

Alterations Produced by Sertaconazole on the Morphology and Ultrastructure of *Candida albicans*

Sertaconazol-bedingte Veränderungen der Morphologie und Ultrastruktur von *Candida albicans*

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Summary: We studied the changes produced in *Candida albicans* yeast cells after treatment in vitro with sertaconazole, with light microscopy, transmission and scanning electron microscopy. Four different concentrations were evaluated (10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M) and two times of action (12 and 17 h) for each concentration. This new antimycotic agent shows a considerable destructive effect in *C. albicans* cells. The alterations produced by sertaconazole were already manifested after 12 h at the concentration of 10^{-6} M and a strong fungicidal activity recorded at 10^{-4} M.

Zusammenfassung: Es wurden die Sertaconazol-bedingten Veränderungen an der Hefephase von *Candida albicans* in vitro lichtmikroskopisch, raster- und transmissionselektronenmikroskopisch bei Einwirkung von vier unterschiedlichen Konzentrationen (10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} M) und zwei verschiedenen Einwirkungszeiten (12 und 17 h) untersucht. Dieses neue Antimyzetikum zeigt einen erheblichen Zerstörungseffekt auf *C. albicans*-Zellen. Die Veränderungen waren bereits 12 h nach Ein-

wirkung von 10^{-6} M Sertaconazol manifest. Fungizide Aktivität wurde bei 10^{-4} M Sertaconazol-Einwirkung beobachtet.

Introduction

The imidazoles are antimicrobial agents of broad spectrum; they basically act at the plasmalemma level inhibiting the synthesis of ergosterol (23). With the exception of gramnegative bacteria all the rest of human pathogenic microorganisms are affected by the imidazole by-products (8). Some of the morphofunctional alterations produced by the imidazoles for a number of cellular compounds are known, but new studies are required to achieve a better knowledge of their action. *Candida albicans* is one of the most common pathogenic yeasts and has been used as a model to study the mechanisms of action of many antimicrobial agents including the imidazoles (1, 2, 3, 6, 9, 10, 11, 12, 13, 14, 21). However, the differences between the particular activity of each of them are in some cases blatant, e.g. De Nollin & Borgers (1976) (11) indicated

remarkable differences between the action of clotrimazole and miconazole. Sertaconazole (a derivative of benzo-[b]-thiophene) is a new antifungal agent synthesised by the Research Center "Grupo Ferrer" (16). The activity of this product in vitro has been evaluated for several fungi, including *C. albicans* (20), although their ultrastructural effect is unknown. The present work studies the effects of sertaconazole, at different concentrations and times, on the morphology of the yeast phase of *C. albicans*, using light microscopy and electron microscopy techniques.

Material and Methods

A purified preparate of sertaconazole nitrate (MW = 500.78) was provided by Ferrer International, Spain. 20 mg of this substance was dissolved in 1 ml of N,N-dimethylformamide (DMF) and further diluted up to 10 ml with distilled water. The *Candida albicans* strain used (ATCC 10231) was cultured on Sabouraud glucose agar at 25°C. A loopful of *C. albicans* was transferred to an Erlenmeyer flask containing 100 ml of the following medium: yeast extract 3 g/l, peptone 5 g/l and glucose 10 g/l (pH 6.5). After 16 h of incubation at 37°C (70 % T at 546 nm), 25 ml of this suspension (containing ca. 2.5×10^6 cells/ml) were transferred to 4 Erlenmeyer flasks, each of which contained 1000 ml of the same culture medium and the following concentrations of sertaconazole: 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M respectively. They were cultured under constant stirring

at 37°C. From each of them 300 ml were sampled after 12 and 17 h of incubation. The samples were centrifuged at 3500 rpm for 10 min. The pellets were prepared for observation with the light (Nomarski) and electron (transmission and scanning) microscopes. Control cultures containing equal amounts of solvent were incubated for the same times.

Interference contrast microscopy

For the observation of the samples using this technique a Leitz Dialux 20 microscope with a Nomarski dispositive was used. The cells were spread on a microscope slide and examined without staining.

Transmission electron microscopy

The fixative used was 2 % glutaraldehyde in 0.1 M phosphate buffer adjusted to pH 7.4 for 2–20 h. The material was rinsed with several changes of buffer and post-fixed with 1 % buffered OsO_4 for 1 h. Both fixations were carried out at 5°C. All material was dehydrated in ethanol series and embedded in Spurr's (18) epoxy resin. Ultra-thin sections were stained with 1 % uranyl acetate and poststained with Reynolds's (17) lead citrate. Sections were examined with a Zeiss EM 10C microscope.

Scanning electron microscopy

The specimens were prepared according to the conventional technique, described in a previous paper (4).

Figures 1-7: *Candida albicans*. Control and specimens treated with sertaconazole (Interference contrast microscopy) x 2032

Figure 1: Control

Figure 2: Concentration 10^{-6} M (12 h)

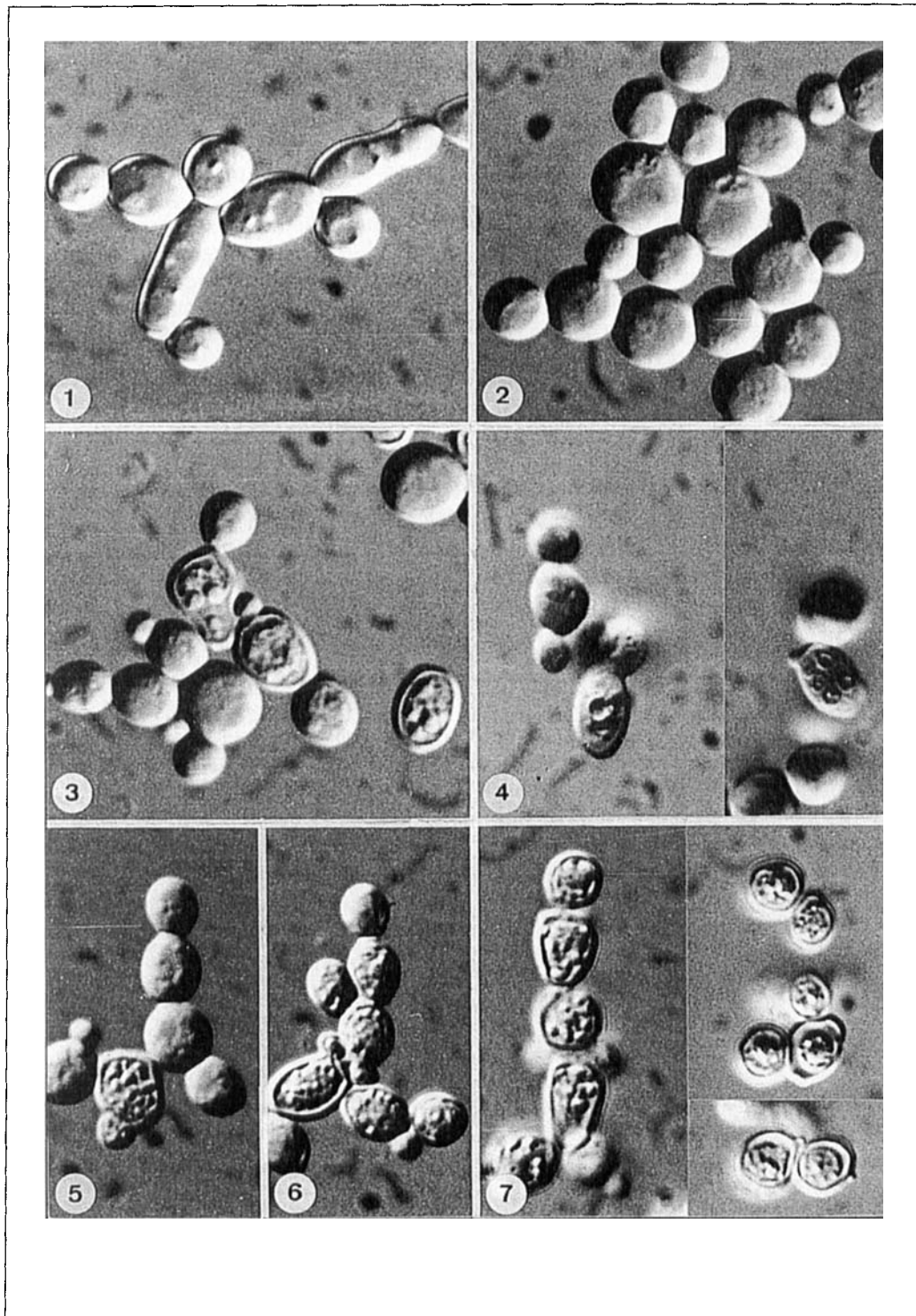
Figure 3: Concentration 10^{-5} M (12 h)

Figure 4: Concentration 10^{-5} M (17 h)

Figure 5: Concentration 10^{-4} M (12 h)

Figure 6: Concentration 10^{-4} M (17 h)

Figure 7: Concentration 10^{-3} M (17 h)



Results

Light microscopy (Interference contrast)

One of the first modifications, induced by the antimycotic, was the lack of capacity to produce pseudomycelium; the cells appear in small groups. In comparison the control specimens after 17 h of incubation carry on producing pseudomycelium.

The concentration of 10^{-6} M, showed cells that became more round shaped and swollen than the control ones, which were subglobose, or oval-shaped (Fig. 1). Some cells also exhibited a slightly convoluted surface (Fig. 2). No significant modifications in the morphology of the cells were observed at the two different times evaluated. At the concentration of 10^{-5} M most cells showed a remarkable deterioration in their external morphology, i.e. they lost their spherical form and swell, becoming more elongated and angular. Their cytoplasm presented big vacuoles (Figs. 3, 4). These changes were already visible after 12 h (Fig. 3) and they remained after 17 h (Fig. 4). The cells kept their capacity for budding, although the new cells probably did not reach maturity because they separated earlier from the mother cell. That is why so many small cells were observed. The samples exposed to an antifungal concentration of 10^{-4} M showed almost all of their cells deteriorated after 12 h. Most of these cells were smaller in size and showed great changes in their cytoplasm (Figs. 5, 6). Few

differences were observed between 12 and 17 h, because the cells after 12 h were already very degenerated. At this concentration we believe that sertaconazole possesses fungicidal properties because the number of cells that survived were much smaller than in the former concentrations. The concentration of 10^{-3} M possessed a strong fungicidal activity since very few cells grew and these were much altered in their external morphology as well as in their cytoplasmic content (Fig. 7). It is even possible that these cells are those of the inoculum.

Scanning electron microscopy

The control cells of *C. albicans* presented a smooth surface and a sub-spherical or oval shape, with a great capacity for producing blastospores (Fig. 8). At the concentration of 10^{-6} M after 12 h of exposition to the antifungal, the cells did not show any appreciable alteration and they kept on budding. Nevertheless, deep surface folds, or some collapsed cells, were detectable after 17 h (Fig. 9), although the cells went on dividing (note in Fig. 10 the scars of division in the mother cells and some gemmations). Occasionally some broken cells could be observed (Fig. 11). After 12 h the concentration of 10^{-5} M showed slightly damaged cells and others markedly deformed (Fig. 12). After 17 h a great number of deteriorated cells and others completely destroyed appeared, together with cellular debris (Fig. 13).

Figures 8-23: *Candida albicans*. Control and specimens treated with sertaconazole (Scanning electron microscopy)

Figure 8: Control (x 2397)

Figure 9: Concentration 10^{-6} M (17 h) (x 2111)

Figure 10: Concentration 10^{-6} M (17 h) (x 5127)

Figure 11: Concentration 10^{-6} M (17 h) (x 7842)

Figure 12: Concentration 10^{-5} M (12 h) (x 3058)

Figure 13: Concentration 10^{-5} M (17 h) (x 6635)

Figure 14: Concentration 10^{-4} M (12 h) (x 3164)

Figure 15: Concentration 10^{-4} M (12 h) (x 1518)

Figure 16: Concentration 10^{-4} M (12 h) (x 1154)

Figure 17: Concentration 10^{-4} M (17 h) (x 1942)

Figure 18: Concentration 10^{-4} M (17 h) (x 8828)

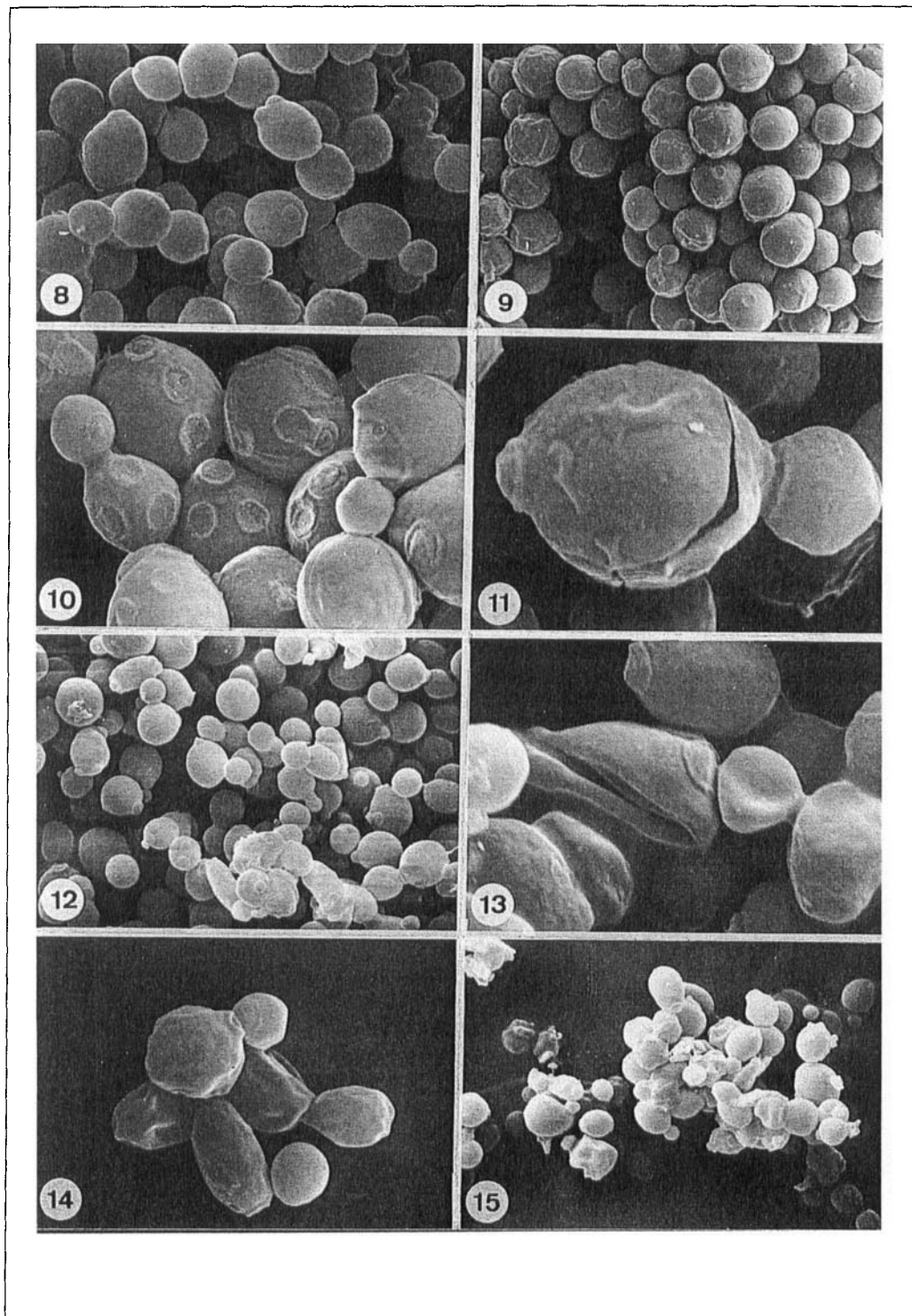
Figure 19: Concentration 10^{-3} M (12 h) (x 1481)

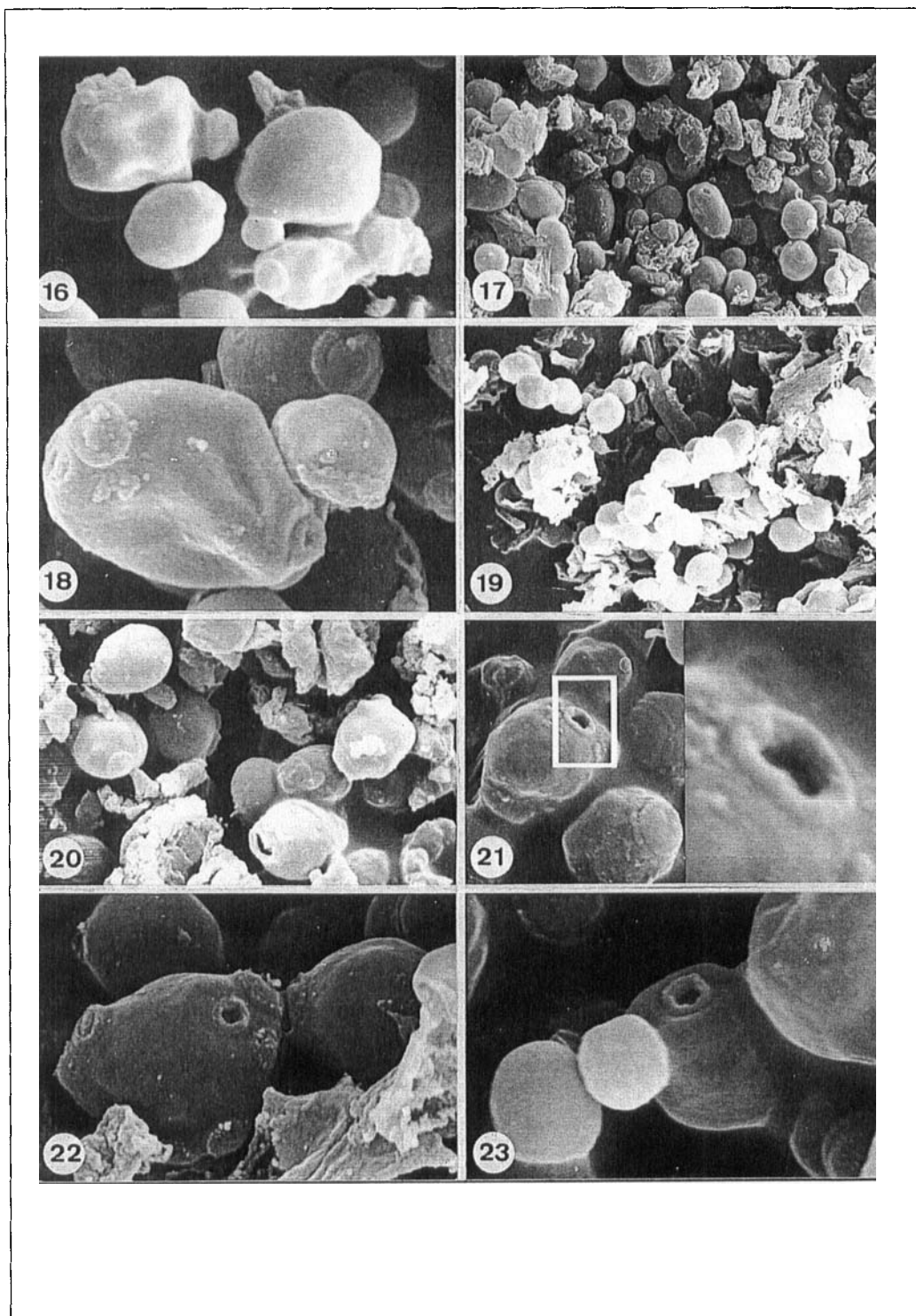
Figure 20: Concentration 10^{-3} M (17 h) (x 2984)

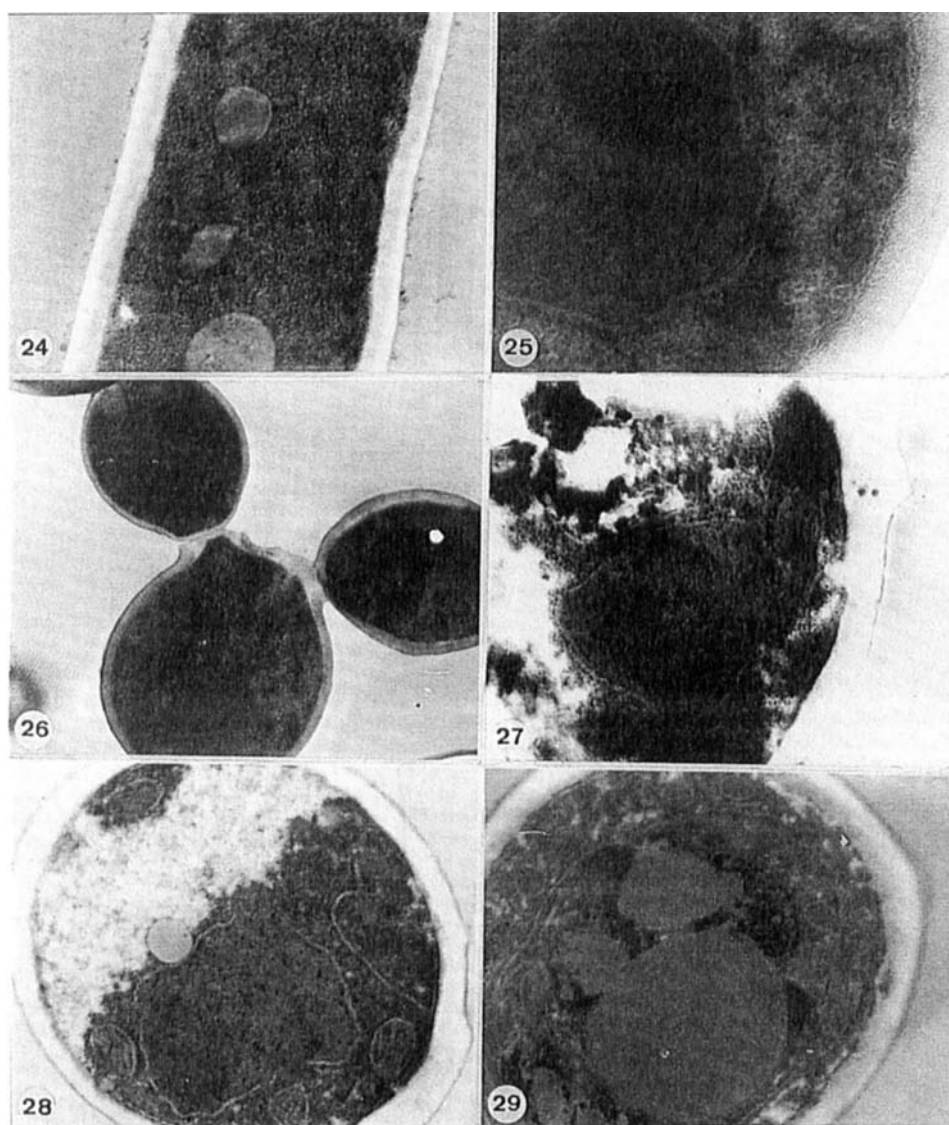
Figure 21: Concentration 10^{-3} M (17 h) (x 5591) (x 22364)

Figure 22: Concentration 10^{-3} M (17 h) (x 6473)

Figure 23: Concentration 10^{-3} M (17 h) (x 8828)







Figures 24-33: *Candida albicans*. Control and specimens treated with sertaconazole (Transmission electron microscopy)

Figure 24: Control (x 26670)

Figure 25: Control (x 42333)

Figure 26: Control (x 21166)

Figure 27: Concentration 10^{-6} M (12 h) (x 33866)

Figure 28: Concentration 10^{-6} M (17 h) (x 21166)

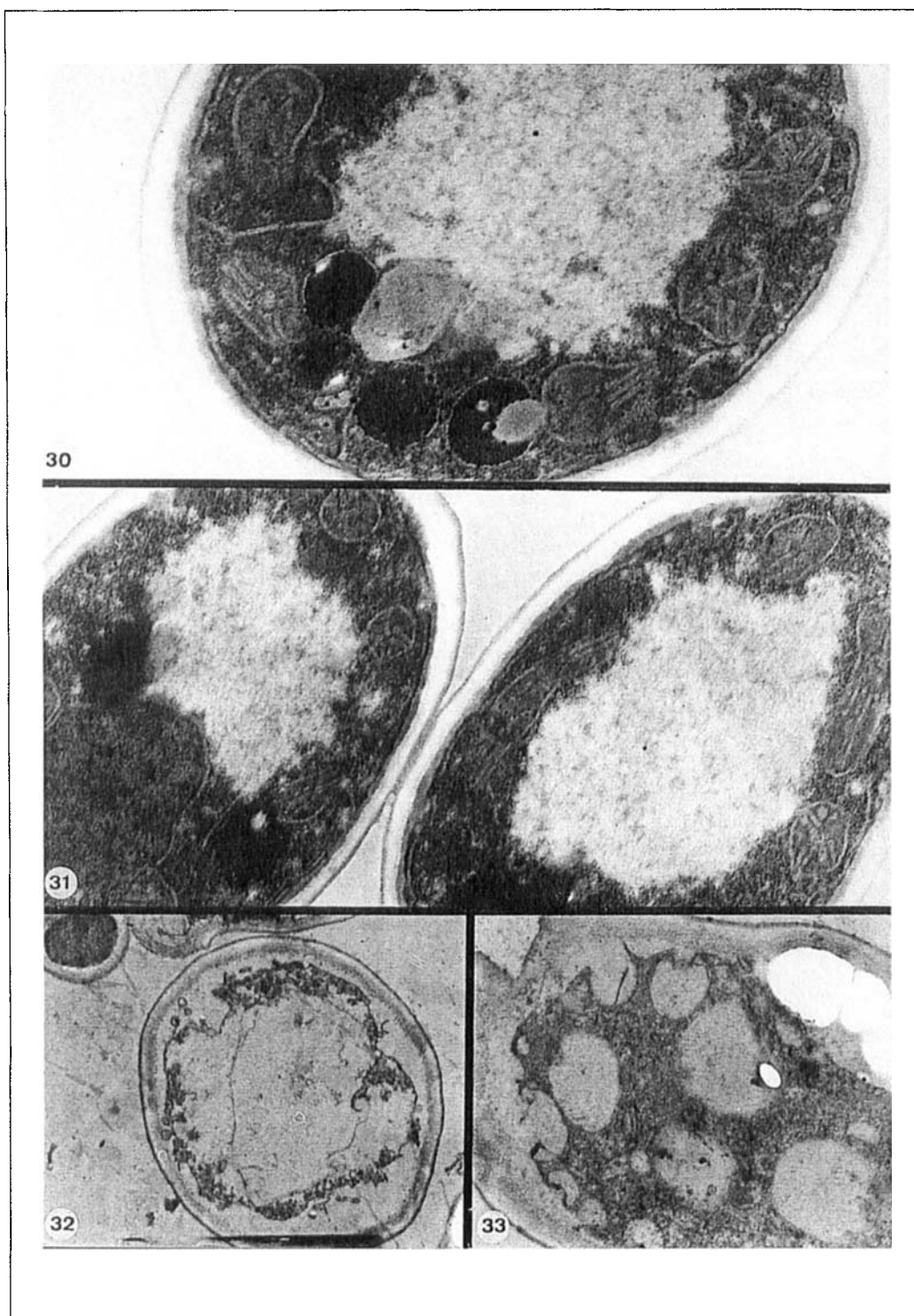
Figure 29: Concentration 10^{-5} M (12 h) (x 16933)

Figure 30: Concentration 10^{-5} M (17 h) (x 44775)

Figure 31: Concentration 10^{-5} M (17 h) (x 35820)

Figure 32: Concentration 10^{-4} M (12 h) (x 10176)

Figure 33: Concentration 10^{-4} M (12 h) (x 20352)



The cells exposed to a 10^{-4} M concentration were already very altered after 12 h. They were collapsed and showed deep surface folds. Some of them lost their sub-spherical morphology becoming polygonal in shape (Figs. 14–16). After 17 h the cells also showed clear degradations and appeared surrounded by cellular debris (Figs. 17, 18).

At the concentration of 10^{-3} M very few cells grew and almost all of them were destroyed. After 12 h we basically found some solitary very altered cells and cellular rests (Fig. 19). The same happened after 17 h (Figs. 20–23). The presence of holes in the gemmation scars is noteworthy. They are probably due to a defect in the regeneration of the new cell walls.

Transmission electron microscopy

The cells not exposed to sertaconazole showed a well preserved morphology during all the sampling periods and revealed essentially the same details of fine structure as found by previous authors. The hyphae, as well as the cells after 17 h of culture, showed an internally well-preserved ultra-structure (Figs. 24–26). The cell wall was well delimited and in intimate contact with the plasmalemma. The cytoplasmic content presented a high electron density as result of its high metabolic activity. The typical organelles of the eucariotic cells i.e. nucleus, nucleoli, mitochondria, endoplasmic reticulum, ribosomes etc. were observed.

At the concentration of 10^{-6} M after both 12 h and 17 h, normal cells were observed, together with others with remarkable cytoplasmic alterations. Such alterations were basically observed at the level of the plasmalemma, which was discontinuous (Fig. 27) and in the swelled and elongated mitochondria that possessed poorly defined cristae (Fig. 27).

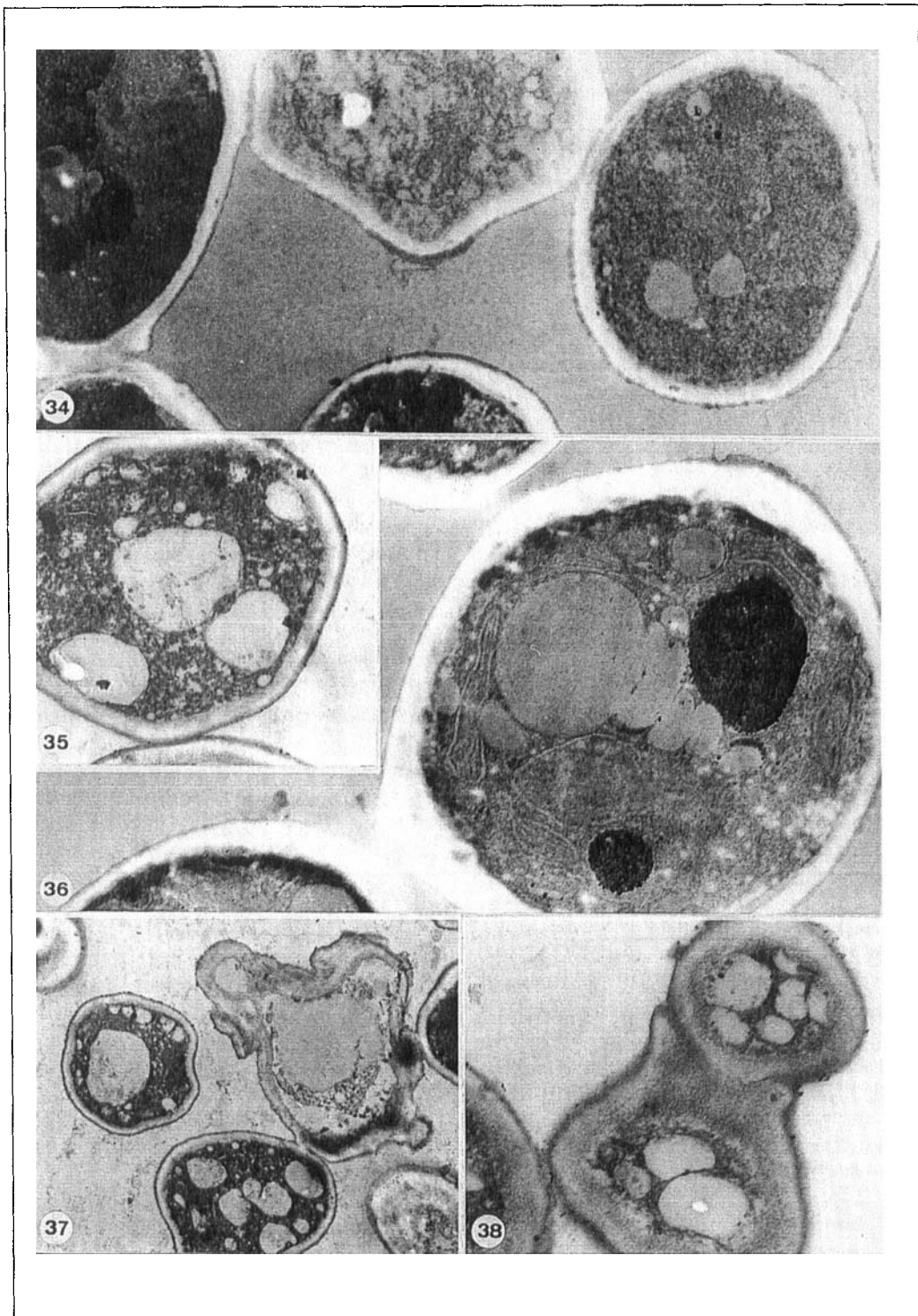
Other cells presented different cytoplasmic electron density zones, which could probably indicate an irregular distribution of the metabolic activity (Fig. 28). The

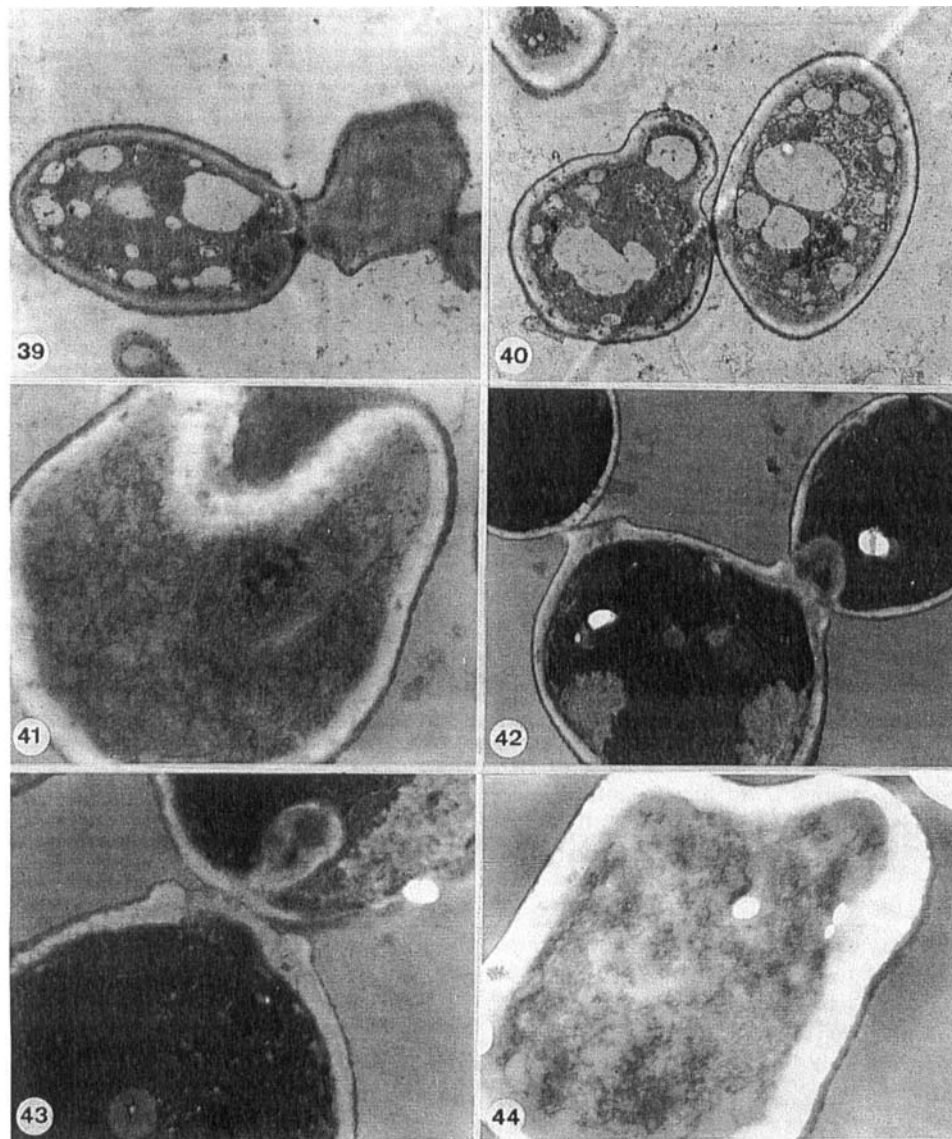
presence of electron-dense granules could be observed, especially between the membranes (i.e. mitochondrial cristae, plasmalemma, nuclear membranes etc.) as well as certain plasmolysis (Fig. 28). Despite all the internal changes mentioned the cells kept their typical external morphology.

At the concentration of 10^{-5} M, already after 12 h, the most striking feature was the presence of big lipidic vacuoles in more or less all the cells, that filled up most of the cytoplasm (Fig. 29). A great number of cells were already totally degraded and neither the plasmalemma nor the organelles could be identified, having also lost their external morphology. Even the apparently externally well – preserved cells showed internally big lysis zones (Figs. 30, 31) and disrupted mitochondria (Fig. 30).

After 12 h at 10^{-4} M, most of the cells showed a much altered cytoplasm. They lost their plasmalemma and it was difficult to recognize any organelles. Externally they were also deformed, having lost their typical morphology (Figs. 32–40). The few cells with a normal external morphology, showed a remarkable internal alteration, i.e. broken and discontinuous plasmalemma, deformed mitochondria and big vacuoles and plasmolysis of all cytoplasmic structures, etc. (Figs. 35, 36). Some cells maintained their budding capacity, but the daughter cells presented the same degenerations as the mother cells and were hardly viable (Figs. 37–40). After 17 h the same level of degradation was detected; most of the observed cells fitted the image illustrated in Fig. 41. A typical characteristic of the few cells that went on dividing was the deformation and invagination of the cell wall in the gemmation scars (Figs. 42, 43). This could be correlated with the holes observed in the same regions with the scanning electron microscope.

At the concentration of 10^{-3} M, as previously indicated, it was difficult to find cells and the few observed were morphologically altered externally as well as internally (Fig. 44).





Figures 34-44: *Candida albicans*. Control and specimens treated with sertaconazole (Transmission electron microscopy)

Figure 34: Concentration 10^{-4} M (12 h) (x 22826)

Figure 35: Concentration 10^{-4} M (12 h) (x 16601)

Figure 36: Concentration 10^{-4} M (12 h) (x 36522)

Figure 37: Concentration 10^{-4} M (12 h) (x 10375)

Figure 38: Concentration 10^{-4} M (12 h) (x 20751)

Figure 39: Concentration 10^{-4} M (12 h) (x 10375)

Figure 40: Concentration 10^{-4} M (12 h) (x 10375)

Figure 41: Concentration 10^{-4} M (17 h) (x 20751)

Figure 42: Concentration 10^{-4} M (17 h) (x 20751)

Figure 43: Concentration 10^{-4} M (17 h) (x 20751)

Figure 44: Concentration 10^{-3} M (12 h) (x 20751)

Discussion

From the results obtained we can indicate that sertaconazole has a considerable destructive action on the cells of *C. albicans*, which is already evident after 12 h in all the tested concentrations. Some fungicidal action at 10^{-4} M, being maximal at 10^{-3} M, was also observed. Torres-Rodriguez et al. (1986) (20) had previously demonstrated the strong antimycotic activity of sertaconazole in vitro on *C. albicans*, stronger even than that of miconazole.

The first changes observed at the low doses of sertaconazole is probably manifested at the level of the plasmamembrane, as already indicated by other authors using other imidazoles (9, 10, 11, 12). All these drugs produce changes on the permeability of the plasmalemma (7, 22) which induces modifications of the cells that become round-shaped and increase in size. Other internal changes such as the swellings of mitochondria and the accumulation of vesicles that also appeared at lower concentrations could not be externally detected. We did not observe the accumulation of membranes between the plasmalemma and the cell wall described by other authors in the same fungus with other imidazoles (11). The presence of dense granules between the membranes is worthy of comment. They were not reported in other works. At high concentration a breaking of the plasmamembrane was clearly observed, which induced a loss of elasticity of the cells and a destruction of its cytoskeleton adopting a polygonal morphology in a lot of cases. Internally a plasmolysis of all cytoplasmic structures occurred. Those important alterations started to appear at the concentration of 10^{-5} M and were presented by all the cells at the concentration of 10^{-4} M. At the concentration of 10^{-3} M, as we previously indicated, it was very difficult to find cells and the few observed were badly destroyed and probably corresponded to the inoculum.

It must be emphasized that the presence of holes in the surface of the cells exposed to

a concentration of the antimycotic of 10^{-3} M corresponds exactly with the gemmation scars. It is possible that the mother cells after having originated blastospores by budding could not form the second septum (1) due to a defect in the synthesis of some compounds of the cell wall. Through this hole the cell will lose its cytoplasmic contents and die. A similar phenomenon was described by Hopsu-Havu & Nevalainen (1979) (5) exposing *C. albicans* to the action of natamycin, and by Steinmetz et al. (1988) (19) exposing the same fungus to the action of rosemary essential oils and to eucalyptol.

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