Aspects of the effect of bile salts on *Candida albicans*

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Cholic acid, chenodeoxycholic acid, deoxycholic acid, glycocholic acid, glycodeoxycholic acid, hyodeoxycholic acid and lithocholic acid as their sodium salts, were fungistatic to the growth of *Candida albicans*. Of the compounds tested, cholic acid, deoxycholic acid and chenodeoxycholic acid were the most active. In combination with other antifungal agents only cholic acid exhibited synergism with amphotericin B, whilst the imidazole antifungal agents inhibited the action of the bile salts. The bile salt minimal inhibitory concentrations were close to the critical micelle concentrations. Even though the compounds are surface active they did not cause loss of intracellular K⁺ and were without effect on oxygen consumption. The bile salts, particularly cholic acid, produced morphological changes that gave rise to swollen cells.

The inherent antibacterial activity of the bile salts has been used in selective media to facilitate the isolation of specific bacteria from mixed cultures [15, 27]. A combination of ox bile and crystal violet has been used to limit the surface spreading of growing fungal colonies [22]. Structurally related to the bile salts, several androgenic and oestrogenic steroid hormones have been shown to stimulate the growth of microorganisms [13, 20] and structural requirements for this growth stimulation have been proposed [3, 20]. Conversely, however, the inhibition of microbial growth by some steroids has been demonstrated. Deoxycortisone, for example, inhibits the growth of both Gram-negative and Gram-positive bacteria and of several fungi [2]. Similarly prednisolone [19], hydrocortisone and corticosterone [14] have been shown to inhibit the growth of bacteria. This work investigates the effects of cholic acid and related compounds on *Candida albicans*.

METHODS

**Fungi and growth media**

*Candida albicans* A39, originally a clinical isolate, was obtained from The Boots Co. pic., Nottingham and *C. albicans* NCYC 597 for some comparative experiments.

Yeasts were maintained on medium (NM) containing (g/l): maltose 38, yeast extract (Lab M, Burnley, U.K.), 2-5; mycological peptone (Lab M), malt extract (Lab M), 2; and agar (Lab M), 18. A chemically defined medium (CDM) was used...
experimentally (unless specified otherwise) and contained (gl °): ammonium tartrate, 5; ammonium nitrate, 1; KH₂PO₄, 0-5; NaCl, 0-1; CaCl₂, 0-1; glucose, 100; biotin, 1 x 10⁻⁵; Tris, 1-2; and boric acid, 66 fig ammonium molybdate, 288 fig FeCl₃, 6H₂O, 1 ML mg; CuSO₄ 5H₂O, 444 fig- MnCl₂ 4H₂O, 89 fig and ZnCl₂, 2-2 mg. Glucose and ammonium tartrate were omitted to give a medium (CFM) without a carbon source and in this medium the concentrations of ammonium nitrate and potassium dihydrogen ortho-phosphate were each raised to 2 gl⁻¹. For testing the sensitivity of C. albicans to the steroids a nutrient agar (NA) was used containing (gl⁻¹) meat extract (Lab M), 10; sodium chloride, 5; and agar, 15.

**Chemicals**

The sodium salts of cholic acid, deoxycholic acid, glycocholic acid, glycodeloxycholic acid, chenodeoxycholic acid, lithocholic acid and hyodeoxycholic acid, amphotericin B, clotrimazole and miconazole were obtained from Sigma Chemicals Co. Ltd, Poole. Aqueous or ethanolic solutions of all these compounds were sterilized by membrane filtration. Other chemicals used were of analytical reagent grade, unless specified otherwise.

**Growth conditions**

Cells were grown in CDM at 30°C for 18 h at 140 rev.min⁻¹ in an orbital incubator (Gallenkamp) and 5 ml of this culture was used to inoculate 100 ml quantities of fresh CDM containing bile salts which were then incubated at 30°C and 140 rev.min⁻¹. Growth was monitored over 8 h by measuring the optical density of the suspensions at 420 nm. The suspensions were examined using light microscopy to determine if germination of the yeast cells had taken place.

**Zones of inhibition**

Ten ml of nutrient agar medium was overlaid with 5 ml of seeded agar and sterile filter paper discs (Whatman A.A, 0-5 cm) impregnated with steroids were placed on the surface of the dried NA plates. After 2 h pre-diffusion at 4°C the plates were incubated at 30°C for 18 to 24 h and any zones of inhibition measured. Alternatively, wells of 8 mm diameter were cut in seeded agar, and the solution of bile salt introduced. The zones of inhibition were measured after 18 h incubation at 37°C.

**Determination of minimum inhibitor concentrations (MICs)**

MICs were determined from doubling tube dilutions in CDM. The inoculum was 30 fil of a 6 h CDM broth culture. The MIC was determined after 24 h incubation at 30°C. An approximate minimum candidical concentration was obtained by subculturing a loopful from all tubes showing no growth to fresh CDM medium and incubating for 24 h at 30°C.

**Effect of steroids on culture viability**

After 18 h incubation C. albicans was resuspended in fresh CDM to give 10⁹ c.f.u. ml⁻¹. The appropriate steroid was added and the suspension incubated at 30°C (140 rev.min⁻¹). Samples were removed at intervals and viability estimated by the method of Miles & Misra [26]. The effect of the steroids on the viability of non-growing cells was examined by suspending C. albicans, (10⁵ c.f.u. ml⁻¹) in either CFM or peptone water containing steroids and maintained at 30°C. Viability was assessed as previously. All systems were studied over a 6 h period and microscopic examinations made at hourly intervals.
Determination of critical micelle concentrations (CMC) of the steroids

The CMCs were determined by dye solubilization and by surface tension measurement. (a) The solubilization of Sudan Black by solutions of bile salts in CMD was studied by adding 10 mg of Sudan Black to tubes of bile salt solutions. The tubes agitated for 2 h at 20°C. The mixtures were centrifuged at 3750 g for 10 min to remove undissolved dye after which the absorbance of the supernatant liquids was measured at 620 nm; (b) The surface tension of a series of solutions of bile salts at 20°C was measured with a Du Nouy tensiometer (White Instrument Co. Ltd.).

Scanning electron microscopy (SEM)

Cells were prepared for SEM by the method of Bulman & Stretton [4]. Samples were coated with 10 nm of gold and viewed in an Alpha 9 SEM (International Scientific Instruments) with a beam angle of 45° and accelerating voltage of 15 kV. Photographs were taken on Ilford FP4 film.

Measurement of oxygen utilization

*C. albicans* was grown for 24 h in CDM at 30°C (140 rev.min⁻¹) then harvested by centrifugation at 1600 g for 20 min, washed twice with 0-01 M phosphate buffer pH 7 and resuspended in 50 ml of CFM per 100 ml of original culture volume. To reduce endogenous respiration, air was bubbled through the suspension for 3 h at 30°C. The cells were then washed and resuspended in CFM at a concentration of 3-75 mg dry weight ml⁻¹. Two ml of this suspension was added to 2 ml of CFM containing the required concentration of bile salt in the chamber of an oxygen electrode (Rank Bros., Bottisham). The suspension was stirred throughout and maintained at 30°C.

Estimation of K⁺-release

*C. albicans* was suspended at 1-0 mg dry weight ml⁻¹ in 20 ml of 0-03 M-tris-HCl buffer (pH 7-5) at room temperature (20°C) and stirred at constant speed with a magnetic flea. Potassium was estimated with a K⁺-sensitive electrode (Russel pH Ltd, Fife, Scotland). The electrode was immersed in the suspension until a steady rate of K⁺ release was obtained, then the cholic acid salt or amphotericin B was added and the K⁺ release followed for 20 min.

Synergism and antagonism

The effect of possible synergists on the action of bile salts was examined using the method reviewed by Maccacaro [23], One filter paper strip soaked in a 1% solution of steroid and another at right angles, impregnated with a 500 µg/ml solution of a possibly synergistic antifungal agent, were placed on solidified CDM seeded with *C. albicans*. Control strips soaked in water were used to ensure that the filter paper was not inhibitory and to quantify any 'wash out' effects. Also, the checkerboard method [18] was used to examine further any combination of compounds that exhibited either synergism or antagonism.

Proportion of yeast and mycelial forms

Ten ml portions of cell suspension in CDM or CFM were agitated with a Vortex mixer to break up clumps of cells. The total dry weight in this sample was obtained by filtering the suspension through pre-weighed glass microfibre filters (Whatman GF/F), washing the residue with several volumes of sterile distilled water and drying it to constant weight with an infra-red lamp. Another 10 ml sample was passed through a 20 µm metallic filter (Endecote Ltd) to retain the filamentous cells. The retained cells
were washed three times with distilled water and the filtrate and washings (yeast cells) were filtered through pre-weighed glass microfibre filters and dried to constant weight.

**RESULTS**

The compounds studied were apparently fungistatic having MIC's (tube dilution) which ranged from 0-06 to 20 mgml⁻¹ (Table 1). Deoxycholic acid was the most active usable compound, with an MIC of 1-3 mgml⁻¹. However, it did not give rise to the largest zone of inhibition in agar diffusion tests. This may reflect differences in diffusion coefficients between individual bile salts. All the bile salts had little effect on the growth rate of cultures of *C. albicans* A39 over the concentration range 1-10 mgl⁻¹. As the concentration of cholic acid and glycocolcholic acid was increased from 1 to 10 g1⁻¹ there was a progressive decrease in the growth rate (Fig. 1). Deoxycholic acid was also inhibitory, but optical density measurements were difficult because of precipitation of the compound after 3 h at 30°C. Direct microscopic counts showed that with deoxycholic acid at 3 gl⁻¹ there was no increase in cell numbers. Viable counts showed that the compounds were inactive against cells suspended in CFM but reduced viable numbers when the cells were in a nutrient medium (Fig. 2). Microscopic examination showed that for both OD measurements and viable counts, germination of the yeast cells with production of hyphae was not a significant problem. However, *C. albicans* NCYC 597 was the most suitable isolate for these studies, because it grew in the yeast form with separate cells, whereas *C. albicans* A39 gave results with a similar trend but the picture was complicated by its tendency to form hyphae.

<table>
<thead>
<tr>
<th>Compound, as sodium salt</th>
<th>Minimum fungistatic concentration (g1⁻¹)</th>
<th>Minimum fungicidal concentration (g1⁻¹)</th>
<th><em>Diameter of zone of inhibition (mm)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenodeoxycholic acid</td>
<td>0-06</td>
<td>&gt;2</td>
<td>14-7</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>0-5</td>
<td>&gt;2</td>
<td>13-2</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>0-125</td>
<td>0-6</td>
<td>9-1</td>
</tr>
<tr>
<td>Glycocholic acid</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>12-6</td>
</tr>
<tr>
<td>Glycodeoxycholic acid</td>
<td>1</td>
<td>&gt;2</td>
<td>6-5</td>
</tr>
<tr>
<td>Hyocholic acid</td>
<td>1</td>
<td>&gt;2</td>
<td>9-5</td>
</tr>
<tr>
<td>Hyodeoxycholic acid</td>
<td>0-5</td>
<td>&gt;2</td>
<td>6-5</td>
</tr>
<tr>
<td>Lithocholic acid</td>
<td>1</td>
<td>&gt;2</td>
<td>6-5</td>
</tr>
</tbody>
</table>

None of the compounds examined could be utilized as the sole source of carbon by *C. albicans* in an otherwise carbon-free medium. Even at low concentrations in medium CDM none of the bile salts stimulated growth.

Only one drug-steroid combination showed synergism, this was with amphotericin B and the sodium salt of cholic acid (Fig. 3). In several cases the drug antagonized the inhibitory effect of the steroid (Fig. 4) but there was no effect on the activity of the drug. Clotrimazole antagonized the inhibitory action of deoxycholic acid and hyocholic acid, amphotericin B antagonized the action of hyodeoxycholic acid, and miconazole and amphotericin B both antagonized the action of hyocholic acid (Table 2). When cells were incubated in the presence of the bile salts there was little loss of intracellular K⁺ when amphotericin B was added. Also, growth in the presence of or contact with the bile salts did not affect oxygen consumption significantly.

The CMC values determined by both the Du Nouy tensiometer and the dye solubilization methods were of the same order. For cholic acid and for deoxycholic acid the CMC value was 2-25 mgml⁻¹ and for glycocolcholic acid 2-5 mgml⁻¹.
BILE SALTS EFFECTS ON C. ALBICANS

FIG. 1. Growth of C. albicans A39 in CDM, pH 6.5 at 30°C and 140 rev.min⁻¹, estimated by optical density at 420 nm. • — • control; O — o in the presence of (3 gl⁻¹) cholic acid, as sodium salt; • A in the presence of (4 gl⁻¹) cholic acid, as sodium salt.

FIG. 2. Effect of bile salts on the viability of C. albicans CYC 597 suspended in CDM, CFM or peptone water at 30°C and 140 rev.min⁻¹. A A in GFM or peptone water, either alone or in the presence of cholic acid (0.5 gl⁻¹) as sodium salts. O — o in CDM with cholic acid (2.5 gl⁻¹) as sodium salt. • • in CDM with deoxycholic acid (1.3 gl⁻¹) as sodium salt. • • in CDM with deoxycholic acid (2.5 gl⁻¹) as sodium salt.

There was an increase in the percentage of cells present in the yeast form with increasing concentrations of all steroids studied, the effect being most pronounced when glycocholic acid was used at 3 gl⁻¹. In this case, 90% of the cell mass was in the
yeast form (Fig. 5). When *C. albicans* was grown in the absence of the bile salts, both yeast and mycelial forms were present (Fig. 6).

Cells that were grown in the presence of concentrations of sodium cholate higher than 1 g l⁻¹ for 6 h showed morphological changes; namely, incomplete separation and the presence of some swollen cells (Fig. 7). When deoxycholic acid was added to the growth medium little morphological change was seen in the 6 h period.

Chenodeoxycholic acid in the growth medium gave elongated yeast forms or short hyphae and a few large spherical cells (Fig. 8). Lithocholic acid gave rise to swollen
TABLE 2. Synergism and antagonism between the bile salts and anti-fungal agents, using NA plates seeded with *C. albicans*, filter paper strips soaked in steroid solutions 10 gl−1 or in drug solution (500 gl−1)

<table>
<thead>
<tr>
<th>Bile Salt</th>
<th>Amphotericin B</th>
<th>Clotrimazole</th>
<th>Miconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenodeoxycholic acid salt</td>
<td>N</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>Cholic acid salt</td>
<td>S</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Deoxycholic acid salt</td>
<td>N</td>
<td>A</td>
<td>N</td>
</tr>
<tr>
<td>Glycocholic acid salt</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<tr>
<td>Glycodeoxycholic acid salt</td>
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<td>Hyocholic acid salt</td>
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<tr>
<td>Hyodeoxycholic acid salt</td>
<td>A</td>
<td>N</td>
<td>N</td>
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<tr>
<td>Lithocholic acid salt</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

N, no effect
A, drug antagonizes action of steroid
S, synergism

FIG. 5. The percentage of yeast form of *C. albicans* A39 after (a) 6 h and (b) 24 h incubation at 30°C, and collapsed cells (Fig. 9); all other compounds had no obvious effect on morphology save for the shift to the yeast form.

DISCUSSION

The bile salts used in this study have many of the structural features required for growth stimulation [3, 20] rather than for inhibition. These include a side chain at C-17, a hydroxyl group at C-3 and methyl groups at C-10 and C-13. However, no stimulation of growth was observed. Indeed, all the bile salts examined in this study inhibit the growth of *C. albicans* and are fungistatic rather than rapidly fungicidal. The preferred features favouring inhibition seem to be a 3a and a 12-a hydroxyl group, and a carboxylic acid present in a side chain at C-17. There was no inhibition of general metabolism and even at high concentrations they did not affect the uptake of oxygen.

Cholic acid was synergistic with amphotericin B. Low concentrations of amphotericin-
FIG. 6. Scanning electron micrograph of *C. albicans* A39 grown for 6 h in CDM at 30°C, 140 rev.min⁻¹ x900.

FIG. 7. Scanning electron micrograph of *C. albicans* grown in the presence of cholic acid salt (4 g l⁻¹) in CDM at 30°C, 140 rev.min⁻¹, of cells showing the incomplete separation of cells and the presence of cellular debris x 9000.
Amphotericin B may increase the membrane permeability to cholic acid allowing greater access to the sensitive site. Antagonism between antifungal agents has been shown with amphotericin B and clotrimazole [5], econazole [5, 11], ketoconazole [29] and miconazole [5, 28]. The antagonism with ketoconazole was considered to be due to depletion of ergosterol in *C. albicans*, caused by subinhibitory levels of ketoconazole and giving rise to polynye resistance [29]. Similarly altered membrane sterols may be responsible for resistance to the bile salts.

The bile salts possess surface active properties, shown by their ability to reduce surface tension and by dye solubilization. Such agents would be expected to exert an antimicrobial action by causing membrane damage with a consequent loss of intracellular material, similar to that seen with amphotericin B. However, as judged by the loss of intracellular K⁺, this is not the case. The CMC value of the bile salts is close to the MIC value and it is possible that the bile salts could effectively remove a vital medium component e.g. biotin. However, the observation that the presence of sodium dodecyl sulphate has no influence on cell growth and that surface active agents, in low concentration, generally stimulate growth [24] suggests that this is unlikely to be a primary lethal factor. Also, it would seem unlikely, in view of a lack of antimicrobial action of non-ionic surface active agents [24], that essential surface components are being removed into micelles or that a surface coating is interfering with normal exchange mechanisms. The monomer is often the important molecular species in antimicrobial action and a relationship between CMC and antimicrobial activity was seen with an homologous series of primary aliphatic amines where toxicity fell off after the *Cₙ* member because each higher homologue contributed fewer and fewer
FIG. 9. Scanning electron micrograph of C. albicans A39 grown in the presence of lithocholic acid salt (2 g/l) for 6 h in CDM at 30°C, 140 rev/min, showing the presence of some large cells with wrinkled surfaces x 1800.

molecules of monomer even in moderately dilute solutions [12, 16]. The morphological changes observed in individual cells grown in the presence of cholic acid sodium salt were similar to those produced by imidazole antifungal agents. Econazole, fenicozole, isoconazole and miconazole all caused convolutions and wrinkles in the cell wall [6]. The folds in the cell walls of miconazole-treated cells may correspond to focal areas where membranous material had been deposited between the cell wall and the plasmalemma [8, 10]. Another feature of the action of miconazole is that chains of interconnected cells were observed with pronounced necks [9] and this was also observed with cholic acid-treated cells (Fig. 7). This probably results from an inability of the cells to divide properly rather than to the formation of yeasts from segments of the mycelium. A further point of similarity is that, like low concentrations of the imidazoles [2], the bile salts promote formation of the yeast form of C. albicans. The phenomena were not produced by growth of C. albicans in the presence of sodium dodecyl sulphate, either above or below the CMC, and so are not merely the result of a lowering of surface tension. The morphological changes induced by cholic acid salt resemble the changes brought about by miconazole [9, 10] even to the extent of causing cell lysis. Miconazole affects ergosterol biosynthesis in C. albicans [30], and altering membrane permeability by altering the internal viscosity and molecular motion of the lipids [7].

Incubation of C. albicans with low concentrations of miconazole causes an accumulation of sterols with a methyl group at C-14 and so the drug inhibits demethylation at C-14. Morphological changes observed in cultures grown in the presence of bile salts may reflect an interaction at an important step in sterol biosynthesis or by competitive insertion in the membrane. Alternatively, the compounds may act on membrane-bound enzymes involved in cell wall biosynthesis and so affect cell size and shape.
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REFERENCES


