Anticandidial Effect of Phenylbutene Derivatives and Their Interaction with Ergosterol

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This paper reports the effect of phenylbut-3-en-2-one, of its analogues, bearing 3-nitro, 4-nitro, 4-chloro- and 4-dimethylamino substituents at the phenyl moiety, and of the hydrazide, phenylhydrazide and oxime of 4-nitrophenylbut-3-en-2-one on the growth and germ-tube formation of *Candida* spp., as well as their ability to interact with ergosterol in water/dimethylformamide (DMF) solution and their acute toxicity for mice. 3-Nitro-, 4-nitro- and 4-chlorophenylbut-3-en-2-ones inhibit candidial growth *in vitro* in concentrations ranging from 0.01 to >0.4 mM and their activity is comparable to that of ketoconazole (in mg/l) and lower than that of amphotericin B. The rest of the compounds are inactive at >0.4 mM. Germ-tube formation of *C.albicans* is inhibited at 0.04 mM 4-nitrophenylbut-3-en-2-one and at 0.005 mM of the 3-nitro isomer. A decrease in the absorption maxima in ergosterol mixtures with 4-dimethylamino, 3-nitrophenylbut-3-en-2-one and the oxime of the 4-nitrophenylbut-3-en-2-one was observed, indicative of interaction in water/DMF solutions, while no changes in the UV spectra of the remaining compounds were detectable. That suggests that the growth inhibiting effect is not in correlation with their ability to interact with ergosterol, despite the resemblance to polyenes. LD_{50} for mice is 367 mg/kg for 4-nitrophenylbut-3-en-2-one and 398 mg/kg for the 3-nitro isomer.

The increase in the incidence of infections, caused by yeast-like fungi from species *Candida* (Pfaller, 1994; Miller and Wenzel, 1987) , the emerging of strains resistant or cross-resistant towards currently available drugs (e.g. 5-fluorocytosine, fluconazole, (Speller and Davis, 1972, Nolte *et al*., 1997)), the narrow therapeutic index of agents for the treatment of systemic infections (e.g. amphotericin B, 5-fluorocytosine (Jullien *et al.,* 1990)), as well as the low serum concentrations of some azole derivatives prompted the search for anticandidial drugs for either systemic or local application, belonging to new chemical classes (Graybill, 1992).

We were interested in the anticandidial effect of a series of phenylbutene derivatives, a group of compounds that have a system of conjugated double bonds in which the delocalised pi-electrons originate both from the aliphatic chain and the benzene ring. It was previously reported that some of them (4-nitro- and 3-nitrophenylbut-3-en-2-one) exhibit an antibacterial effect and inhibit protein synthesis in *E. coli* (Tabakova *et al.,* 1994). These two nitro derivatives were also effective against *S. cerevisiae* (Tabakova, unpublished data) and it was expected that they may act against yeast-like fungi as well. Since the nitrophenylbutenones did not affect protein synthesis in the yeast strains it was obvious that the mechanisms of growth inhibition in bacteria and baker's yeasts would be different. The unsaturated character of the phenylbutene derivatives makes them similar to pecilocin – a polyene antibiotic (Reiner, 1982) and one possibility is that the phenylbutenes interact with ergosterol and that the anti-yeast effect of this series is associated with the unsaturated character of the molecule.

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To the best of our knowledge, the anticandidial effect of phenylbutene derivatives has not been investigated so far and the objective of this work is to compare the anticandidial effect of phenylbutene derivatives with different number of conjugated double bonds and substituents and their ability to interact with ergosterol, as determined by UV absorption spectra.

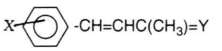
For this purpose we have determined *in vitro* the minimal inhibitory concentration (MIC) of phenylbut-3-en-2-ones, with different substituents at the phenyl ring and of the hydrazide, phenylhydrazide and oxime derivatives of 4-nitrophenylbut-3-en-2-one for standard strains of *Candida spp.* and clinical isolates from patients and their ability to interact with ergosterol in solution. The results are presented in Table I. The effect of 4-nitrophenylbut-3-en-2-one on germ tube formation in *C.albicans* was studied, as well as the acute toxicity of the compound for mice.

The MIC values presented in Table I are the mean ± SD for the strains taken from three independent experiments and the range.

Only the nitro- and chlorosubstituted analogues were effective in concentration of 0.4 mM or lower, while the unsubstituted and dimethylamino substituted compounds had no anticandidial effect. The concentration of the compounds that inhibited the formation of germ tubes for *C. albicans* ATCC 90028 strain is 0.04 mM for 4-nitrophenylbut-3-en-2-one and 0.005 mM for the 3-nitroanalogue.

Table I. Interaction with ergosterol (decrease in the specific sterol absorption maxima) and the minimal inhibitory concentrations in mM (mean ± SD and range) of phenylbutene derivatives.

Compound **	X	H	4-$(CH_3)_2N$	4-NO_2-	3-NO_2-	4-Cl-	4-NO_2	4-NO_2-	4-NO_2-	Ketoconazole	Amphotericin B
	Y	O=	O=	O=	O=	O=	=N-NH_2	=NHN-C_6H_5	=NOH		
Decrease at $\lambda_{(nm)}$			295 Fig. 1	Fig. 1	271,281	261	261		271		
Strain											
C.albicans	ATCC 90028	>0.4	>0.4	0.4	0.2	0.4	>0.4	>0.4	>0.4	0.05	0.005
C.albicans (9)*	clinical isolates	>0.4	>0.4	0.19±0.17 (>0.4–0.05)^r	0.20±0.14 (0.4–0.125)^r	0.33±0.21 (>0.4–0.1)^r	>0.4	>0.4	>0.4	0.0389±0.051 (0.1–0.0015)^r	0.00311±0.00267 (0.008–0.00015)^r
C.parapsilosis	ATCC 22019	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	0.1	>0.016
C. parapsilosis (4)*	clinical isolates	>0.4	>0.4	0.53±0.27 (>0.4–0.2)^r	0.56±0.24 (>0.4–0.2)^r	0.56 ±0.24 (>0.4–0.2)^r	0.4	>0.4	>0.4	0.1167±0.068 (>0.1–0.1)^r	0.02867 ±0.01453 (>0.016–0.001)^r
C.glabrata	ATCC 90050	>0.4	>0.4	>0.4	>0.4	0.4	>0.4	>0.4	>0.4	0.05	0.001
C.glabrata (1)*	clinical isolate	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.4	>0.1	<0.0005
I.orientalis	ATCC 6258	>0.4	>0.4	0.4	0.2	0.4	>0.4	>0.4	>0.4	0.0125	0.002
C. krusei (2)*	clinical isolate	>0.4	>0.4	0.33±0.09 (0.4–0.2)^r	0.36±0.09 (0.4–0.2)^r	0.3 ±0.09 (0.4–0.2)^r	>0.4	>0.4	>0.4	0.1416±0.173 (>0.5–0.0125)^r	0.04875 ±0.02011 (0.08–0.025)^r
C.tropicalis (2)*	clinical isolate	>0.4	>0.4	0.46±0.33 (>0.4–0.1)^r	0.63±0.05 (>0.4–0.2)^r	0.66±0.18 (>0.4–0.4)^r	>0.4	>0.4	>0.4	0.0412±0.032 (0.1–0.006)^r	0.01472±0.01420 (>0.016-<0.0015)^r

* Number of strains.
r – range.
** Structural formula 

It appears that the antifungal activity of the compounds is associated with the different distribution of the electronic density at the aromatic ring, rather than with the number of conjugated double bonds *per se*, since the chlorosubstituted analogue was insignificantly less active than the nitroderivatives.

Increasing the number of double bonds by derivatisation of the aliphatic chain decreased the anticandidial effect and in fact only the carbonyl derivatives were active.

The UV spectra of the compounds in ergosterol solution indicated that only the 3-nitrophenylbut-3-en-2-one, the 4-dimethylaminophenylbut-3-en-2-one (Fig. 1.) and the oxime of the 4-nitrophenylbut-3-en-2-one (decrease of the specific absorption maxima of ergosterol at 261, 271, 281 or 295 nm and amphotericin B (decrease in the ratio of absorption of the characteristic peaks of amphotericin B – Fig. 2.) interacted under these conditions. There was no correlation between the anticandidial effect of the phenylbutene derivatives and their behaviour in ergosterol mixtures, which makes unlikely our initial hypothesis that they act similarly to polyenes.

There are data that nitro- and chlorosubstituted phenyls are substrates for mammalian CYP 450 and beta unsaturated ketones are substrates for mammalian glutathione-S-transferases (Boobis *et al.*, 1989). Assuming that *Candida* has biotransformation enzymes with analogous substrate specificities, it may be suggested that the toxicity for fungi of the nitro- and chlorosubstituted phenylbut-3-en-2-ones is produced by mechanisms, similar to those of cellular toxicity. Investigations to verify this hypothesis have started and will be reported in a separate article.

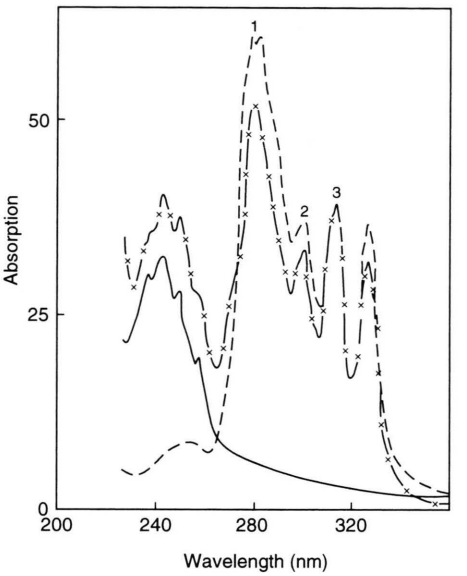


Fig. 2. Interaction of amphotericin B with ergosterol is indicated by the decrease in the ratio of the absorption of peaks 1/3 of amphotericin B (by the method described by Norman *et al.*, 1972). (———) – ergosterol (0.2 mM) alone; (-------) – amphotericin B (0.05 mM) alone; (---x---x--) – mixture of ergosterol (0.2 mM) and amphotericin B (0.05 mM).

The nitro- and chlorophenylbut-3-en-2-ones have an activity (in mg/l) similar to that of ketoconazole, but are less active than amphotericin B. However, the LD_{50} for mice of the 3- and 4-nitrophenylbut-3-en-2-ones (398 and 367 mg/kg after

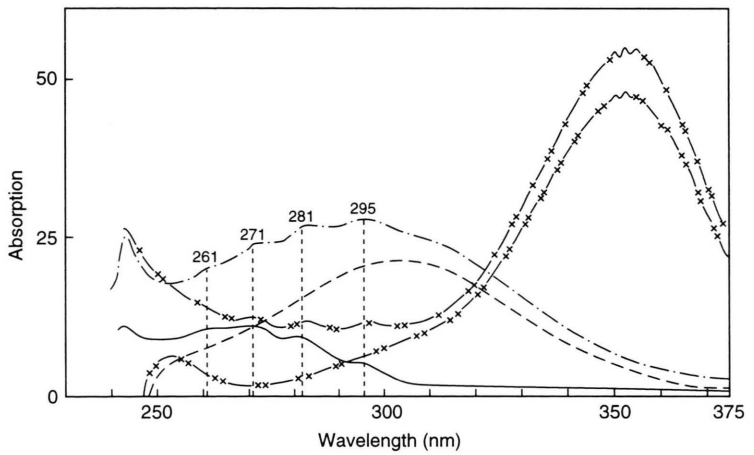


Fig. 1. UV absorption spectra of ergosterol 0.05 mM(———), 4-nitrophenylbut-2-en-3-one alone 0.2 mM (---xx---xx-----), ergosterol (0.05 mM) in mixture with 4-nitrophenylbut-2-en-3-one (0.20 mM) (---x---xx---x--) The UV absorption of the mixture is equal to the sum of the absorption of the components and indicates no interaction between the components. 4-dimethylaminophenylbut-2-en-3-one (0.2 mM) (-----) interacts with ergosterol (0.05 mM), as indicated by the decrease at one of the specific maxima for ergosterol at 295 nm in the absorption spectrum of the mixture (-·-·-).

intraperitoneal application, respectively) is considerably lower than that for amphotericin B (6 mg/kg (Reiner, 1982)). The fact that they are also effective against bacteria may make them useful in mucocutaneal candidiasis or mixed infections.

Experimental

Compounds: The structural formulae of the compounds are given in Table I and the synthesis and identification was carried out as described previously (Tabakova *et al.*, 1994). Ketoconazole and amphotericin B were commercial products. Standard *Candida* strains are listed in Table I and clinical isolates were from adult patients with a mucocutaneal form of the disease. The strains were characterised to subspecies by their ability to metabolise carbohydrates, as described by Baker (1967).

The growth-inhibiting effect of the phenylbutene derivatives has been assessed *in vitro* by the minimal inhibitory concentration (MIC) of the compounds, determined by the microtitre broth dilution method (Radetsky *et al.*, 1986) in Sabouraud broth with 1% glucose. MICs are equal to the lowest concentration of the compound, at which no visually detectable growth occurred after a 48-h incubation at 30 °C. In brief, the compounds were dissolved in a small volume of ethanol and these solutions were further diluted with the respective nutrient medium, the concentration of ethanol being less than 1% in the final dilutions. 0.1 ml aliquots of the drug-containing media were dispatched in the wells. Inoculation was carried out by adding to each of the wells 0.1 ml suspension of the respective *Candida* strain, containing $0.5-1x10^5$ culture forming units(cfu)/ml. The suspensions were prepared by diluting overnight cultures of the yeast-like fungi to an optical density of 0.125 (0.5 McFarlan standard) by adding sterile medium and subsequent 10-fold dilution.

Effect on germ-tube formation: Candida albicans strains were inoculated ($1x10^6$ cfu/ml) in TC 199 medium (Difco) containing different concentrations of the drugs. After 4–6 h of incubation at 30 °C the presence of germ-tubes was determined microscopically and recorded.

Interaction with ergosterol was studied as described by Norman *et al.* (1972). Solutions of ergosterol, the phenylbutene analogues and mixtures of ergosterol and the respective phenylbutene derivatives were prepared in 0.1 ml dimethylformamide (DMF), diluted to 2 ml with distilled water and were left to stand for 30 min. The UV spectra were recorded on a Beckman DU-600 and were compared.

Acute toxicity of the compounds was determined after intraperitoneal application for mice, using the LD_{50} criterion.

Acknowledgements

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