factors (RIFs) against 26 isolates of *Candida* species, eight isolates of *Aspergillus* species and six isolates of dermatophyte fungi. Eight of the new compounds were azole antifungals, the ninth was a phenylmorpholine derivative. Against *Candida* species, all the novel compounds gave RIFs that were of a similar order to RIFs for established imidazole compounds. Two topical antifungals, butoconazole and terconazole, and two systemic antifungals, itraconazole and vibunazole, gave mean RIFs <60% in tests with *Candida* species, and therefore matched clotrimazole, ketoconazole and tioconazole in terms of RIF. However, none of the new compounds gave RIFs as low as amphotericin B against the *Candida* isolates. Against *Aspergillus* isolates, itraconazole, with a mean RIF of 25%, was even more active *in vitro* than amphotericin B. Vibunazole was as active as ketoconazole against *Aspergillus* isolates. All the new antifungals except Bay l-9139 gave very low RIFs against dermatophyte isolates, and thus matched established imidazole antifungals for inhibitory effects *in vitro*. In terms of RIF data, all the nine new compounds tested appear to offer reasonable potential for antifungal chemotherapy *in vivo*. A similar conclusion would not have been drawn from minimal inhibitory concentration data, which tended to show most of the new antifungals in a very poor light. Tests with amphotericin B, 5-fluorocytosine and ketoconazole showed that RIF can vary substantially with the pH of the test medium. For amphotericin B and ketoconazole the best activity was seen at neutral pH values; for 5-fluorocytosine the greatest inhibitory activity was found at lower pH values.

Introduction

There is presently considerable effort within the pharmaceutical industry to produce antifungal agents that are more effective, safer and easier to administer than compounds already established for the therapy of mycoses. Most of the drugs currently available for antifungal chemotherapy are members of the polyene or imidazole/triazole derivative groups. Griseofulvin and 5-fluorocytosine (5FC) are the major exceptions, being sole representatives of their chemical types and having unique modes of action (Speller, 1980). The great majority of newly developed antifungals are imidazole or triazole derivatives. They fall into two categories: agents for topical

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therapy of superficial mycoses, of which genital *Candida* infections and dermatophytoses are the most common examples, and agents that are absorbed systemically when given by mouth, and can therefore be used for treatment of systemic as well as superficial mycoses.

Preclinical assessment of antifungal agents presents problems because many animal models of mycotic diseases are less than ideal, and, in the case of imidazole derivatives, because the results of tests for antifungal activity *in vitro* are highly variable. Minimal inhibitory concentrations (MICs) of imidazole and triazole antifungals vary markedly according to the test protocol, particularly with respect to the growth medium used and the size of the inoculum (Hoeprich & Huston, 1976; Kitahara *et al.*, 1976; Odds, 1980; Plempel *et al.*, 1974; Galgiani & Stevens, 1976); moreover, MIC end-points are difficult to assess subjectively because the newer antifungal agents cause a gradual diminution of growth with increasing drug concentration rather than a sharp, clear-cut end point.

In an attempt to overcome the problems of classical MIC tests *in vitro*, we recently devised a novel test method for antifungals, in which the areas under a fixed sector of an antifungal dose-response curve were expressed as a percentage of the area under the dose-response curve of a theoretical, non-inhibitory drug. This figure, the relative inhibition factor (RIF), approaches 100% for a drug that is non-inhibitory for a given fungus, and it approaches 0% for a drug to which a fungus is exquisitely sensitive (Odds & Abbott, 1984). The tests are performed in Eagle's minimal essential medium with added serum under 5% CO_2 in air—conditions that are likely to mimic those in tissues *in vivo* more closely than conventional mycological media—and growth is measured by ATP bioluminescence photometry, which assesses biomass independent of cell shape. The dose-response curve of antifungals is measured over a drug concentration range of 0·01–10 mg/l. For most antifungals this range covers clinically achievable doses. Moreover, this range was previously found to cover the regions of inflexion in the dose-response curves for four antifungals (Odds, 1982) yet its upper limit is low enough to avoid the problems of drug insolubility that sometimes confound tests *in vitro* (Plempel, Regel & Buchel, 1983). Because the conditions are standardized throughout, the method allows cross comparison between effects of different antifungals and effects on different fungi. It also takes account of the partial inhibition phenomena seen with imidazole and triazole antifungals.

In preliminary tests with several well-established antifungal agents, RIF data correlated well with MIC data for polyenes, 5-fluorocytosine and griseofulvin, where MICs are generally thought to relate well to inhibition *in vivo*, but they showed no correlation with MICs for agents of the imidazole derivative type, and therefore offer an alternative measure of azole effects on fungi to MIC determinations (Odds & Abbott, 1984). In the present study, we have determined RIFs for ten new antifungal agents against a variety of common fungal pathogens, to offer a data base for comparison with other preclinical tests involving these new agents. We have used the RIFs for nine antifungals already established for clinical use as a reference base for the new compounds. We have also investigated the effect on RIF of variation in the pH of the culture medium for three antifungal agents.

Of the ten new antifungals tested, three are compounds that are absorbed systemically after oral administration: BAY l-9139 (an imidazole derivative), vibunazole (BAY n-7133) and R 51211 (both triazole derivatives; the latter has been given the WHO proposed name itraconazole). The seven other compounds are intended for

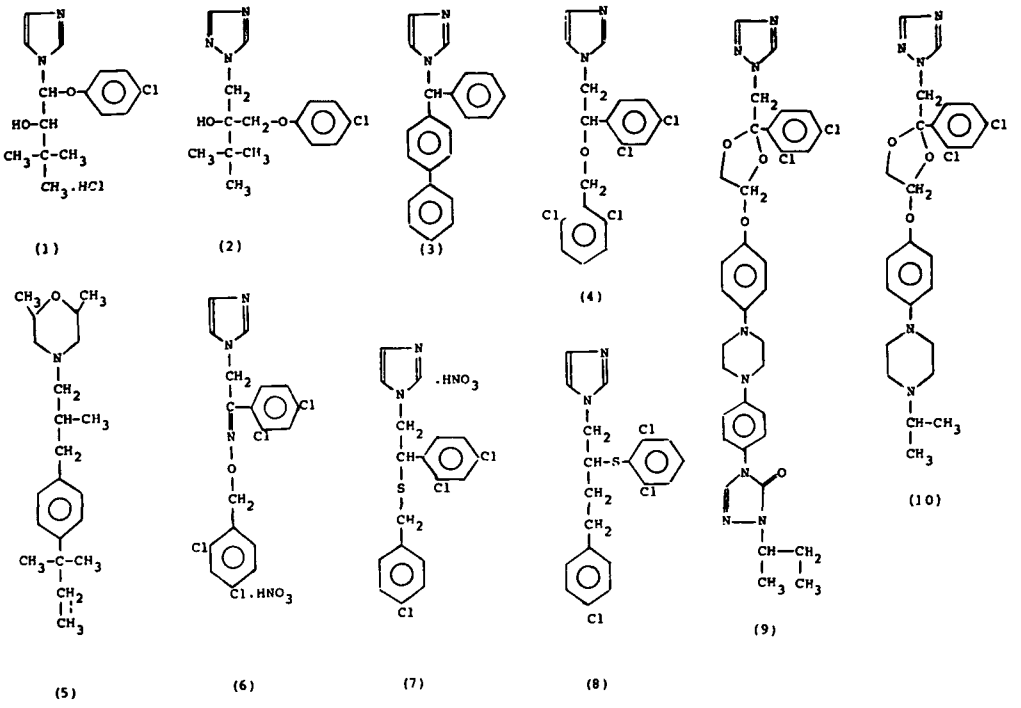

Figure 1. Structural formulae of antifungal compounds. (1) BAY 1-9139, (2) vibunazole (BAY n-7133), (3) bifonazole, (4) isoconazole, (5) Ro 4767/002, (6) oxiconazole, (7) sulconazole, (8) butoconazole, (9) itraconazole (R 51211), (10) terconazole.

topical use. Ro 14-4767/002 is a phenylmorpholine derivative, and the other six compounds, bifonazole, butoconazole, isoconazole, oxiconazole, sulconazole and terconazole, are all imidazole or triazole derivatives. The structural formulae of the ten new test compounds are given in Figure 1. The nine reference antifungals tested were amphotericin B and nystatin (polyenes,) clotrimazole, econazole, ketoconazole, miconazole and tioconazole (imidazole derivatives), 5-fluorocytosine (5FC) and griseofulvin.

Methods

Antifungals

The sources of the antifungals tested were as follows: amphotericin B, as 'Fungizone' intravenous preparation, was purchased from E.R. Squibb & Sons, Ltd.; nystatin, griseofulvin and 5FC were purchased from Sigma Chemical Co.; BAY 1-9139, bifonazole, clotrimazole and vibunazole were the gift of Bayer AG; miconazole, ketoconazole, terconazole and itraconazole were the gift of Janssen Pharmaceutica; oxiconazole and Ro 14-4767/002 were the gift of Hoffmann La Roche Co.; econazole was the gift of F.A.I.R. Laboratories; butoconazole and sulconazole were the gift of Syntex Pharmaceuticals International Limited; isoconazole was the gift of Schering Pharmaceuticals; and tioconazole was the gift of Pfizer U.K. Ltd.

Series of stock concentrated dilutions of the antifungals were prepared as previously described (Odds & Abbott, 1984) in the following solvents: bifonazole, butoconazole, clotrimazole, econazole, isoconazole, miconazole, oxiconazole, Ro 14-4767/002, sulconazole, terconazole, tioconazole and vibunazole in acetone; BAY 1-9139 and 5FC in water; griseofulvin, nystatin and itraconazole in DMSO; ketoconazole in 50% acetone and amphotericin B in 0·4% sodium deoxycholate. The stock concentrates were stored at −20°C when not in use. Dilutions of the antifungals were prepared in the appropriate solvents as previously described (Odds & Abbott, 1984).

Test fungi

Representatives of pathogenic yeast species, *Aspergillus* species and dermatophytes were tested. They comprised *Candida albicans* (14 isolates), *Can. glabrata* (3), *Can. guilliermondii* (2), *Can. krusei* (2), *Can. parapsilosis* (2), *Can. pseudotropicalis* (1), *Can. tropicalis* (2), *Asp. fumigatus* (6), *Asp. flavus* (2), *Microsporum canis* (1), *Trichophyton mentagrophytes* (3), *Trich. rubrum* (2). All the isolates originally came from clinical material. Nine of the 26 *Candida* isolates were known to be resistant to 5FC. All isolates were maintained by subculture on Sabouraud dextrose agar (Oxoid). The *Can. albicans* isolates included the strain known as *Can. albicans* Darlington (Warnock *et al.*, 1983), which is known to be resistant to several azole antifungals (Odds & Abbott, 1984). During the course of the experimental work, subcultures of two further azole-resistant *Can. albicans* isolates, AD and KB (Ryley, Wilson & Barrett-Bee, 1984) were supplied to us. These were not included in the full series of RIF tests, but were treated with azole antifungals only.

Determination of RIF

The test protocol was as previously described (Odds & Abbott, 1984). Briefly, fungal inocula were standardized to 66 nM ATP (yeasts and dermatophytes) or 6·6 nM ATP (*Aspergillus* species) in double-strength Eagle's minimal essential medium buffered with HEPES and $NaHCO_3$. Inoculated media in 0·5 ml amounts were mixed with equal volumes of antifungal solutions diluted to twice their final concentration in water. By this procedure an initial fungus concentration equivalent to 33 or 3·3 nM ATP was achieved in the wells of plastic 'Repli-dishes' (Sterilin Ltd.). The cultures were incubated at 37°C under 5% CO_2 in air (yeasts and *Aspergillus* species) or at 30°C in air (dermatophytes) to the mid-logarithmic phase of growth (6–8 h for yeasts, 14–17 h for *Aspergillus* species, 24–36 h for dermatophytes). Details for sampling of the cultures, measurement of ATP concentration and calculation of RIF were given previously (Odds & Abbott, 1984).

To determine the extent of variation of RIF with the pH of the culture medium, RIFs were determined as described above for amphotericin B, 5-fluorocytosine and ketoconazole except that the pH of the culture medium was altered by addition of differing amounts of $NaHCO_3$.

Determination of MIC

For a small number of fungal isolates and antifungals the MIC of the antifungals was determined. The MIC test was done in yeast nitrogen base/glucose broth (Odds & Abbott, 1984) with inocula of 10^5 yeasts or conidia per ml.

Results

Relative inhibition factors

A summary of the RIF data for the 19 antifungals and 40 fungi tested is given in Table I. Against pathogenic *Candida* species, mean RIFs ranged from 20% (amphotericin B) to 98% (griseofulvin). Of the three new orally active compounds, itraconazole gave the lowest mean RIF (47%) and BAY 1-9139 the highest (65%). Among the seven new topical antifungals, the lowest RIFs were seen with terconazole and butoconazole (56 and 59%, respectively) and the highest with bifonazole (77%). Overall, the range of mean RIFs seen for the new antifungals (47–77%) was broadly similar to that seen for the established imidazole-type antifungals (54–69%). For each imidazole or triazole antifungal, although the ranges of RIFs for individual *Candida* isolates were fairly wide, the upper extremes (>80%) resulted entirely from the inclusion among the test isolates of one particular strain, *Can. albicans* Darlington (Warnock *et al.*, 1983; Odds & Abbott, 1984), which is uniformly resistant to imidazole and triazole derivative antifungals.

Against the *Aspergillus* isolates, the highest inhibitory activity was seen with one of the new orally active compounds, itraconazole (mean RIF was 25%). Griseofulvin, 5FC and Ro 14-4767/002 were essentially noninhibitory against *Aspergillus* species (Table I). The activity of the topical antifungals against these fungi is of academic interest only, since *Aspergillus* infections are systemic. Among the orally active antifungals, ketoconazole and vibunazole were virtually identically inhibitory in terms of RIF (means of 56% and 55% respectively); BAY 1-9139 was less active.

Only 5FC appeared to be noninhibitory against dermatophytes in terms of RIF (Table I). All the new compounds were highly inhibitory, with mean RIFs ranging from around 10–12% (itraconazole and sulconazole) to 26–29% (isoconazole and Ro 14-4767/002). The only exception was BAY 1-9139, with a mean RIF of 49%, but even this was lower than the mean RIF for the established anti-dermatophyte antifungal, griseofulvin (58%).

Variation in RIF with pH of the test medium

For amphotericin B, 5FC and ketoconazole there was a marked variation in RIF against eight yeast isolates with changes in pH of the test system (Figure 2). For amphotericin B and ketoconazole, the RIF fell as the pH rose from 4·5 to 8·0. The opposite trend was seen with 5FC, where the RIF increased with pH: this change was seen with resistant as well as sensitive isolates.

RIFs of azole antifungals for three azole-resistant isolates of Can. albicans

For three *Can. albicans* isolates known to be resistant to several azole antifungals, RIFs were high for all 14 azole antifungals included in the present study (Table II). The data show that in terms of RIF, azole resistance was greatest overall for *Can. albicans* Darlington, and least for *Can. albicans* AD (mean RIFs were: Darlington, 91%; KB, 86%; AD, 80%). Only itraconazole gave RIFs less than 80% for all three isolates.

MICs of the antifungals tested

MIC tests, done in a 'conventional' agar plate system, were performed only for a small number of fungi and antifungals, since previous experience had shown that

Table I. Relative inhibition factors of 19 antifungal compounds tested *in vitro* against 40 isolates of pathogenic fungi

Antifungal	Activity against 26 isolates of *Candida* spp.				Activity against 8 isolates of *Aspergillus* spp.				Activity against 6 dermatophyte isolates			
	amean*	gmean**	median	RIF range	amean	gmean	median	RIF range	amean	gmean	median	RIF range
amphotericin B	20±4	19	18	13–33	37±7	36	36	26–49	17±10	13	15	6–31
BAY 1-9139	65±13	63	57	40–88	81±8	81	83	63–90	49±11	48	48	38–69
bifonazole	77±13	76	79	37–98	71±9	70	72	53–86	23±8	22	22	15–37
butoconazole	59±15	57	60	26–95	87±14	85	91	60–100	16±7	15	17	6–22
clotrimazole	57±15	55	57	33–92	48±8	47	46	37–58	21±15	15	20	4–45
econazole	68±16	66	66	39–98	49±8	48	47	39–60	19±8	15	20	3–31
5-fluorocytosine	49±20	44	45	13–95	92±8	92	96	79–100	92±7	92	91	83–100
griseofulvin	98±3	98	99	92–100	97±3	97	98	91–100	58±15	56	57	41–80
isoconazole	73±13	72	75	48–94	58±6	58	58	51–69	26±9	25	25	16–43
itraconazole	47±13	45	44	30–79	25±4	25	26	19–30	12±4	11	13	5–18
ketoconazole	54±12	53	53	33–84	55±9	54	55	43–71	18±7	17	19	8–30
miconazole	69±17	66	68	34–98	68±10	67	68	54–81	26±14	22	21	11–51
nystatin	52±6	52	54	34–61	68±5	67	68	59–75	46±12	44	50	22–60
oxiconazole	67±18	64	68	26–95	72±6	71	69	66–84	20±5	19	19	13–27
Ro 14-4767/002	67±17	65	69	39–93	93±7	93	96	76–100	29±10	26	33	9–42
sulconazole	69±17	67	65	30–98	71±7	71	70	61–82	12±5	11	14	5–18
terconazole	56±18	53	52	29–94	75±9	75	74	63–94	17±7	16	18	7–27
tioconazole	59±17	56	58	28–95	69±10	68	71	53–79	15±11	10	11	2–31
vibunazole	59±18	56	54	34–97	56±6	55	55	45–66	24±10	22	22	13–44

*Arithmetic mean ± standard deviation.
**Geometric mean.

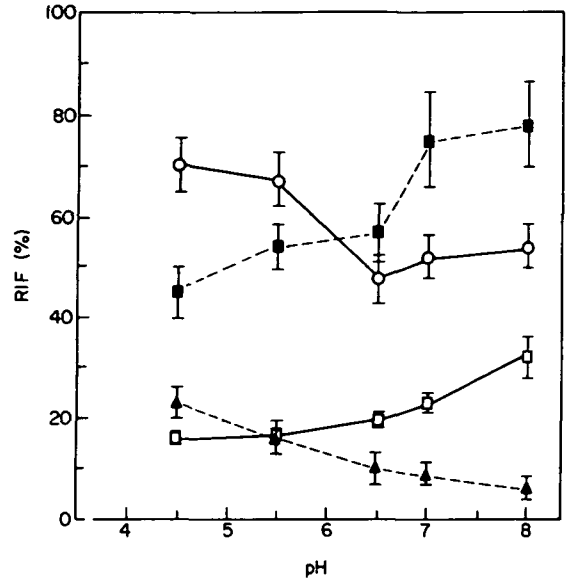

Figure 2. Variation of RIF against eight yeasts with pH of Eagle's medium. ▲, Amphotericin B; ○, ketoconazole; □, 5-fluorocytosine (resistant isolates); ■, 5-fluorocytosine (sensitive isolates). Points indicate means and standard deviations.

Table II. RIFs of 14 azole antifungals against three azole-resistant *Can. albicans* isolates from patients with chronic mucocutaneous candidosis

Antifungal	RIF (%) vs. *Can albicans:* Darlington	KB	AD
BAY 1-9139	88	83	78
bifonazole	98	100	82
butoconazole	95	88	71
clotrimazole	82	87	79
econazole	95	91	94
isoconazole	93	93	80
itraconazole	79	70	68
ketoconazole	84	85	64
miconazole	96	91	93
oxiconazole	82	95	90
sulconazole	98	94	100
terconazole	94	75	64
tioconazole	95	82	78
vibunazole	90	73	83

the MIC test system gave unrealistically high MICs for many azole antifungals (Odds, 1980; Odds & Abbott, 1984). The new azole antifungals usually gave very high MIC figures: they were in the range 1–33 mg/l for bifonazole and butoconazole, 33 to >100 mg/l for BAY 1-9139, terconazole and vibunazole, and nearly all isolates tested were not inhibited by itraconazole at 100 mg/l.

Discussion

With the exception of Ro 14-4767/002, all the new antifungals tested in this study were imidazole or triazole derivatives. It is clear that since azole antifungals continue to be developed by several independent manufacturers this class of compounds still shows the greatest potential for new developments in antifungal chemotherapy.

Azole antifungals can be assigned to 'generations' of development, according to their structure and approximate time of invention. The first generation compounds, which include clotrimazole, miconazole and econazole, were all imidazole derivatives and strongly hydrophobic. The second generation is represented by compounds such as ketoconazole and BAY 1-9139—imidazole derivatives with greater water solubility than the first generation compounds and thus the potential for systemic absorption after oral administration. The third generation of azole compounds is less easy to define, but in general it is based on a triazole rather than an imidazole, nucleus, and there are two clear subdivisions among the triazole compounds according to their ability to be absorbed after oral administration (e.g. itraconazole, vibunazole) or their value for topical use only (e.g. terconazole).

Despite the proliferation of different azole antifungals, one property has remained fairly consistent among those that have reached the point of clinical evaluation—the cure rates attributed to them in comparable superficial mycoses are very similar. Thus, for example, clinical and mycological cure rates of azole antifungals in vaginal *Candida* infections—an application where clotrimazole, econazole, isoconazole, ketoconazole, miconazole and tioconazole have all been tested—usually range from about 85–95% in optimal trial protocols (Heel, 1982; Heel *et al.*, 1978; Henderson, 1983; Hoffbrand, Allen & Good, 1974; Odds, 1977; Sawyer *et al.*, 1975*a, b;* Walther, 1982). It is therefore no surprise that the 14 azole antifungals tested in the present study show broadly similar RIFs against the three groups of test fungi.

The RIFs certainly appear to mirror the similar antifungal efficacy of azoles *in vivo* more reliably than MICs, which were almost always excessively pessimistic, especially for the third generation azole antifungals. Although there has not yet been direct experimental demonstration of correlation between azole RIF and effective doses of antifungals *in vivo*, the likelihood of such a correlation is exemplified by the unusually high RIFs for all the azole antifungals against the three azole-resistant *Can. albicans* isolates from patients with chronic mucocutaneous candidosis (Table II). The resistance of two of these isolates to azoles *in vitro* has been throughly investigated in several test systems (Ryley *et al.*, 1984) and in all three cases the patients from whom the isolates were obtained failed to respond to azole antifungals *in vivo.*

The correlation of RIF with MIC for 5FC has been demonstrated previously (Odds & Abbott, 1984). Since as many as one-third of the *Candida* isolates included in the present study were known to be resistant to 5FC, the overall mean RIF for this compound (Table I) gives an unfair representation of its activity. The mean RIF for 5FC-sensitive *Candida* isolates was 42% and for 5FC-resistant isolates it was 62%. Higher concentrations of 5FC than those tested in the present study have been shown to inhibit *Aspergillus* spp. *in vitro* (Wain, Polak & Florio, 1981) and some clinical cases of pulmonary aspergillosis have responded to treatment with this drug (Scholer, 1981). This suggests that RIF data for 5FC against *Aspergillus* isolates may not always correlate well with the activity of the drug *in vivo*, although Scholer (1980) did not consider 5FC to be a valid monotherapy for aspergillosis.

Variations in antifungal susceptibility with the pH of the test medium have

previously been investigated in detail only for ketoconazole (Minagawa, Kitaura & Nakamizo, 1983), and our RIF data (Figure 2) confirm that the inhibitory effects of ketoconazole are superior at a pH around neutrality to those at a lower pH. The fact that for 5FC and amphotericin B the RIF/pH curves sloped in opposite directions suggests that pH effects may be mutually cancelling for these two drugs in combination so that they will act harmoniously against fungi over a wide range of environmental pH. This observation lends further support to the common clinical usage of these two antifungals in combination.

Our RIF data with several of the new antifungals tend to agree with published reports of their activity in MIC tests done under conditions designed or selected to reveal their activity optimally. Thus, the high activity of bifonazole against dermatophytes and its lower potency against yeast pathogens confirm the data of Plempel *et al.* (1983), Shadomy, Dixon & May (1982) and Yamaguchi, Hiratani & Plempel (1983*a*). Plempel *et al.* (1983) pointed out that the very low solubility of bifonazole may adversely affect its inhibitory performance in tests *in vitro*. It is possible that differences in solubility may account to some extent for differences in RIF noted for several azole antifungals, for relatively few of them have good water solubility.

The comparable behaviours of vibunazole and ketoconazole *in vitro* were also reported by Fromtling, Yu & Shadomy (1983) and Yamaguchi *et al.* (1983*b*). Polak (1982, 1983) found oxiconazole to be slightly more effective against *Candida* spp. *in vitro* than clotrimazole, econazole or miconazole: the RIF data (Table I) suggest its activity is of the same order as these compounds. Only the previously described very high activity of Ro 14-4767/002 (Polak, 1982, 1983) has not been confirmed in RIF tests. RIF tests have not previously been conducted with phenylmorpholine derivatives; the relevance of the RIF data for these compounds therefore remains to be established.

Acknowledgements

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