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Modes of Antifungal Action of Alkanols against *Saccharomyces cerevisiae*

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Abstract—Primary aliphatic alcohols from C_6 to C_{13} were tested for their antifungal activity against *Saccharomyces cerevisiae*. Undecanol was found to be the most potent fungicide followed by decanol. The time–kill curve study showed that undecanol was fungicidal against *S. cerevisiae* at any growth stages. This fungicidal activity was not influenced by pH values. The alcohols tested inhibited glucose-induced acidification by inhibiting the plasma membrane H^+-ATPase. The primary antifungal action of amphipathic medium-chain (C_9–C_{12}) alkanols comes mainly from their ability as nonionic surfactants to disrupt the native membrane-associated function of the integral proteins. Hence, the antifungal activity of alkanols is mediated by biophysical process, and the maximum activity can be obtained when balance between hydrophilic and hydrophobic portions becomes the most appropriate.

Introduction

In our previous report on the structure–antimicrobial activity relationship (SARs) study with a series of long chain alcohols, we described that their maximum antimicrobial activity depends on the hydrophobic alkyl (tail) chain length from the hydrophilic hydroxyl group (head).[1] In other words, microorganisms having different membrane structure showed different susceptibilities to alcohols having different chain lengths. It was proposed that the hydrophilic head binds with an intermolecular hydrogen bond like a 'hook' attaching itself to the hydrophilic portion of the membrane, and then the hydrophobic tail portion of the molecule is able to enter into the membrane lipid bilayer. This creates, as a result, disorder in the fluid bilayer of the membrane. The result obtained can be explained by their nonionic surface-active properties and also by their non-specific activity. The anesthesia cutoff phenomenon among alkanols is a more well known example and a long standing problem.[2] It is well established that the hydrophobicity is often associated with biological action.[3] However, the rationale for this observation, especially the role of the hydrophobic portion, is still poorly understood and widely debated.

In our continuing challenge to this long standing question, we found that alcohols disrupt glucose-induced acidification by inhibiting the plasma membrane H^+-ATPase of *Saccharomyces cerevisiae*. Hence, we investigated the effect of a series of primary alcohols (hereafter referred to as alkanols for simplicity) on the plasma membrane H^+-ATPase of *S. cerevisiae* as a model because this enzyme is the most abundant plasma membrane protein,[4] constituting over 20% of the total membrane protein in *S. cerevisiae*.[5] The effects of the different chain length of alkanols in relation to their activity upon the membrane enzyme H^+-ATPase were studied. The alkanols have been among the most widely studied compounds in general SAR research, no doubt because of their stability and availability. In addition, the inhibition mechanism of the membrane-bound enzyme H^+-ATPase by alkanols can also be used as a model for their anesthetic action and drug toxicity.[6]

Materials and Methods

Chemicals

Alcohols were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). For the experiment, all compounds were first dissolved in *N,N*-dimethylformamide (DMF) which was purchased from EM Science (Gibbstown, NJ, USA). The concentration of DMF in each medium was always 1%.

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Test strain and media

The test strain, *S. cerevisiae* ATCC 7754 used for this study was purchased from American Type Culture Collection (Rockville, MD, USA). ME (2.5% Malt extract) and YPD (1% Yeast extract, 2% Bacto Peptone, 2% Dextrose) media were used for the assay. Cells were inoculated into ME broth and incubated with shaking for 16 h at 30 °C prior to the antimicrobial activity assay.

Antifungal assay

Broth macrodilution minimum inhibitory concentrations (MICs) were determined as follows. Serial 2-fold dilutions of the test compounds were made in DMF, and 30 μL of each dilution was added to 3 mL of ME broth. These were inoculated with 30 μL of 16 h culture of *S. cerevisiae*. The cultures were incubated without shaking at 30 °C for 48 h. The MIC was the lowest concentration of test compound that demonstrated no visible growth. After the MIC was determined, 30 μL of each tube was added to 3 mL of fresh ME broth, and the cultures were incubated without shaking at 30 °C for 48 h. The minimum fungicidal concentration (MFC) was the lowest concentration of antifungal compound that demonstrated no visible growth in the fresh media.

Growth curves

Time–kill studies were performed to examine the effects of combinations of compounds in more detail. The culture cells were prepared as described above and incubated at 30 °C for 16 h. A 30-μL aliquot of the 16-h culture was inoculated into 3 mL of ME broth containing appropriate concentrations of the test compounds. The initial population size was about 10^6 colony forming units (CFU) per mL. Samples were taken at selected times, and serial dilutions were made in sterile saline before the samples were plated onto YPD agar plates. The plates were incubated at 30 °C for 1 day before the number of CFU was determined.

Acidification measurement

The cultured cells were incubated at 30 °C for 24 h in YPD broth. A 100 mL of the 24-h culture was centrifuged, the pellet was washed twice by distilled water, and readjusted to 100 mL with distilled water. The initial population size was about 10^8 CFU/mL. Then, 18 mL of the cell suspension was transferred to Erlenmeyer flasks and 200 μL of the appropriate concentrations of the test compounds were added. After incubation at 30 °C for 5 min, 2 mL of 20% glucose was added and acidification was started. The pH of each sample was checked at selected times. During the reaction, samples were incubated at 30 °C. The inhibition ratio (%) was calculated as follows; $(1-[H^+]_{\text{inhibitor}}/[H^+]_{\text{inhibitor free}})\times 100$.

Cycloheximide treatment

The culture cells were prepared as described above, incubated at 30 °C for 16 h and diluted 100 times by ME broth. A 200 μL aliquot of the diluted culture was inoculated into 20 mL of fresh ME broth and incubated for 14 h at 30 °C by shaking. Then, 1 mL of cycloheximide (50 μg/mL) or undecanol (25 μg/mL) was added in the cell culture, and incubated at 30 °C by shaking. Two hours later, 1 mL of undecanol (25 μg/mL) was added to one of the flasks which contained 50 μg/mL of cycloheximide. Samples were taken at selected times, and serial dilutions were made in sterile saline before the samples were plated onto YPD agar plates. The plates were incubated at 30 °C for 1 day before the number of CFU was determined.

Results and Discussion

The MICs of a series of alkanols from C_6 to C_{13} against *S. cerevisiae* were previously reported,[1] and their MFCs have been obtained. The results are listed in Table 1. In agreement with many other studies of the homologous series of alkanols, the antifungal activity of the alkanols increased with number of carbons in the chain until dodecanol and undecanol which had the best MIC and MFC, in this experiment. Noticeably, the activity disappeared after the chain length reached the best MIC and MFC, known as the so-called 'cutoff' phenomenon. For example, dodecanol (C_{12}) was the most effective with an MIC of 12.5 μg/mL, while tridecanol (C_{13}) showed no activity up to 1600 μg/mL. In other words, dodecanol is the most effective fungistatic but did not show any fungicidal activity up to 1600 μg/mL. It should be noted that the MIC of dodecanol slows growth for the first 24 h, but the growth recovered shortly after and becomes no longer different from the control. The reason for this remains unclear. The cutoff point may migrate by the slight difference in growth conditions such as inoculum size of yeast cells or medium composition. Namely, it seems to be important that the cutoff point exists but not crucial where is the cutoff point. Alkanols are capable of forming hydrogen bonds with water and as a result, simple alkanols are fairly soluble in water. However, as the hydrocarbon content increases especially to more than six carbons there is a general decline in solubility. As the hydrocarbon chain becomes longer, its hydrophobic properties come to dominate the properties of the molecule so that the medium-chain (C_9–C_{11}) alkanols are amphipathic molecules.

Table 1. Antifungal activity of alkanols against *S. cerevisiae*

Alkanols tested	μg/mL (48 h)	
	MIC	MFC
C_6	>1600	>1600
C_7	800	>1600
C_8	200	800
C_9	100	200
C_{10}	50	50
C_{11}	25	25
C_{12}	12.5[a]	>1600
C_{13}	>1600	>1600

[a]The value is variable.

Among the alkanols tested, undecanol (C_{11}) was found to be the most potent against *S. cerevisiae* with an MFC of 25 μg/mL (0.15 mM). No differences in MIC and MFC were noted, suggesting that its activity was fungicidal. This fungicidal effect was confirmed by a time–kill curve method as shown in Figure 1. Cultures of *S. cerevisiae*, with a cell density of 6×10^5 CFU/mL, were exposed to two different concentrations of undecanol. The number of viable cells was determined following different periods of incubation with undecanol. The results show that $\frac{1}{2}$MIC slows growth; however, the final cell count is not significantly different from the control. At the MFC lethality occurs quickly, within the first 8 h, indicating a membrane disruptive effect. Similar results were obtained with hexanol but fungicidal activity was not seen until 24 h, indicating that short-chain alkanols act in somewhat different ways.

It is known that *S. cerevisiae* produces the acidification of the external medium during growth on glucose. This external acidification is closely associated with the metabolism of the sugar and its magnitude depends on the buffering capacity of the growth medium. The H^+-ATPase is important not only in the regulation of internal pH but also the energy-dependent uptake of various metabolites.[4] Interestingly, alkanols were found to inhibit this acidification process by inhibiting the H^+-ATPase. As a result, it seems that the antifungal activity of alkanols is, at least in part, due to their inhibition of the H^+-ATPase and the inhibition ratio is shown in Figure 2. Interestingly, the potency of the inhibition of each alkanol differs and the cutoff phenomenon does not occur. The alkanols of the chain length less than C_8 and longer than C_{12} exhibited much weaker inhibition activity. This inhibition pattern is not specific to only alkanols but also that of alkenals and fatty acids. The longer chain ($>C_{12}$) alkanols are soluble in the membrane phospholipid, and is thought to be incorporated into the hydrophobic domain of the membrane. In contrast, the shorter chain ($<C_9$) alkanols enter the cell by passive diffusion across the plasma membrane.[7] It seems that only amphipathic medium-chain (C_9–C_{11}) alkanols act as surface-active compounds (surfactants). It should be remembered that dodecanol exhibited fungistatic activity with MIC of 25 μg/mL but did not show any fungicidal activity up to 1600 μg/mL. This alkanol inhibited the external acidification when tested after 5 min but not after 20 h. More specifically, the acidification inhibitory activity of fungicidal undecanol was gradually enhanced, whereas cells treated with fungistatic dodecanol gradually recovered with time, as shown in Figure 3. Yeast cells appeared to adapt to dodecanol stress, eventually recovering and growing normally, similar to that of other stress.[8]

Based on the data obtained, it seems logical to assume that alkanols act at the lipid–protein interface of H^+-ATPase as nonionic surfactants. It is possible that the absence of a functioning state of the H^+-ATPase could be due to its relative sensitivity to denaturation by alkanols. The binding of alkanols as nonionic surfactants can only involve relatively weak headgroup interactions such as hydrogen bonding. It is suggested that the intrinsic proteins of the membranes are held in position by hydrogen bonding, as well as by hydrophobic and electrostatic forces. As proposed above, hydrogen bonds are formed or broken by alkanols and then redirected. As a result, the conformation of the membrane protein may change. In particular, the H^+-ATPase could lose its proper conformation. In addition

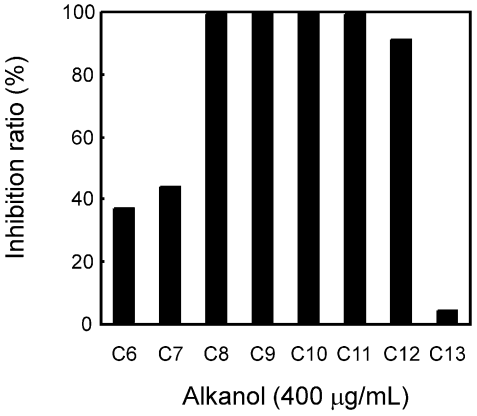

Figure 2. The inhibition of medium acidification by alkanols (400 μg/mL) for short-time incubation. The acidification was assayed for 10 min. The inhibition ratio (%) was calculated as follows: $(1-[H^+]_{inhibitor}/[H^+]_{inhibitor\ free})\times100$.

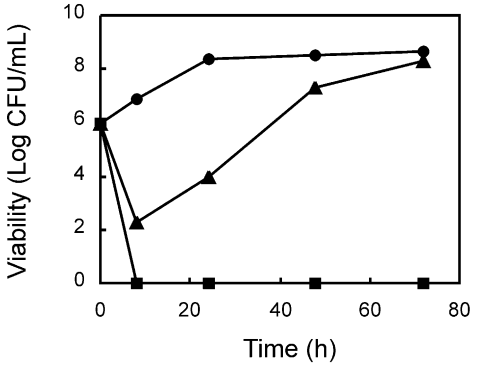

Figure 1. Time–kill curve of undecanol against *S. cerevisiae*. A 16-h culture was inoculated into ME broth containing 0 μg/mL (▲), 12.5 μg/mL (■), and 25 μg/mL (●) of undecanol.

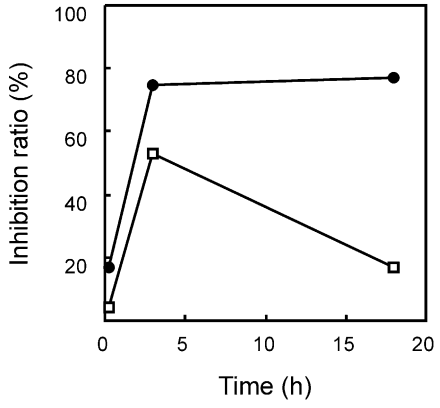

Figure 3. The inhibition of medium acidification by undecanol (●) and dodecanol (□) (400 μg/mL) during long time incubation. The inhibition ratio (%) was calculated as follows: $(1-[H^+]_{inhibitor}/[H^+]_{inhibitor\ free})\times100$.

to H^+-ATPase, alkanols may destroy the native membrane-associated functions of the integral proteins, such as ion channels and transport proteins. This can be supported, for instance, by the fact that alkanols inhibit the uptake of glucose and other nutrients by *S. cerevisiae* in a noncompetitive way. It appears that alkanols, as well as the corresponding alkanals and (2*E*)-alkenals, are nonionic surfactants. Because of the lack of specificity, alkanols act in an unspecific manner on the lipid–protein interaction. This can be explained as the amphipathic medium-chain alkanols are considered as more appropriately balanced nonionic surfactants and more strongly disrupt the lipid-protein interface in *S. cerevisiae*. The shorter-chain ($<C_9$) alkanols enter the cell by passive diffusion across the plasma membrane.[7] In contrast, the longer-chain ($>C_{12}$) alkanols are more soluble in the membrane phospholipid and is thought to be incorporated into the hydrophobic domain of the membrane[9] without perturbing the lipid.[10] This can be supported by the partitioning of radiolabelled long-chain alcohols into biological membranes[11] and lipid bilayers.[12] The cutoff in antifungal activity observed could be due to a corresponding cutoff in the absorption of long chain alcohols into lipid-bilayer portions of membranes.[9] It should be noted that the carbon number for cutoff slightly varies by experimental conditions, tridecanol shows no antifungal activity against *S. cerevisiae* under any conditions. The precise explanation for the role of alkyl chain length—which must be related to antifungal activity—still remains obscure.

It appears most likely that the nonspecific antimicrobial mechanism of alkanols is due to their nonionic surface-active properties. The common nature among these alkanols should be considered in that the electron negativity on the oxygen atom forms an intermolecular hydrogen bond with a nucleophilic group in the membrane, thereby creating disorder in the fluid bilayer of the membrane. The fluidity of the cell membrane can be disturbed maximally by hydrophobic compounds of particular hydrophilic hydroxyl group. They could enter the molecular structure of the membrane with the polar hydroxyl group oriented into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned into the lipid phase by dispersion forces. Eventually, when the dispersion force becomes greater than the hydrogen bonding force, the balance is destroyed and the activity disappears.[1] In connection with this, the hydrophobic bonding energy between an average fatty acid ester and a completely hydrophobic peptide is approximately 12 kcal/mol. Addition of a hydrogen bond between a peptide and a fatty ester's carbonyl adds another 3–6 kcal/mol. Furthermore, alkanols first approach the binding site with the electron negativity of the hydroxyl oxygen atom. This hydrogen bond acceptor will affect the hydrogen bonds that regulate the permeability of the lipid bilayer. For example, in the lipid bilayer, the hydroxyl group of ergosterol resides near the membrane-water interface and is likely to bind to the carbonyl group of phospholipids.[13,14] Alkanols may function by disrupting and disorganizing these hydrogen bonds. Ergosterol is a major component of the plasma membrane of *S. cerevisiae* and owes its membrane-closing properties to its rigid longitudinal orientation in the membrane. Since ergosterol has a profound effect on membrane structure and function, cell function will be impaired if the hydrogen bond is broken. The similar hydrogen bond-breaking concept was proposed to explain the anesthesia cutoff phenomenon.[2]

Further support for the surfactant postulate was also obtained in experiments that showed a rapid decline in the number of viable cells after the addition of undecanol at the exponential growth-phase as shown in Figure 4. In addition, the effect of undecanol was tested during holding viable cell number in the presence of cycloheximide. This drug is known to restrict cell division by inhibiting protein synthesis; its effect against *S. cerevisiae* cells is fungistatic. Undecanol rapidly killed *S. cerevisiae* cells in which cell division was inhibited by cycloheximide. This observation excludes several modes of action for undecanol such as inhibition of DNA, RNA, protein, or cell-wall component synthesis in vivo. The result observed indicates that the antifungal mechanism of undecanol is due primarily to its surfactant property, although it is not possible to confirm that membrane damage is the only cause of the lethal effect. It can be concluded that the medium-chain alkanols target, in part, the extracytoplasmic region as surfactants. This is highly desirable since they do not need to enter the cell, thus avoiding most resistance mechanisms based on cellular pump.

The time–kill curve study showed that undecanol was fungicidal against *S. cerevisiae* at any stage of growth. Figure 5 shows the effect of undecanol at various growth stages. Undecanol at MFC rapidly reduced the number of viable cells when added to the culture at the exponentially growing culture (10^6 CFU/mL) within the first 4 h. Thus, no viable cells were detected within 2 h after adding undecanol. However, undecanol was not fungicidal when added to the stationary growing culture (10^8 CFU/mL). This alcohol rapidly reduced the number of viable cells, but slowed thereafter and complete

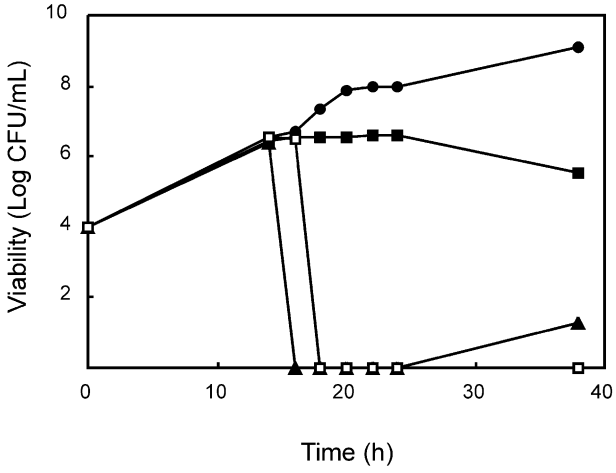

Figure 4. Fungicidal effect of undecanol in cycloheximide treated cells. After 10^4 cells were incubated in ME broth for 14 h, compounds were added as follows; 50 μg/mL cycloheximide (■), 25 μg/mL undecanol (▲), no compound (●). After further 2 h incubation, undecanol was added in cycloheximide treated cells (□).

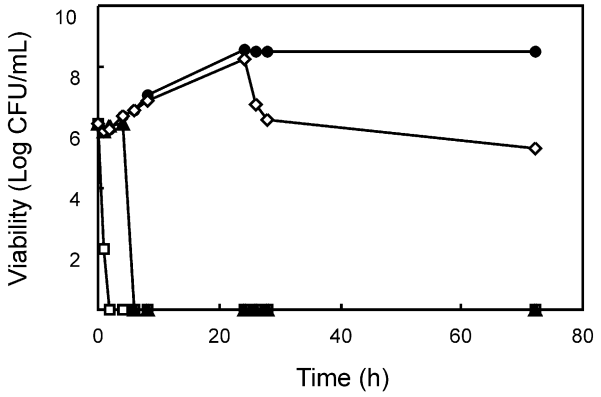

Figure 5. Effect of undecanol (25 μg/mL) at the various growth stages of *S. cerevisiae*. A 16 h culture was inoculated into ME broth. Undecanol was added at 0 h (□), 4 h (▲), 24 h (◇), and control (●).

lethality did not occur. As the number of viable cells increases, the amount of drug's molecules needs to be increased in order to retain the fungicidal activity. The results indicate that undecanol disrupts the lipid–protein interface nonspecifically as a surfactant rather than in a direct interaction with specific target proteins such as cell-surface receptors or signal transduction proteins.

The same series of alkanols were recently found to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver. The potency increased with increasing chain length up to undecanol. Given each alkanol's nearly identical effect on State 3 and uncoupled respiration, action is not directly on ATP synthetase, but earlier in the respiratory process. Hexanol and decanol were also assayed against freeze-thawed (broken) mitochondria to distinguish effects on the mitochondrial substrate carrier from that on the electron transport chain. Both alcohols were only weak inhibitors of respiration in broken mitochondria, suggesting that inhibition originates from interference with the dicarboxylate carrier which must transport succinate across the mitochondrial membranes. Alkanols may inhibit this transporter in the inner membrane as nonionic surfactants. The concentrations found to inhibit mitochondrial respiration are slightly lower than those causing fungicidal activity against *S. cerevisiae*, prompting the question whether decreased mitochondrial functions might in some way contribute to *S. cerevisiae* death. It is uncertain, however, if the respiratory inhibition mechanism is the primary mode of antifungal action of alkanols against *S. cerevisiae* because we do not know if alkanols can reach the mitochondria in vivo. The results with mitochondria also support the alkanols' nonionic surfactant concept because enzyme systems related to transport of solutes and electron transfer are located in the inner membrane of the cell envelope.[15]

On the other hand, *S. cerevisiae* is a facultative anaerobic organism that is able to survive without a functional respiratory chain, by falling back on the fermentation of sugars to supply its energy demand. This latter mechanism is used preferentially, since when a combination of fermentable and non-fermentable carbon sources is available, respiration is greatly reduced and fermentation accounts for the major fraction of sugar catabolism. *S. cerevisiae* is thus able to rapidly adjust its metabolism to its environment and in particular to the availability of carbon sources. Alkanols also inhibit the growth of *S. cerevisiae* growing on non-fermentable carbon sources such as ethanol-, lactate-, acetate- and glycerol-containing media. As surfactants, alkanols exhibit fungicidal activity when *S. cerevisiae* is growing both fermentable and non-fermentable condition.

Table 2. pH Effect of fungicidal (MFC) activity (μg/mL) of undecanol, sorbic acid, geraniol against *S. cerevisiae*

pH	Undecanol	Sorbic acid	Geraniol
3	12.5	800	800
5	25	1600	800
7	12.5	>1600	800
9	12.5	>1600	800

Safety is a primary consideration for *S. cerevisiae* control agents, especially concerning their use in food products that may be utilized in unregulated quantities on a regular basis. The phytochemicals characterized as antifungal agents against *S. cerevisiae* from edible plants should be superior to non-natural preservatives. Incidentally, alcohols are among the most versatile of all organic compounds; free and esterified alcohols occur widely in nature. In addition, alcohols have another superior property as antifungal agents compared to commonly used antifungal agents such as sorbic acid and benzoic acid. As a weak acid antifungal agent, the activity of sorbic acid is pH dependent and increases as the pH of the substrate decreases[16] as shown in Table 2. At higher pH values (>5), sorbic acid did not show any antifungal activity up to 1600 μg/mL due to a higher degree of dissociated molecules. In contrast, undecanol and geraniol are not influenced by pH values.

The surfactant concept can be extended to answer many other questions related to membrane-bound proteins. For example, there has been a long-standing debate on whether alkanols produce their effects in the central nervous system by acting on lipids or on proteins.[17] Anesthesia involves many membrane-bound proteins such as synaptosomal ATPases and acetylcholine receptor.[18,19] Alkanols may alter these membrane-bound proteins in similar fashion, by disrupting and disorganizing the hydrogen bonds at the lipid bilayer–protein interface.

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