CHAPTER IV

DISCUSSION

The antifungal drugs available in the present market have various mode of actions. Amphotericin B and other polyene antifungal agents such as nystatin and pimaricin are fungistatic or fungicidal, depending on the concentration of the drug and the sensivity of the fungus. Their mode of actions are at least in part dependent on their binding to a sterol moiety, primary ergosterol, present in the membrane of sensitive fungi causing pores or channels formation in cell membrane which increased the permeability of the membrane and allowing leakage of a variety of small molecules (Bastide *et al*., 1982; Sande and Mandell, 1985). Flucytosine or 5-fluorocytosine, the fluorinated pyrimidine is converted in fungal cells to 5-fluorouracil (5-FU) by the enzyme cytosine deaminase. 5-Fluorouracil is then metabolized to 5-fluorodeoxyuridylic acid. 5-FU is incorporated into RNA causing malfunction while 5-fluorodeoxyuridine monophosphate inhibits thymidylate synthetase enzyme (Wain and Polak, 1979 ; Sande and Mandell, 1985). Arai *et al*. (1977) ascribed the morphological changes induced by flucytosine to an inhibitor of DNA synthesis and Polak and Wain (1977, 1978, 1979) have shown that total DNA increase, hyphal nuclear division and thymidine incorporation into DNA are all inhibited by flucytosine. Polak and Wain (1978,1979) also showed the abnormal yeast cell shape (Arai *et al*., 1977; Polak and Wain, 1978) were due to abnormal, accelerated carbohydrate syn-

thesis. Griseofulvin is fungistatic in vitro for various species of the dermatophytes. Young actively metabolizing cell may be killed by the drug, but older,more dormant elements are only inhibited. A prominent morphological manifestation of the action of griseofulvin is the production of multinucleate cells as the drug inhibits its fungal mitosis. Griseofulvin causes disruption of the mitotic spindle by interacting with polymerized microtubules, its binding sites on the microtubular protein are distinct (Sande and Mandell, 1985; Gray et al., 1982). The imidazole derivatives are fungicidal if the concentration is sufficiently high. They act primarily at the cell membrane by inhibiting the incorporation of acetate into ergosterol, and they also inhibit lanosterol demethylase. The cell membrane is disorganization and alters its permeability. The uptake of essential nutrient is impaired (Sreedara Swamee,et al., 1974; Voigt, 1978; Van den Bossche et al., 1980; Pye and Marriott, 1982; Borger et al., 1983; Sande and Mandell, 1985). At the ultrastructure level, the imidazole derivatives cause structural alterations of cell wall (De Nollin et al.,1977 and Bastide et al., 1982) with abnormal membranous inclusions inside (De Nollin and Borgers, 1974 and Borgers et al., 1983). The intracytoplasmic organelles are disorganized and finally get plasmolysis. There are lipid droplets accumulation in necrotic cells (Iwata et al., 1973; De Nollin and Borgers, 1974; De Nollin et al., 1977; Voigt, 1978; Borger et al., 1983).

The undecanoic acid is primarily fungistatic activity may be observed with long exposure to high concentrations of the agent. The drug inhibits phosphatides in the fungus (Das and Banejaree, 1982).

Some antifungal drugs not available in the markets are under study of the mechanism of action such as aculeacin A and papulacandin B, the cell wall active antifungal agents (Bozzola et al., 1984), Naftifine, which interferes with fungal lipid metabolism and causes cell wall alterations (Meingassner et al., 1981) and Ambrutricin which inhibit ^{14}C uridine and ^{14}C leucine incorporation in fungus (Ringel, 1978).

The antifungal drugs described above are either antibiotics or synthetic substances but there are some antifungal active agents derived from the medicinal plants, such as Allicin, from *Allium sativum L.* (Yamada and Azuma, 1977), and Eugenol, from Clove oil (*Eugina caryophyllata Thunb.*) (Boonchird and Flegel, 1982). There is still no study about their mode of action to the fungus.

Tea seed cake (Ch'a-tzu-ping or Tae kow) is one of the medicinal plants with antifungal property. Tea seed cake extract (TK) was reported to act against some pathogenic fungi. (Lohakhajornpan, 1979; Laohapaibul et al., 1981 and Thongvichai, 1983). Tosukhovong et al. (unpublished data, 1984) tried to purify TK by column chromatography.

The thin layer chromatographic patterns (figure 3.1) showed that both light precipitate obtained from partial purification of TK (P) and TK were not pure substances. The light precipitate (P) was composed of at least 4 components while TK was composed of at least 5 components.

From the MIC/MFC determinations (modified Shadomy et al.)

TK had higher antifungal activity against *Candida albicans* ATCC 10231 and *Arthroderma benhamiae* JCM 01886 than the light precipitate (P) (Table 3.1, 3.2). It seemed that some antifungal active ingredients had lost during column chromatography. *A. benhamiae* JCM 01886, the filamentous fungi was more susceptible to the drugs, P, TK and miconazole than *C. albicans*, the yeast-like fungi when compared in the same media, SDA or SDB .

The MIC/MFC of the same drug against the same organism was different according to the kind of culture medium and its pH. This result agrees well with those from Sande and Mandell (1985) and Odds (1979) who stated that the in vitro testing of susceptibility of organisms were dependent upon factors such as culture medium, pH and the size of inoculum. The MIC of miconazole obtained from this study (12.5 µg/ml in YNB and buffered YNB and 3.13 µg/ml in SDB and SDA) was a little bit higher than that was previously reported (0.1-10 µg/ml) (Sande and Mandell, 1985; Odds, 1979; Mcginnis, 1980 and Shadomy et al., 1980), because of the heavier inoculum size used and that the miconazole used in this experiment might not be quite as pure. Furthermore, the method for determining the MIC of antifungal drugs are varied from drug to drug or even between the individual drug, by means of culture medium, pH and inoculum size (Odds, 1979; Shadomy et al., 1980; Mcginnis, 1980 and Holt, 1975), Sande and Mandell stated in 1985 that the results of susceptibility testing were difficult to interpret, and there were no firm indications for performance of this testing in vitro. In addition, the data was not useful for predicting clinical responses. However, the MIC/MFC still serves as the important para-

meter to determine the antifungal activity of drugs and the susceptibility of fungus strains. Therefore, the susceptibility testing of of antifungal drugs against certain organisms should be observed both in vivo and in vitro for reliable results. Any way, the MIC/MFC data clearly showed that TK had higher antifungal activity than P, thus TK was chosen to be further studied of the antifungal action.

The kinetics of inhibition of growth and killing of organisms illustrated that TK was both fungistatic and fungicidal to *Candida albicans* and *Arthroderma benhamiae* depending on the dosage used. The 200 µg/ml TK was fungicidal to *C. albicans* (inoculum size used: $2x10^6$ cells/ml) while it was fungistatic to *A. benhamiae* (inoculum size used; 3 mg dry weight/ml) but the 1,000 µg/ml TK was fungicidal to both of the organisms. The onset of action of TK was two hours for *C. albicans* and one day for *A. benhamiae*.

The light microscopic study illustrates that TK is fungicidal to both *C. albicans* and *A. benhamiae* because the dead cells are observed since the 2-hour incubation for *C. albicans* and since the 1-day incubation for *A. benhamiae*. The major sites of action should be the cell membrane and the cell wall of the fungi causing cellular content leakage in *C. albicans* and bulging mycelium in *A. benhamiae*. Finally, the *C. albicans* blastoconidia are broken with drastic cellular content liberation and only cell fragments remained while in *A. benhamiae* , the necrotic hypha are almost tattery and microconidial,macroconidial formation are inhibited.

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By scanning electron microscopy and transmission electron microscopy, TK appears to be fungicidal to both organisms and exerts its primary effect on the cell membrane and cell wall. The intracytoplasmic organelles are also involved and plasmolysis occurs. The extrusion of cellular contents and alteration in shape of the cells are probably due to an osmotic imbalance provoked by a drug-induced alteration in cell membrane permeability and the destruction of the cell wall itself. Moreover, focal disruption and partial dissolution to complete loss of the cell membrane were noted while the cell wall deformation was markedly observed in various stages from focal thickening and dark bands formation to cell wall rupture in the cells exposed to higher dose and longer incubation. The events of drug induced ultrastructural changes are difficult to interpret because the material is processed with conventional electron microscopic procedures, since it has not, so far been possible to obtain adequate detail morphology of untreated control cells for comparison.

TK also interferes with cellular metabolism of both organisms since it inhibits ^{3}H-thymidine monophosphate incorporarion into DNA and also inhibits ^{14}C-glucose uptake into the fungal carbohydrate. The inhibition of DNA biosynthesis may cause the defect in cellular reproduction, RNA biosynthesis or even protein synthesis in the fungal cells while the inhibition of carbohydrate biosynthesis may cause the defect in cell wall formation. The biochemical changes of the TK treated cultures agree well with the morphological studies which illustrates the deformed cell wall and disappearance of intracytoplasmic organelles of the fungal cells.

All of the studies demonstrated lead to a conclusion that TK appeared to be fungistatic or fungicidal agent depending on its dosage and the sensitivity of the fungi. The drug may exert its primary effect at the cell membrane and cell wall of the fungi while intracytoplasmic organelles were also involved. However, other biochemical changes of the TK treated cultures should be further studied, such as the RNA biosynthesis, protein biosynthesis, sterol biosynthesis, the analysis of carbohydrate biosynthesis and cellular respiration, etc. These data will probably pin point the antifungal action of TK.

Although TK is not a pure substance, it has an interesting fungistatic or fungicidal action described above. It should be further purified to substance with more potent antifungal activity and the study should be done both in vivo and in vitro. Thereafter, the toxicity test and clinical trial must be studied before clinical use will be possible.

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