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Original Research Article

Analytical assay and morphological study of fungal cells treated with coumarin derivatives

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ABSTRACT

In this research, synthesized coumarin derivatives were evaluated as antifungal drugs. Scanning electron microscopy (SEM) and transmittance electron microscopy (TEM) techniques were used to investigate morphological changes of fungal cells. In the cells treated with subinhibitory concentrations of compound 61, cell wall was rugged. However, the compound 23 and AmB groups had very few cells with a wrinkled surface. TEM results showed changes in vacuoles of the cells treated with coumarins. Our findings suggest that this increase is due to the phosphodiesterase (PD) inhibitory activity of coumarins.

Keywords: Fungal cells, morphological study, coumarin derivatives

INTRODUCTION

Since many of the important antifungal drugs like azoles act on the ergosterol biosynthetic pathway and inhibit ergosterol biosynthesis at the 14-demethylation step, it was intended to evaluate the effect of coumarins on this metabolic pathway. Scanning electron microscopy (SEM) is a technique that is utilized to look at the surface of organism. In transmittance electron microscopy (TEM) of the cells, more details

of intracellular changes are observed. Sterols, especially ergosterol, are very important for most fungal cells. Mutants defective in sterol transmethylation had a sixfold reduction in tryptophan uptake. Cholesterol, lanosterol, and ergosta-7,22-dienol could satisfy the bulk of the sterols required, however, a sterol with Δ-5- unsaturation is required for growth. Heme-deficient cells, did not grow on defined medium using respiratory substrates such as glycerol and ethanol. The sterol content of the cell can affect respiration [1]. Ethanol and glycerol are respiratory substrates. The sensitivity of antifungal agents that are inhibitors of sterol biosynthesis is enhanced when cultures are grown on respiratory substrates [2]. Cytochrome P-450 isozymes are iron-containing hemoprotein which catalyze the monooxygenation of a broad spectrum of lipophilic substances, such as fatty acids, sterols, sex steroids, glucocorticoids, mineralocorticoids, vitamin D, leukotrienes, prostaglandins and retinoic acid. A lipophilic compound can bind tightly to the apoprotein of cytochrome P-450 [3,4]. Therefore, lipophilic compounds may influence P450 enzymes. Coumarin or 4-methylcoumarin are 3-hydroxylated by rat-liver microsomes and 4-methylcoumarin given orally has induced microsomes [5]. Inhibition of one of the enzymatic steps in the metabolic pathway can cause accumulation of substances before that step. This is the case for azoles, which inhibit 14-demethylase in ergosterol biosynthesis. In the case of test compounds no sign of squalene or lansoterol accumulation was observed in the angelicin and compound 61 [33] treated *C. albicans*.

Azole antifungals are shown to induce hepatic CY P450 isozymes [6]. As coumarins are substrates for a variety of P450 enzymes [7], it could be possible that in a way they lead to induction of this type of enzymes. In fact, it has been reported that coumarin and some compounds with electrophilic centres induce phase II enzymes (like glutathione-S-transferase, GST) which participate in the reaction for detoxification of carcinogens [8]. If this theory is true, the rise in ergosterol level after treatment with coumarins can be easily explained. In addition, the reason for antagonism of coumarins with azoles can be understood. However, an increased level of ergosterol could be enough to reduce the inhibitory activity of azoles, which depend on depleting the ergosterol pool in the fungal cell [9].

Other factors could be involved in the rise of ergosterol in fungal cells. One is pH, since lanosterol production is shown to be favored at the optimum pH levels. The other factor is

sulphur metabolism as cysteine acted as an elicitor at lower concentrations tested (0.9 mM) for several triterpenes and ergosterol. Allicin, and methionine elicited production of the C-21 aldehyde derivative. Cysteine is not inhibitory to sterol biosynthesis; in contrast it has a stimulatory effect on ergosterol production. This suggests that cysteine plays some role in sterol biosynthesis, from lanosterol to ergosterol, via certain intermediates including the alkylation (C-24) of the side chain by S-adenosyl methionine. Ergosterol is also affected by variables such as fungal species, O_2 availability, temperature, growth substrate, and the method of analysis used [10]. In this study, we intend to report analytical assay and morphological study of fungal cells treated with cumarine derivatives.

MATERIALS AND METHODS

Chemicals used in this study as antifungal (except the drugs) were of coumarin types synthesized**.** A group of other drugs and chemicals were used as cAMP analogs, db-cAMP (ICN®), or phosphodiesterase inhibitors, including theophylline, IBMX, pentoxyfylline**.** Ergosterol was purchased from Sigma. Squalene, lanosterol/dihydrolanosterol (a mixture of 59.3% and 40% respectively) were purchased from Nakalai (Japan).

Fungal species and growth condition

C. albicans ATCC 14053 was cultured on SDA slants for 24 h at 25^o C. After this period, fungus was suspended in saline solution to a concentration of 75-77% T at 530 nm. The test compounds were dissolved in water or acetone and added to the medium, RPMI 1640, adjusted to pH = 7.0. The final fungal suspension is equal to 5×10^3 CFU/ml. The standing cultures were kept at 30^o C for 21 h.

Sterol extraction

The fungal cells were harvested by centrifugation at 2600 rpm at 0^o C for 20 min. They were then washed three times with chilled distilled water and recollected by centrifugation. The cells were divided into two equal sets, one for dry weight measurement and the other for sterol analysis. The first set of cells was dried at 60^o C. The second set of cells was suspended in methanol (4 ml) and n-hexane (2 ml). The mixture was sonicated for 1 h. The extraction was repeated twice with a fresh hexane layer each time. Hexane fractions were collected and evaporated in a centrifugal concentrator [6].

Trimethlylsilyl derivatization of the remaining sterols performed in the presence

of BSA (30 µl) [*N*', *O*'-bis-(trimethylsilyl) trifluroacetamide] at 60 ºC for 1 h [7].

Ergosterol binding assay

To estimate the quality of possible interaction between coumarins and ergosterol, a spectrophotometric method described originally for AmB was used. AmB (Sigma) and coumarins were dissolved in DMSO and methanol, respectively, at a concentration of 10^{-3} M for AmB, and 10^{-2} for compounds 28 and 23 [33]. Ergosterol and cholesterol (Sigma) stock solutions were prepared in $CHCl_3$ at a concentration of 10^{-2} M and stored for a few days in a refrigerator. Sterol working solutions were prepared daily by mixing one part of stock solution with 9 parts of ethanol. Desired amounts of antibiotic and sterol solutions were added to required volumes of distilled water. Spectrophotometric measurements were performed 1 h after mixing. After this period, no changes in the spectra were observed in the control groups. In all experiments, the concentrations of AmB, angelicin, and compound 23 were 1.8, 12.5 and 6.25 µg/ml. The free form of AmB has an absorption band at 409 nm, which does not exist in the spectrum of the complex form, and has a high absorption coefficient. The measurements for angelicin and compound 23 were done at their proper λ_{max} 302 and 317 nm respectively, which did not interfere with sterols absorption region. The results are the average of three separate experiments.

Electron Microscopy

Preparation of the cells for electron microscopy

C. albicans cells, cultured on SDA plates, were used to make an inoculum of 10^6 (CFU/ml) in saline, equal to a transmittance of 75-77% at 530 nm, and transferred to tubes containing RPMI 1640 medium to make a final cell count of 5×10^3 (CFU/ml) in the tubes. The antifungal agents were added at subinhibitory concentrations to allow the cells to grow and make enough cells for the microscopic examination. The final concentrations of fluconazole, AmB, angelicin, compounds 61, and 23 [33] in the medium were 1, 1, 42, 20, and 2.5 µg/ml, respectively. Cells were incubated at 25ºC. To the control groups equal volume of solvent used for antifungal agents, methanol (33 µl), was added.

SEM picture of samples

After appropriate incubation and treatment regiments, the cultures were centrifuged and pelleted cells washed with isotonic phosphate buffer (pH 7), fixed in 2.5 % glutaraldehyde at 4 ºC for 12 h, and washed again in 0.214 (M) Millonig phosphate buffer (pH 7.4). They were then postfixed with 2 % osmium

tetroxide for 2 h. The cells were dropped onto a poly-L-lysin coated coverslip (12 mm in diameter) and stayed for 10 min, and dehydrated in an increasing series of ethanol solutions. The samples were put in a critical point drier, and coated with gold in argon atmosphere using an Edwards Sputter Coater S150B (England). The specimens were examined with a Hitachi S-2500 scanning electron microscope (Tokyo, Japan).

TEM picture of samples

Aliquots of the centrifuged pellets (10 min, 10 000 rpm) of *C. albicans*, cultured as described above, were prefixed in a mixture of 2.5% (v/v) glutaraldehyde-0.2 M Millonig's phosphate buffer, pH 7.4, then postfixd with 2% (w/v) osmium tetroxide for 3 h at room temperature. In the specified cases, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 6% tannic acid (pH was adjusted to pH 7.4 by NaOH) was used for the purpose of fixation and staining. Samples were dehydrated in ethanol series and embedded in Spurrs resin. Specimens were cut on an ultramicrotome as 0.1 μm thickness and collected on 200-mesh copper grids with a formvar supporting film (Sigma). Thin sections were stained with uranyl acetate and lead citrate. Sections were examined by a Hitachi H-7000 transmission electron microscope (Tokyo, Japan) operated at 75 kV.

Assay for c AMP phosphodiesterase

Samples were dissolved in DMSO and tested for their activity against phosphodiesterase of beef heart (Sigma) according to [11]. The standard reaction mixture (500 μl) containes Tris-HCl (pH 7.5; 0.05 M), $MgCl_2$ (1mM), bovine serum albumin (250 μg), [^{3}H]-cyclic AMP (0.01 mM; 1.2×10^6 dpm), beef heart phosphodiesterase (2.25 mU) and the sample (12.5-100 μg). The reaction was initiated by the addition of [^{3}H]-cyclic AMP. The reaction mixture was incubated for 30 min at 37^o C and was stopped by immersing the test tube in boiling water for 3 min. Snake venom nucleotidase (500 μg) was added to the cooled reaction mixture and incubated for 30 min at 37^o C. A 50% suspension of Dowex Agl-X8 resin was then added to the reaction mixture. The resin, which absorbed unchanged [^{3}H]-cyclic AMP, was precipitated by centrifugation and the radioactivity of an aliquot of the supernatant containing [^{3}H]-adenosine, resulted in the assay reaction, was measured with liquid scintillation counter. All assays were performed in duplicate. IC_{50} value is the concentration of compound required to give

50 % inhibition of phosphodiesterase activity.

RESULTS

Morphological changes

SEM examination of fungal cells is a powerful way to study morphological changes. SEM is utilized to look at the surface of organism. SEM pictures of *C. albicans* cells treated with angelicin, compound 61 and 23 [33], nitroangelicin (Figure1-5) Although there is not a clear change of structure in the angelicin group, others show significant changes. In the cells treated with subinhibitory concentrations of compound 61, cell wall is rugged. However, the compound 23 and AmB groups have very few cells with a wrinkled surface.

TEM of the *Candida* cells with details of intracellular changes are observed (Figure 6-10). However, by a quick look at the cells treated with angelicin and compound 23 two big changes are evident.

The first change can be observed in the vacuoles. It looks like the vacuolar membrane invaginates to form an intravacuolar membrane matrix that eventually breaks down, leaving clumps of densely stained and amorphous material surrounded by electron lucent areas. Invagination of vacuole may be cutting the vacuole into several smaller compartments. The second change is observed in the cell membrane, which is quite fuzzy in the angelicin group and thin or unclear in compound 23 group. In this group, as expected, the cell membrane is disrupted and various abnormalities appear in the organelles.

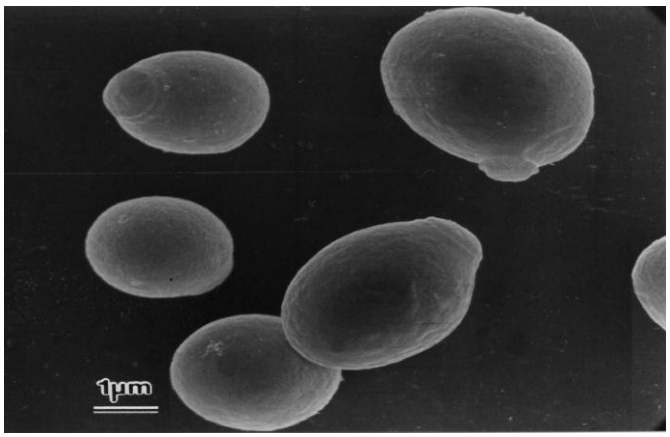

Figure 1. SEM picture of *C. albicans* cells exposed to angelicin (7.8 μg/ml).

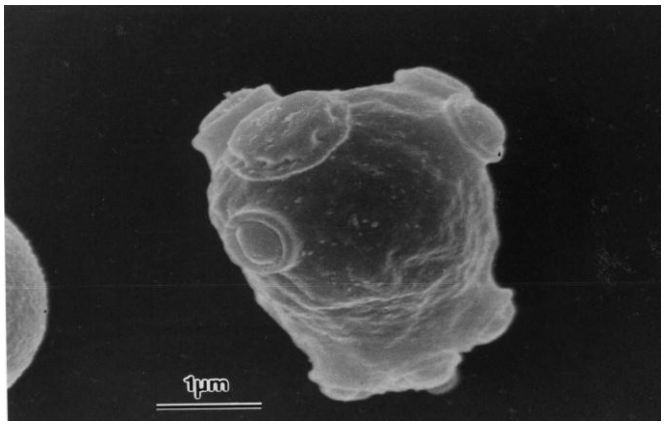

Figure 2. SEM picture of *C. albicans* cells exposed to compound 61 (15.6 μg/ml) [33], grown in RPMI containing citrate buffer at 37° C for 24 h.

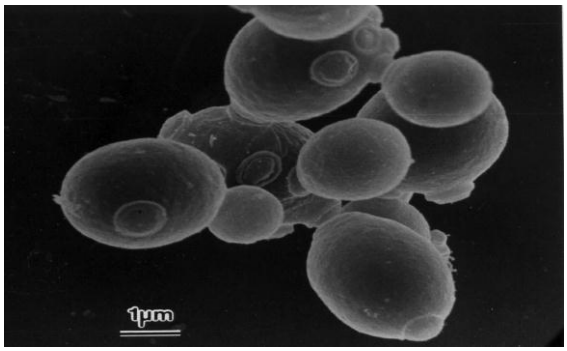

Figure 3. SEM picture of *C. albicans* cells exposed to compound 23 (3.9 μg/ml) [33].

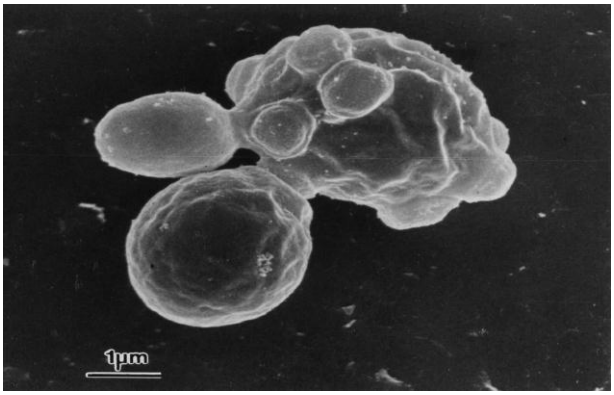

Figure 4. SEM picture of *C. albicans* cells exposed to nitroangelicin (15.6 μg/ml).

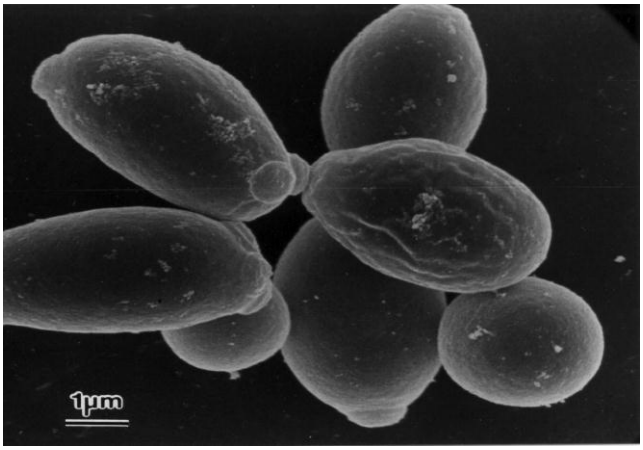

Figure 5. SEM picture of *C. albicans* cells exposed to AmB (2.5 µg/ml).

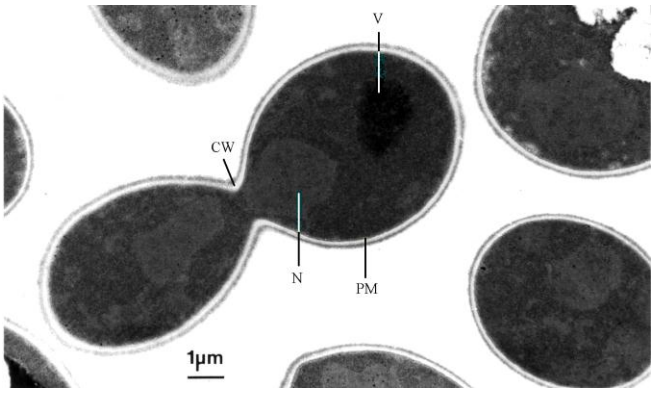

Figure 6. TEM picture of the control *C. albicans* cells, grown in RPMI at 25 ºC for 24 h.×2100. CW, cell wall; PM, plasma membrane; N, nucleus; V, vacuole.

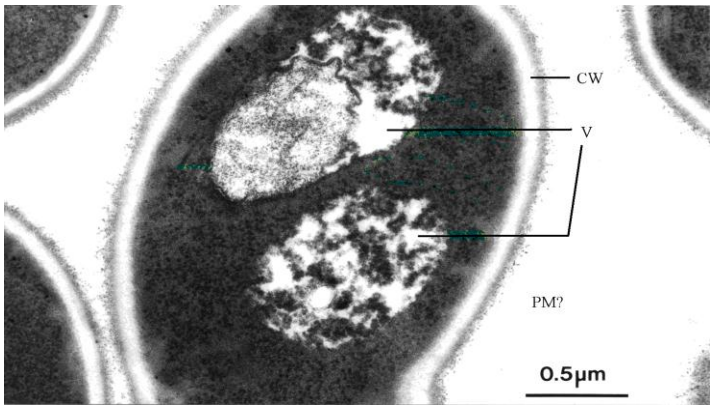

Figure 7. TEM picture of *C. albicans* cells, exposed to angelicin, grown in RPMI at 25 ºC for 24 h×6000. CW, cell wall; PM, plasma membrane; V, vacuole.

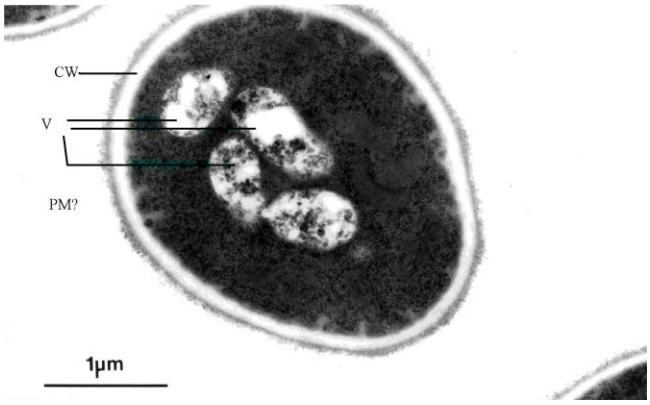

Figure 8. TEM picture of *C. albicans* cells, exposed to angelicin, grown in RPMI.

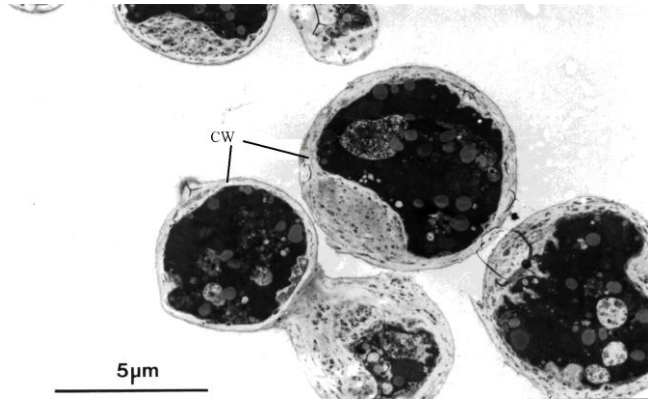

Figure 9. TEM picture of *C. albicans* cells, exposed to fluconazole.

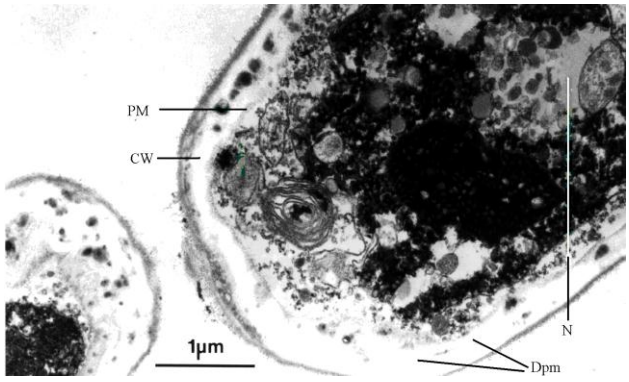

Figure 10. TEM picture of *C. albicans* cells, exposed to fluconazole.

DISCUSSION

Most of the pictures taken by TEM technique showed changes in vacuoles of the cells treated with coumarins. It was suggested earlier that coumarin treatment changes the pH of vacuoles and also this action might be mediated through inhibition of vacuolar H^+-ATPase. Here, we try to focus on the role of vacuolar ATPase and consequences of its inhibition.

Vacuolar, or V-type, proton translocating ATPase is distributed in bacteria, yeasts and mammalian cells. In higher Eukaryotae, these proton pumps are found in numerous intracellular organelles of both the constitutive sorting and specialized secretory pathways. In addition, these enzymes participate in urinary acidification as well as osteoclast mediated bone reabsorption. From a pathophysiologic standpoint, vacuolar proton pumps are essential to nutrient uptake and processing in the food vacuole of *Plasmodium falciparum*, and evidence suggests that V-type proton pumps may confer resistance to chemotherapeutic agents in cultured tumor cells [12].

Vacuolar ATPase is sensitive to bafilomycin A1, N-methylmaleimide, KNO_3, and resistant to oligomycin, azide and vanadate. The vacuolar ATPase utilizes the energy generated by hydrolysis of ATP to pump protons into the vacuole lumen. This results in a calculated electrochemical potential difference of protons on the order of 180 mV contributing to both a decreased pH and a membrane potential of approximately 75 mV for *S. cerevisiae*. The primary mechanism for transport of storage molecules into the vacuole appears to rely on a proton antiport system. Although the vacuolar ATPase is the major energy donor for these transport systems, there is some evidence that a pyrophosphatase activity is associated with the vacuole membrane which may be responsible for a PPi-dependent formation of a pH gradient. By altering the ion conductivity of the vacuolar membrane, proton uptake activity can be blocked or enhanced while the ATPase activity is relatively unaffected. Treatment of yeast cells with bafilomycin A1 causes an increase in vacuolar pH, which can be demonstrated by the abolition of quinacrine accumulation in the vacuole.

One of the most prominent features of eukaryotic cells is the reliance on subcellular compartmentalization. The presence of distinct membrane enclosed organelles allows the cell to spatially separate otherwise competing reactions. The various catabolic and anabolic reactions in the cell may be controlled partly by separating them from

each other; as doing chemical reactions in different containers and then storing the products to different containers after reactions are completed. The vacuole is the main storage organelle for a variety of metabolically important compounds and ions. This role in storage is not a passive one. The observation that some yeast mutants, which lack a normal vacuole, are pH sensitive suggests that vacuoles may play a role in homeostasis of the intracellular pH. These mutants also show some degree of osmo-sensitivity, indicating an additional role in osmoregulation. In addition other mutants defective in vacuolar protein sorting have extremely large vacuoles, which may reflect a defect in osmo-regulatory capabilities. The membrane potential-dependent cation channel may afford some control. Since the vacuole and cytosol are isotonic, this channel may be an osmotic regulator, which acts to balance the osmotic potential differences resulting from the uptake of cations into the vacuole [13]. In addition to the changes observed in TEM pictures, SEM photos show some wrinkles in the cell wall of the cells. IBMX at 1 and 10 μM concentrations caused an increase in intracellular cAMP and stimulated the production of endoglucanase [14]. A change in the sterol composition of the membrane is shown to lower overall specific activity of the enzyme, chitin synthase [15]. The cAMP has important roles in fungal physiology, such as yeast to hyphae transition [16-19]. Also, cAMP can induce translation of some genes and can affect the cell cycle in fungi, and vertebrates [20]. The cAMP in *C. albicans* rises during yeast to mycelial form transition, even at lower temperatures. Cysteine, which suppressed germination, also reversed the increase in intracellular cAMP (possibly through activation of cyclic nucleotide phosphodiesterase as in *E. coli*). Cysteine promotes yeast like growth via, for example, reduction of disulfide linkages in glucomannan protein. In *M. rouxii* the addition of db-cAMP or cAMP induced the yeast like morphology. *Mucor racemosus* is similar to *M. rouxii*, however, *Histoplasma capsulatum,* like *C. albicans* tends to be in a mycelial form in this condition [18]. Although no mycelial form was observed in our study, the increased cAMP could be responsible for some of the morphologic changes seen in the SEM study.

Up to this study, there has been no method available for producing only one major triterpene or sterol as the chief product of fungal metabolism. However, coumarins, like sulphur compounds, may be noteworthy elicitors at specific concentrations. In

addition to what mentioned above, the level of ergosterol and its biosynthesis, as for any other biomolecule, should have regulatory systems. Although this system has not been proven to date, there are studies suggesting ergosterol may function in wild type yeast as a feedback regulator of sterol biosynthesis [21-24]. Therefore, regardless of the involvement of cAMP in such regulation, coumarins may be disrupting the feedback control of ergosterol biosynthesis. The role of cAMP in phosphatidylcholine biosynthesis was determined by the uptake of precursors in aminophylline and atropine grown cells. Incorporation of methionine in total lipids of *Microsporum gypseum* cells was higher in the presence of aminophylline. Aminophylline (4 mM) led to a four-fold increase and atropine (2 mM) led to a 2.3 fold decrease in cAMP levels in *M. gypseum*. Methylation of lipids is shown to be affected by cAMP. In our experiments the phosphodiesterase inhibitory (PDIs) were co-incubated for 24-48 h with the fungal cells and the antifungal agents. The unexpected results in some cases may have been due to the length of exposure of the cells to the PDI. Also the various responses achieved by using different phosphodiesterase inhibitors can be due to different isozymes that interact differently with the inhibitors. The circulatory effects of coumarins have been studied on the basis of the PD inhibitory activity (Figure 11-12). The reports indicate the involvement of cAMP-phosphodiesterase inhibition in coronary vasodilatory effects of acyloxydihydropyrano- and acyloxydihydrofurano-coumarins. Inhibition of platelet aggregation also occurred by increasing intraplatelet cAMP concentration due to the application of coumarins [25].

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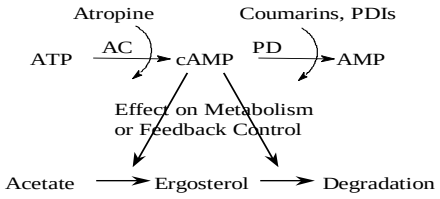

Figure 11. Putative mechanism of the inhibition of PD by coumarins in fungal cell and consequent events. Possible role of cAMP in ergosterol metabolism.

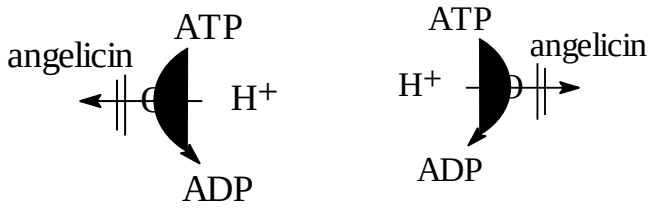

Figure 11. Putative mechanism for the inhibition of glucose induced medium acidification and induction of cytoplasmic pH decrease and vacuolar pH increase by coumarins mediated through H^+-ATPase inhibition.

CONCLUSION

In summary, an increase in the ergosterol level is a novel observation. Although, in some cases, it has been reported that ergosterol can be increase compared to other sterols present in the cell, in all of the studies, the reported increase of ergosterol was infact a "less decrease" as compared to general decrease in sterol levels. However, here we reported an increase of ergosterol on its own based on the cell dry weight. Our findings suggest that this increase is due to the PD inhibitory activity of coumarins. Although there are reports of interaction of coumarins with mitochondria [26-32], no morphological changes in this organelle is evident from TEM pictures. This finding can have significant implications in antifungal research. Clinical consequences might emerge from our findings are as follows: Antifungal compounds especially azole derivatives could be considered to show reduced activity while being used with phosphodiesterase inhibitors like theophylline (drug-drug interaction). A clinical potentiation of activity may occur in concomitant usage of azoles and atropine analogues (combination therapy).

REFERENCES

[1]. Parks LW, Smith SJ, Crowley JH. Biochemical and physiological effects of sterol alteration in yeast- a review. *Lipids,* 1995; 30(3), 227-30.

[2]. Parks LW, Casey WM. Physiological implications of sterol biosynthesis in yeast. *Annu. Rev. Microbiol, 1995;* 49, 95-116.

[3]. Cauwenbergh G, Bossche HV. Terconazole, pharmacology of a new antimycotic agent. *J. Reprod. Med, 1989;* 34(8), 588-92.

[4]. Aoyama Y, Yoshida Y, Sonoda Y, Sato Y. Role of the side chain of lanosterol in

substrate recognition and catalytic activity of lanosterol 14α-demethylase (cytochrome P-450) of yeast. *Biochim. Biophys. Acta, 1991;* 1081, 262-66.

[5]. Feuer G. 3-Hydroxylation of coumarin or 4-methylcoumarin by rat-liver microsomes and its induction by 4-methylcoumarin given orally. *Chem. Biol. Interact, 1970:* 2(3), 203-16.

[6]. Ronis MJ, Ingelman-Sundberg, M, Badger TM. Induction, suppression and inhibition of multiple hepatic cytochrome P450 isozymes in the male rat and bobwhite qual (*Colinus virginianus*) by ergosterol biosysnthesis inhibiting fungicides (EBIFs) *Biochem. Pharmacol*, 1994; 48(10) 1953-65.

[7]. Ito T, Aoyama Y, Ishida K, Kudoh M, Hori K, Tsuchiya S, Yoshida Y. Selectivity of isoprenoid-containing imidazole antifungal compounds for sterol 14-demethylase P450 ($P450_{14DM}$) and 7-ethoxycoumarin O-demethylase P450 of rat liver microsomes. *Biochem. Pharmacol, 1994;* 48(8), 1577-82.

[8]. Talalay P. The role of enzyme induction in protection against carcinogenesis. *Cancer Chemopreven*, 1992; 469-78.

[9]. Ghannoum MA, Spellberg BJ, Ibrahim, AS, Ritchie JA, Currie B, Spitzer ED, Edwards JE, Casadevall A. Sterol composition of *Cryptococcus neoformans* in the presence and absence of fluconazole. *Antimicrob. Aagents Chemother, 1994;* 38(9), 2029-33.

[10]. Kahlos K, Tikka VH. Antifungal activity of cysteine, its effect on C-21 oxygenated lanosterol derivatives and other lipids in *Inonotus obliquus, in vitro*. *Appl. Microbiol. Biotechnol, 1994;* 42, 385-90.

[11]. Nikaido T, Ohmoto T, Noguchi H, Kinoshita T, Saitoh H & Sankawa U. Inhibitors of cyclic AMP phosphodiesterase in medicinal plants. *Planta Med, 1981;* 43(1), 18-23.

[12]. Crider BP, Xiao-Song X, Stone D K. Bafinomycin inhibits proton flow through the H^+ channel of vacuolar proton pumps. *J. Biol. Chem, 1994;* 269(26), 17379-81.

[13]. Klionsky DJ, Herman PK, Emr, SD. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Reviews*, 1990; 54(3), 266-92.

[14]. Sestak S, Farkas V. Metabolic regulation of endoglucanase synthesis in *Trichoderma reesei*: participation of cyclic AMP and glucose-6-phosphate. *Can. J. Microbiol, 1993;* 39(3), 342-47.

[15]. Walker-Caprioglio HM, Casey WM, Parks LW. Saccharomyces cerevisiae membrane sterol modifications in response to

growth in the presence of ethanol. *Appl. Environ. Microbiol, 1990;* 56(9), 2853-57.

[16]. Egidy G, Paveto C, Passeron S, Galvagno MA. In situ measurement of cAMP related enzymes during yeast to hyphae transition in *C. albicans*. *Cell Biol. Int. Rep, 1990;* 14(1), 59-68.

[17]. Larsen AD, Sypherd PS. Cyclic adenosin 3', 5'-monophosphate and morphogenesis in *Mucor racemosus*. *J. Bacteriol, 1974;* 117(2), 432-38.

[18]. Nimi M, Nimi K, Tokunaga J, Nakayma H. Changes in cyclic nucleotide levels and dimorphic transition in *Candida albicans*. *J. Bacteriol, 1980;* 142(3), 1010-14.

[19]. Cho T, Hamatake H, Kaminishi H, Hagihara Y, Watanabe K. The relationship between cyclic adenosine 3',5'-monophosphate and morphology in exponential phase Candida albicans. *J. Med. Vet. Mycol, 1992;* 30(1), 35-42.

[20]. Lane HA, Nigg EA. Cell-cycle control: POLO-like kinases join the outer circle. *Trend. Cell Biol, 1997;* 7, 63-68.

[21]. Lorenz R, Parks L. Physiological effects of fenpropimorph on wild-type Saccharomyces cerevisiae and fenpropimorph resistant mutants. *Antimicrob. Agents Chemother, 1991;* 35(8), 1532-37.

[22]. Venkatramesh M, Guo D, Harman JG, Nes WD. Sterol specificity of the *Saccharomyces serevisiae* ERG6 gene product expressed in *Escherichia coli*. *Lipids*, 1996; 31(4), 373-77.

[23]. Passi S. Possible role of ergosterol oxidation in aflatoxin production by Aspergillus parasiticus. *Mycol. Res*, 1994; 98(3), 363-68.

[24]. Pinto WJ, Lozano R, Nes WR. Inhibition of sterol biosynthesis by ergosterol and cholesterol in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, 1985; 836, 89-95.

[25]. Teng C, Lin C, Ko F, Wu T, Huang T. The relaxant action of osthole isolated from Angelica pubescens in guinea-pig trachea. *Naunyn Schmiedebergs Arch. Pharmacol, 1994;* 349(2), 202-8.

[26]. Zboril P, Dadak V. Hydrophobic interaction of isoprenic coumarin ostruthin with nonphosphorylating mitochondrial fragments. *Arch. Biochem. Biophys, 1973* 159(1), 249-58.

[27]. Sardari S, Nishibe S, Daneshtalab M. Coumarins, the bioactive structures with antifungal property. *Stud Nat Prod Chem*, 2000; 23: 335-93.

[28]. Sardari S, Mori Y. Synthesis and antifungal activity of coumarins and angular furanocoumarins. *Bioorg Med Chem*, 1999; 7(9): 1933-40.

[29]. Rezayan AH, Azerang P, Sardari S, Sarvary A. Synthesis and biological evaluation of coumarin derivatives as inhibitors of Mycobacterium bovis (BCG). *Chem Biol Drug Des*, 2012; 80(6): 929-36.

[30]. Song PP, Zhao J. Evaluation of antifungal activities and structure-activity relationships of coumarin derivatives. *Pest Manag Sci*, 2017; 73(1): 94-101.

[31]. Ayine-Tora DM, Kingsford-Adaboh R. Coumarin antifungal lead compounds from Millettia thonningii and their predicted mechanism of action. *Molecules*, 2016; 21(10): 1369.

[32]. Soltani S, Dianat S, Sardari S. Forward modeling of the coumarin antifungals; SPR/SAR based perspective. *Avicenna J Med Biotechnol*, 2009; 1(2): 95-103.

[33]. Sardari S, Daneshtalab M, G. Micetich R. ^{31}P-NMR study for determination of coumarin antifungal mechanism of action. *HBB* journal, 2017; 1(3): 37-51.