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Alkamides from *Echinacea* disrupt the fungal cell wall-membrane complex

I. Cruz [a], J.J. Cheetham [a], J.T. Arnason [b], J.E. Yack [a], M.L. Smith [a,*]

[a] Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada
[b] Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario K1N 6N5, Canada

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ABSTRACT

We tested the hypothesis that alkamides from *Echinacea* exert antifungal activity by disrupting the fungal cell wall/membrane complex. *Saccharomyces cerevisiae* cells were treated separately with each of seven synthetic alkamides found in *Echinacea* extracts. The resulting cell wall damage and cell viability were assessed by fluorescence microscopy after mild sonication. Membrane disrupting properties of test compounds were studied using liposomes encapsulating carboxyfluorescein. Negative controls included hygromycin and nourseothricin (aminoglycosides that inhibit protein synthesis), and the positive control used was caspofungin (an echinocandin that disrupts fungal cell walls). The results show that yeast cells exposed to sub-inhibitory concentrations of each of the seven alkamides and *Echinacea* extract exhibit increased frequencies of cell wall damage and death that were comparable to caspofungin and significantly greater than negative controls. Consistent with effects of cell wall damaging agents, the growth inhibition by three representative alkamides tested and caspofungin, but not hygromycin B, were partially reversed in sorbitol protection assays. Membrane disruption assays showed that the *Echinacea* extract and alkamides have pronounced membrane disruption activity, in contrast to caspofungin and other controls that all had little effect on membrane stability. A Quantitative Structure-Activity Relationship (QSAR) analysis was performed to study the effect of structural substituents on the antifungal activity of the alkamides. Among the set studied, diynoic alkamides showed the greatest antifungal and cell wall disruption activities while an opposite trend was observed in the membrane disruption assay where the dienoic group was more effective. We propose that alkamides found in *Echinacea* act synergistically to disrupt the fungal cell wall/membrane complex, an excellent target for specific inhibition of fungal pathogens. Structure-function relationships provide opportunities for synthesis of alkamide analogs with improved antifungal activities.

Introduction

The genus *Echinacea* has been revised to four perennial plant species in the Asteraceae family that are native to the plains of central United States and southern Canada as supported by morphometric, molecular and chemosytematic characters (Binns et al., 2002a). The first recorded use of *Echinacea* was by Native Americans to treat infections, inflammation and pain (Shemluck, 1982), and the phytomedicine was readily adopted by North American settlers and Europeans (Bauer, 2000). Today, *Echinacea* is a top-selling herbal product (Cavaliere et al., 2010) that is primarily used as an immunomodulator and to reduce the duration and severity of cold and flu symptoms, as well as to treat upper respiratory tract and urinary tract infections (Woelkart et al., 2008). There is overall support for claims that *Echinacea* preparations modulate immune and inflammatory responses, are antimicrobial and have antioxidant activities (Barnes et al., 2005). However, the antimicrobial mode of action of compounds in *Echinacea* is not well characterized.

The chemical composition of various plant parts from the three *Echinacea* commonly used as medicines, *E. pallida* var *pallida*, *E. pallida* var *angustifolia* and *E. purpurea*, is well established (Binns et al., 2002b). Three groups of compounds in these *Echinacea* species have pharmacological activity: the caffeic acid derivatives (CADs), the lipophilic alkamides, and the highly polar polysaccharides (Bauer, 2000; Clifford et al., 2002). Of these three groups, CADs and alkamides are the most abundant in ethanolic pharmaceutical preparations of *Echinacea*, and therefore they are the focus of many scientific studies (Toselli et al., 2009). The alkamides are of particular interest due to superior bioavailability when compared to the CADs (Matthias et al., 2004; Woelkart et al., 2005).

Alkamides comprise a diverse group of secondary metabolites that occur in at least ten plant families. At least twenty alkamides

* Corresponding author. Tel.: +1 613 520 3864; fax: +1 613 520 3539.
E-mail address: myron_smith@carleton.ca (M.L. Smith).

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are present in root extracts of *E. pallida* var *angustifolia* and *E. purpurea* as major constituents of the lipophilic fraction (Bauer and Remiger, 1989). They are isobutylamides of unsaturated fatty acids, mostly derived from undeca and dodecanoic acids that differ by the length of the carbon chain, the degree of saturation, and position of saturated carbon bonds. The pair of isomers dodeca-2E, 4E, 8Z, 10E/Z-tetranoic acid isobutylamides (hereafter referred as the "tetraenes") are the most abundant alkamides found in ethanolic *Echinacea* preparations (Bauer and Remiger, 1989).

This study focuses on determining the mode of action of *Echinacea* as an antifungal and the role of the alkamides, if any, in *Echinacea*'s antifungal activity. A previous study used an array of *Saccharomyces cerevisiae* gene deletion mutants to demonstrate that strains with mutations in genes involved in cell wall functions were especially inhibited by *Echinacea* extracts (Mir-Rashed et al., 2010). This finding was notable since cell wall structure represents an attractive target for antifungal therapy research (Onishi et al., 2000). The possibility that compounds in *Echinacea*, and more specifically the alkamides, can be exploited as antifungals with selective cytotoxicity or could lead to the discovery of new antifungals is of broad interest for the control of fungi that are pathogens or involved in materials spoilage.

Here, we test whether exposure to *Echinacea* ethanolic extract (ECH) and each of seven chemically synthesized pure alkamides found in *Echinacea* (Matovic et al., 2007, 2011), including the abundant tetraenes, disrupt fungal cell wall structure/functions. Cell wall damage was monitored by an established method involving mild sonication (Ruiz et al., 1999) and fluorescence microscopy with *S. cerevisiae* cells and by a sorbitol protection assay. Since cell wall functions are intrinsically related to the cell membrane in fungi, we also investigated the membrane disruption properties of *Echinacea* extract and the synthetic alkamides using a membrane leakage assay. A classic Hansch approach to QSAR modeling (Hansch and Lien, 1971; Hansch and Clayton, 1973) identified key structural components that correlate with antifungal efficacy of alkamides tested, and can be used to direct studies of synthetic analogs.

Materials and methods

S. cerevisiae growth conditions

S. cerevisiae strain S288C (MATα *SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*) was inoculated from glycerol stocks to minimal synthetic complete (SC) medium [2% glucose (BDH, Toronto, Canada), 0.67% yeast nitrogen base (Becton, Dickson and Co., Sparks, MD)] with or without 2% agar (Bioshop Canada, Burlington, ON).

Antimicrobial agents

Echinacea extract was prepared in 70% ethanol as previously described (Mir-Rashed et al., 2010). Extracts were filtered (0.2 μm, nylon) prior to HPLC separations and alkamides were identified by comparison with reference standards (see Graphic Abstract; Merali et al., 2003). *Echinacea* stock solutions (0.7 mg/l in 70% ethanol) were prepared immediately prior to use. The synthetic alkamides used in this study (Matovic et al., 2011; Table 1), were donated by Dr. R. P. Lehmann (Integria Healthcare, Queensland, Australia). Weighed quantities of alkamides were resuspended in 70% ethanol to a concentration of $\sim 12 \times 10^3$ mg/l and stored in the dark at −80 °C until use. Caspofungin acetate (CAS) was provided by Merck & Co. (Rahway, NJ) and was used as a positive control for cell wall disruptive agents in our experiments. CAS is a FDA-approved member of the echinocandin class of antifungals that disrupts cell wall integrity (Letscher-Bru and Herbrecht, 2003). CAS stock solutions were prepared in distilled water at a concentration of 40 mg/l and stored in the dark at −20 °C until use. Two aminoglycosidic antifungals, hygromycin B (HYG, Roche Applied Science, Mannheim, Germany) and nourseothricin dihydrogen sulfate (NAT, WERNER BioAgents, Jena, Germany), were used as negative controls as their mode of action is not directly related to cell wall stability but, rather, both inhibit protein synthesis (McGaha and Champney, 2007). HYG and NAT solutions were prepared in distilled water immediately before use at concentrations of 8×10^3 mg/l and 10×10^3 mg/l, respectively.

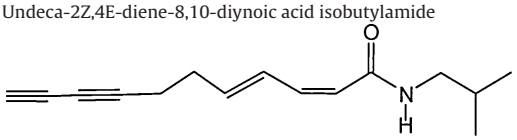
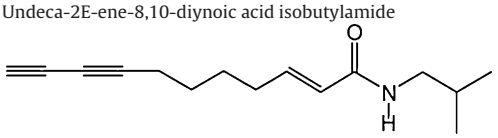
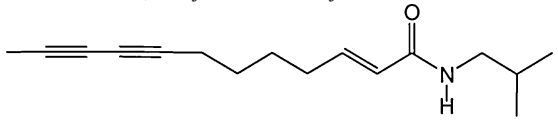
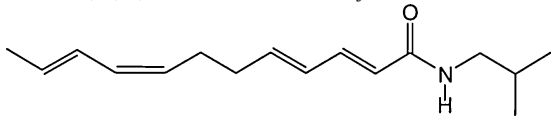
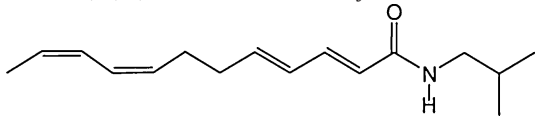
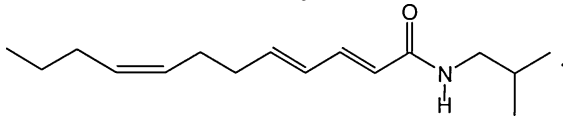
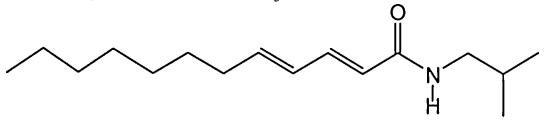
Minimum inhibitory concentration (MIC) determinations

The antimicrobial activities of compounds were tested against 10^3 colony forming units (CFU)/ml of yeast S288C to determine MIC using a microdilution broth assay (Rex et al., 1993). Microplates were incubated stationary for 48 h, at 30 °C before MIC values were recorded as absence of visible growth. MIC assays were performed independently at least three times for each compound.

Cell wall disruption, viability and sorbitol protection assays

Based on preliminary cell wall damage assays using concentrations that caused 20, 40 and 60% growth inhibition, a concentration that resulted in 40% growth inhibition was chosen as optimal for the cell wall disruption assay. Percent growth inhibition was determined by drop out plates, in which yeast cultures were 10 times serially diluting with fresh medium and 10 μl of each dilution was spotted onto agar medium. The ratio of CFU/ml of treated/non-treated samples was then determined after 36–48 h at 30 °C. For the cell wall damage assay, yeast cell suspensions were inoculated from a late exponential phase culture in SC medium and grown to mid-log phase by overnight incubation at 30 °C and 150 rpm, and the cell density was then adjusted to $\sim 10^7$ cell/ml. A 10 μl aliquot was removed for subsequent enumeration of colony forming units by a drop out plate assay. Volumes of 0.4 ml of the cell culture were transferred to 1.5 ml sterile microcentrifuge tubes, and test compound was added to obtain a ~40% growth inhibition. An equivalent volume of 70% EtOH was added to another tube as carrier control. The cells were incubated at 30 °C, 150 rpm for 4 h, after which an aliquot of 90 μl was transferred from each tube to a new microcentrifuge tube and set aside as the non-sonicated control, and a second drop plate assay was subsequently performed to ensure that this 4 h incubation with inhibitors resulted in a 40 ± 5% growth reduction of yeast. The remaining cells were subjected to mild sonication (Vibra Cell VCX130, Sonic & Materials, CT, USA) on ice for 2 min (3 mm microtip probe, 20% amplitude, pulse 15 s, interval 3 s), an established method for monitoring yeast cell wall disruption (Ruiz et al., 1999). After sonication, all samples (non-sonicated and sonicated) were immediately observed by fluorescence microscopy as follows. Ten μl of yeast cell suspension was added to 2 μl of a solution of 0.1% (w/v in distilled water) calcofluor white M2R (American Cyanamid, Bound Brook, NJ, USA) and 0.1% Evans Blue (Fisher Scientific Co., Limited, Toronto, ON, Canada) on a microscope slide. A coverslip was gently applied before microscopic observations were made with an Axio Imager fluorescence microscope (Carl Zeiss, Toronto, ON, Canada). Two fluorescence channel images were obtained in rapid succession. The first was with the DAPI filter to detect the blue-white fluorescence of calcofluor binding mainly to chitin in the fungal cell wall. The second image was obtained with the Rhodamine filter to detect Evans Blue which fluoresces as a red-pink color inside yeast that are unable to pump the dye out of the cell (i.e. cells with compromised metabolic activity or that are dead). These images were captured using Zeiss AxioVision software (Carl Zeiss MicroImaging GmbH, Germany), processed with Image J (Wayne Rasband, National Institutes of Health, USA), and

Table 1
Molecular properties of alkamides used.

Code name	Molecular structures and chemical names	Lipinski's "rule of 5" properties			
		Log $P \pm$ SD	MW	nOH nOHNH	Violations
	Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	3.51 ± 0.37	229.32	2	0
Alk2b				1	
	Undeca-2E-ene-8,10-diynoic acid isobutylamide	3.82 ± 0.39	231.34	2	0
Alk3a				1	
	Dodeca-2E-ene-8,10-diynoic acid isobutylamide	4.42 ± 0.35	245.37	2	0
Alk5				1	
	Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	4.37 ± 0.47	247.38	2	0
Alk8E				1	
	Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide	4.37 ± 0.47	247.38	2	0
Alk8Z				1	
	Dodeca-2E,4E,8Z-trienoic acid isobutylamide	4.70 ± 0.48	249.40	2	0
Alk11				1	
	Dodeca-2E,4E-dienoic acid isobutylamide	5.24 ± 0.35	251.41	2	1
Alk12				1	

transferred to Photoshop (Adobe Systems Inc., San Jose CA, USA) for manual enumeration of cell wall damage. The experiment was repeated at least three times for each compound/treatment tested and approximately 1000 cells were examined from 5 to 8 randomly selected fields for each of the three replicate treatments for a total of at least 3000 cell observations per compound/treatment. Cells were evaluated for cell wall damage and viability, counted and classified in each of three groups: cell wall damaged (CWD), Evans Blue stained (EB+), and healthy cells (HC) that could be budded/multi-budded and single non-divided daughter cells (Fig. 1). Experimental data was subjected to analysis of variance (ANOVA) with a post hoc Tukey HSD test ($\alpha = 0.05$) using JMP (v8.0.2, SAS Institute Inc., Carry, NC, USA).

A sorbitol protection assay (Onishi et al., 2000) was done with three representative alkamides, alk3a, alk5, and alk11, and caspofungin and hygromycin B to further test cell wall disruption activities. For this, a microdilution broth assay was performed with each inhibitor, with and without the addition of 0.8 M sorbitol as an osmotic stabilizer. MICs were determined after 48 h as described above.

Membrane disruption assay

Large unilamellar liposomes (LUVs) were prepared using total yeast lipids or dioleoylphosphatidylcholine (DOPC) (Avanti Polar Lipids, AL, USA), which were dissolved in chloroform/methanol (2:1, v/v) in a glass vial. Solvent was removed by blowing nitrogen gas into the vial followed by desiccation under a vacuum. Lipid films were hydrated with 50 mM carboxyfluorescein (CF) (Molecular Probes, OR, USA). Large unilamellar vesicles were prepared by extrusion through polycarbonate filters (pore diameter 100 nm) in an extrusion device (Avestin, Ottawa, Canada). Nonencapsulated CF was removed by gel filtration on a Sephadex G-75 column (QC, Canada) using iso-osmotic buffer (100 mM NaCl, 10 mM HEPES, pH 7.4). The release of CF from LUVs was measured using a 96 well fluorescence plate reader, with excitation and emission

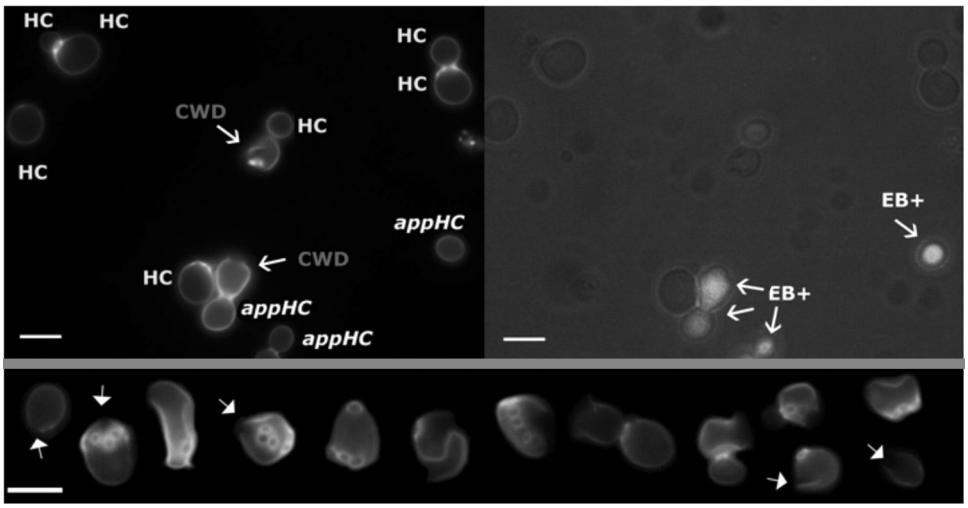


Fig. 1. Exposure of yeast to *Echinacea* extract results in cell wall damage and death. Yeast cells were treated with *Echinacea* extract for 4 h prior to sonication and then simultaneously stained with CFW (top left panel-DAPI filter) and Evans Blue (top right panel-Rhodamine filter) immediately before fluorescence microscopy. In these images the frequency of yeast showing CWD = 2/12 and EB+ = 4/12. HC = healthy cell, appHC = apparently healthy cell (cell wall intact but EB+), CWD = cell wall damage, EB+ = Evans Blue stained. The bottom panel shows a collection of yeast cells stained with CFW displaying cell wall damage due to exposure to *Echinacea* ethanolic extract. Yeast cells showed cell wall damage ranging from small defects (arrows) to complete collapse of cell structure. In all panels, bar = 5 μm.

wavelengths of 485 nm and 520 nm, respectively. Fluorescence intensity was recorded before addition of test compounds, one hour after addition, and after adding Triton X-100. Leakage is expressed as a percentage relative to the total amount of CF released by addition of 1% of Triton X-100, which represented 100% leakage.

Quantitative structure relationship analysis (QSRA)

A classic Hansch approach to QSAR modeling (Hansch and Clayton, 1973) was used to investigate the correlation between predicted lipophilic properties and the *in vitro* antifungal activity of alkamides listed in Table 1. The online tools Molinspiration Cheminformatics and ALOGPS 2.1 were used to calculate the lipophilicity descriptor log *P* (octanol–water partition coefficient) for each alkamide. The log *P* values of alkamides (Table 1) were plotted against the values for the biologic activity measured (log 1/*C*) and subsequently evaluated by regression analysis. In this case, the biologic activity measured (*C*) is the concentration in mol/l to cause a 40% growth inhibition (Table 2). The two sets of data were plotted and the QSAR model was developed using a non-linear regression analysis by the method of least-squares (GraphPad PRISM® version 5, San Diego, CA, USA. www.graphpad.com). The drug-likeness properties of the alkamides used in this study, as defined by Lipinski's rule of 5 (Lipinski et al., 2001), were obtained using the JME Molecular Editor (Molinspiration Cheminformatics, Slovensky Grob, Slovak Republic; http://www.molinspiration.com/) and are summarized in Table 1.

Results

Strains of *S. cerevisiae* with deletions of genes important to cell wall functions were identified as being super-sensitive to *Echinacea* extracts (Mir-Rashed, 2010). It was also established that among the compounds found in *Echinacea* extracts, the alkamides demonstrate pronounced membrane permeability and bioavailability (Matthias et al., 2004; Woelkart et al., 2005). These observations lead us to test whether alkamides from *Echinacea* exert antifungal activity by disrupting fungal cell wall/membrane functions.

Antifungal activity of alkamides

As the first step in this study, the relative growth inhibition caused by each test compound was determined by microdilution and drop out plate assays (Table 2). The pure alkamides tested caused a 40% growth inhibition when concentrations ranged from 105 to 305 mg/l. This was comparable to that of hygromycin (HYG) but higher than the required amount of nourseothricin (NAT) and caspofungin (CAS).

Cell wall disruption activity by test compounds

An established cell wall disruption assay was used (Ruiz et al., 1999), the results of which were corroborated with a sorbitol protection assay (Onishi et al., 2000). The basis of the former assay is that exposure to a cell wall disrupter at a sub-inhibitory concentration will result in yeast that are more susceptible to cell wall damage by mild sonication. Breaks to the cell wall and loss of metabolic activity resulting from the combined exposure to the test compounds and mild sonication were observed and enumerated by fluorescence microscopy using calcoflour white and Evans Blue, respectively (Fig. 1). The frequency of cells with obvious breaks in the cell wall (CWD) and/or that were stained with Evans Blue (EB+) were counted and statistically analyzed (Fig. 2; Table 2) for non-treated/non-sonicated (−T/−S), non-treated/sonicated (−T/+S), treated/non-sonicated (+T/−S) and treated/sonicated (+T/+S) samples. Cell wall damage (CWD) frequencies of less than 0.15% were observed for all non-sonicated samples with the exception of the caspofungin-treated non-sonicated group (casT/−S), where the detected CWD frequency was significantly greater at ~0.8% (data not shown). Caspofungin specifically disrupts fungal cell walls by inhibiting β-1,3-glucan synthase (Letscher-Bru and Herbrecht, 2003), which might explain these significantly higher CWD frequencies in non-sonicated samples treated with caspofungin. With respect to loss of viability, no significant frequency differences in Evans Blue-positive cells were observed in the non-sonicated samples, with the exception again of a significant higher frequency of EB+ in the caspofungin-treated samples (3.9%) and for alk11T/−S samples (1.8%). All other non-sonicated samples had an EB+ frequency of less than 1.5%

Table 2
Antifungal activity of the test compounds against *S. cerevisiae* S288C.

Compound[a]	MIC[b] (mg/l $\times 10^3$)	GI[c] (40%)	Log 1/C[d] (mol/l)	CWD % (+) S[e]	EB+ % (+) S[f]
CTR	–	–	–	12.2 ± 0.4	14.2 ± 0.5
ECH	70–143	58	–	28.7 ± 0.6	19.2 ± 1.2
Alk2b	0.17–0.35	0.152	3.179	28.1 ± 0.7	21.6 ± 1.0
Alk3a	0.29–0.59	0.218	3.026	27.4 ± 0.9	20.3 ± 0.9
Alk5	0.40–0.81	0.310	2.898	22.4 ± 0.9	21.2 ± 0.9
Alk8E	0.03–0.62	0.260	2.978	21.9 ± 0.9	19.8 ± 1.3
Alk8Z	0.32–0.65	0.271	2.960	20.1 ± 0.6	19.8 ± 1.0
Alk11	0.45–0.89	0.340	2.865	21.9 ± 1.3	20.7 ± 0.7
Alk12	0.45–0.89	0.329	2.885	20.7 ± 0.7	21.6 ± 0.8
CAS	0.70×10^{-4}–1.50×10^{-4}	6.1×10^{-5}	–	20.9 ± 0.7	23.3 ± 0.9
HYG	0.25–0.51	0.215	–	13.1 ± 0.7	14.5 ± 0.7
NAT	7.00×10^{-3}–1.50×10^{-4}	0.006	–	12.0 ± 0.5	14.4 ± 0.7

[a] CTR = no compound control; ECH = *Echinacea* extract; Alk2b to Alk12 = alkamides (see Table 1); CAS = caspofungin; HYG = hygromycin B; NAT = nourseothricin.
[b] MICs are given as a concentration range where upper range value was lowest tested concentration that resulted in no visible growth.
[c] GI (40%) = inhibitor concentration that results in 40% growth inhibition of yeast based on enumeration by drop out plates.
[d] Log 1/*C* = logarithm of the inverse of GI (40%) in mol/l.
[e] Percent cell wall damage after sonication and treatment with each compound.
[f] Percent cells staining with Evans Blue after sonication and treatment with each compound.

(data not shown). The overall low frequencies of CWD and EB+ in the non-sonicated samples indicate that the general health of the yeast cells is not greatly compromised by the assay conditions prior to the mild sonication step. A baseline effect of sonication was observed as untreated control cells subjected to mild sonication (−T/+S) showed about 12% cell wall damage (CWD) and 14% decrease in cell viability (EB+, Fig. 2). Therefore, with the mild sonication protocol used, cell wall damage and loss of viability occurs to some extent, whether or not the cells were subjected to a prior treatment with a compound that disrupts cell wall function(s). In relation to the negative controls, treatment with either aminoglycoside HYG or NAT alone, or accompanied by sonication, did not result in a significant increase of CWD or EB+ frequencies when compared to the carrier control (Fig. 2). These observations validate the use of HYG and NAT as suitable negative controls for this study; both compounds inhibit cell proliferation through interference with protein synthesis but under the experimental conditions used here, neither increases cell wall sensitivity to sonication. The caspofungin-treated sonicated group (casT/+S) had CWD and EB+ frequencies that were significantly greater than that observed in the other control groups −T/+S, hygT/+S and natT/+S (Fig. 2).

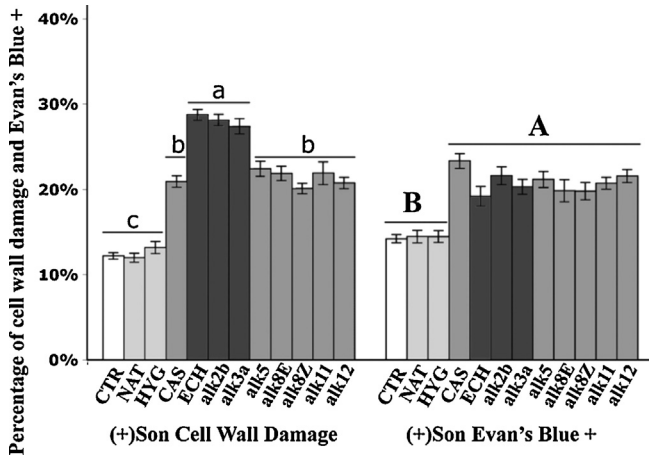


Fig. 2. Effect on yeast cells of exposure to test compound followed by mild sonication. Shown are mean percentages ± SE of cell wall damage (left) and Evans Blue+ cells (right) in sonicated (+)Son test groups with and without compound treatments. Each bar represents at least 3 independent experiments and counts of at least 3000 cells. For each assay, means linked by the same letter are not significantly different from each other (ANOVA, Tukey test $\alpha = 0.05$).

Therefore, caspofungin can be considered a suitable positive control for the cell wall disruption assay.

In comparison to the carrier and negative controls, the group treated with *Echinacea* extract and sonication (echT/+S) showed a significantly greater frequency of CWD and EB+ cells. A CWD of 28.7% was observed in the (echT/+S) group in contrast to 12.2% with the carrier control (−T/+S) as seen in Fig. 2. We then investigated the effect on the yeast cell wall of seven synthetic alkamides that are known to occur in *Echinacea* root extracts. These seven alkamides differ from each other by the length of the carbon chain, the degree of saturation and the positions of saturated carbon bonds along the carbon chain (Table 1). Treatments with each of the seven alkamides followed by sonication (alkT/+S) resulted in a significantly greater frequency of yeast with cell wall damage (CWD) when compared with the carrier control (−T/+S) and negative (hygT/+S and natT/+S) controls (Fig. 2). The percentage of cell wall damage in sonicated samples showed a clear division of the alkamides into two statistically distinct groups: the alkamides alk2b and alk3a together with the crude *Echinacea* ethanol extract (ECH) demonstrated the highest activity, followed by a second group comprising the remaining five alkamides including the "tetraenes" alk8E and alk8Z (Fig. 2).

A sorbitol protection assay further corroborated that alkamides from *Echinacea* disrupt the fungal cell wall. The basis of this assay is that cell wall damage effects should be ameliorated under hyperosmotic conditions. When 0.8 M sorbitol was included in the medium the MICs for caspofungin and all three alkamides tested, alk3a, alk5, and alk11, were respectively increased by 8-, 8-, 4- and 4-fold over no-sorbitol controls. In contrast, inclusion of sorbitol had no effect on the hygromycin B MIC.

QSAR analysis

For the QSAR analysis the predicted lipophilic properties (log *P* – octanol–water partition coefficient) of the seven alkamides studied were plotted against the experimental antifungal activity values (log 1/*C*, where *C* is the alkamide concentration that results in a 40% growth inhibition; Table 2). The data was subjected to a non-linear regression analysis by the method of least-squares resulting in an equation that described ~86% of the activity of the alkamide set (data not shown). The results of the QSAR analysis indicates that log *P* is a key determinant of the pharmacokinetic properties of the alkamides, with the log *P* values of all seven alkamides predicted to be on the more lipophilic side of the curve and with the log *P* values of the most active alkamides (alk2b and alk3a) occurring within the desirable range for orally delivered drug formulations (Hansch

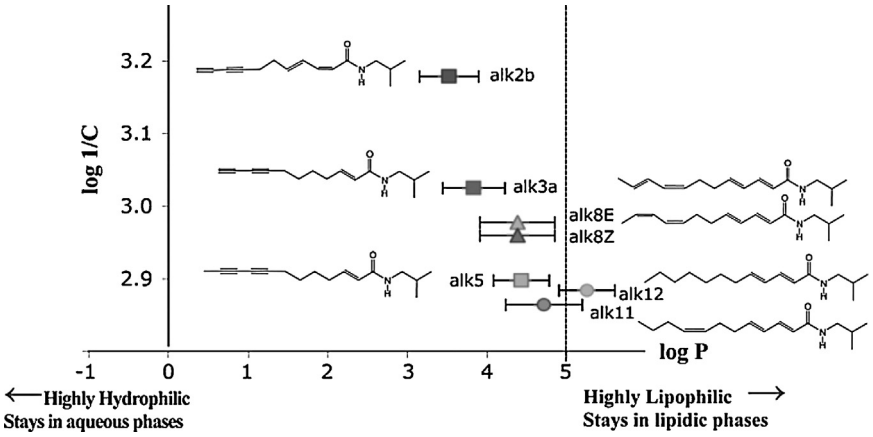


Fig. 3. Relationship between alkamide lipophilic properties (log *P*), structure, and antifungal activity (log 1/*C*). The vertical dotted line indicates where the log $P \leq 5$ of Lipinski's rule of 5 for ideal drugs applies to the alkamide set tested here.

et al., 1968; Vistoli et al., 2008). The alkamides having lowest antifungal activity (alk11 and alk12) are predicted to have log *P* values at the limit of desirable lipophilicity according to Lipinski's rule of 5 (Lipinski et al., 2001).

Membrane disruption by Echinacea and alkamide exposure

The membrane disrupting properties of the test compounds were investigated with a carboxyfluorescein (CF) loaded liposome assay. CF is trapped in liposomes at a self-quenching concentration. In the presence of membrane-disrupting compounds CF is released from liposomes and fluorescence increases due to relief of self-quenching, and can be measured and compared to the fluorescence intensity obtained by complete lyses of the liposomes with a 10% TritonX-100 solution at the end of the experiment. The inset in Fig. 4 shows a concentration-dependent increase in the permeability of liposomes typical of ECH and alkamides. Induced leakage of CF was monitored with liposomes made from DOPC phospholipid, total yeast lipids, and DOPC with ergosterol, a sterol found in fungal cell membranes. Interestingly, *Echinacea* extract was most effective in inducing CF leakage in liposomes made from total yeast lipids suggesting some specificity of ECH toward the disruption of fungal membranes (Fig. 4).

Synthetic alkamides were tested along with CAS, HYG and NAT with liposomes made from total yeast lipids. Whereas little or no leakage was observed with CAS, HYG and NAT controls, the synthetic alkamides resulted in significant leakage of CF from liposomes. This indicates that, while alkamides and CAS both sensitize yeast cell walls to sonication-induced breakage, the alkamides likely have a different mode of action from CAS.

Discussion

Ethanol extracts of *Echinacea* were previously reported to inhibit the growth of fungi, including those associated with mycoses (Merali et al., 2003), and antifungal activity of *Echinacea* extracts is enhanced in yeast strains with deletions of genes involved in cell wall functions (Mir-Rashed et al., 2010). We found that this antifungal activity can be attributed to the presence of alkamides in *Echinacea* extracts, with diynoic alkamides being more effective than dienoic alkamides. We observed that exposure to any of the seven tested alkamides resulted in pronounced sensitivity to sonication-induced cell wall damage that was comparable in effect to caspofungin, an echinocandin antifungal known to interfere with fungal cell wall functions (Letscher-Bru and Herbrecht, 2003). Further supporting that alkamides disrupt the cell wall, similar to caspofungin, sensitivity of yeast to representative alkamides was partly reversed under hyperosmotic conditions. Unlike caspofungin, alkamides from *Echinacea* also had striking activity in membrane disruption assays. Overall these results show that alkamides act as antifungals by disrupting the cell wall/membrane complex.

There is a diversity of alkamides in *Echinacea* (Bauer and Remiger, 1989), and these may offer interesting new insights into how to design compounds that target the fungal cell wall. To further understanding of this structure-activity relationship and the role of substituents in biological activity of alkamides, we carried out a QSAR analysis of the alkamides tested (Fig. 3). The relationship between lipophilicity and biological activity is not usually linear; it is better represented by a parabolic curve where the apex, termed log P_0, represents the ideal balance between hydrophobicity and hydrophilicity, at which maximum biological activity can be achieved when comparing a set of structurally related compounds such as the alkamides (Hansch et al., 1968; Hansch and Clayton, 1973). The parabolic model accounts for the random movement of molecules as they partition in and out of aqueous and lipophilic membrane phases, in moving from the site of application to the site of action (Hansch, 1981).

The first general observation emerging from the structure-activity analysis is that the two alkamides showing the most antifungal activity (alk2b/3a, based on lowest MIC and most cell wall disruption) have an 11-carbon chain (undeca–), as opposed to the less active remaining alkamides that have a 12-carbon chain (dodeca–). This observation indicates that the length of the carbon chain has an effect on the biological activity of alkamides. The alkamides can also be divided in two groups based on molecular structure similarities (Table 1): those carrying diynoic moieties (alk2b, alk3a and alk5) and dienoic alkamides containing two conjugated double bonds (alk8E, alk8Z, alk11 and alk12). Considering that of the three alkamides displaying diyne moieties, alk2b and alk3a were the most effective in inducing cell wall damage, it is intriguing that alk5, differing from alk3a only by an additional methyl group at the diyne terminal, displayed such a drop in activity. In a more detailed analysis of the diynoic group and considering alk2b as the original analog, it is noticeable that a saturation of the double bond at C4 results in the alk3a molecular structure and promotes an increase in lipophilicity and a decrease in antifungal activity. This is also observed when a methyl group is introduced in the diynoic terminal of the alk3a carbon chain resulting in the alk5 molecular structure. In comparing the dienoic alkamides to alk2b, the substitution of the diynoic moiety for the dienoic moiety in conjunction with the terminal methylation has a highly negative

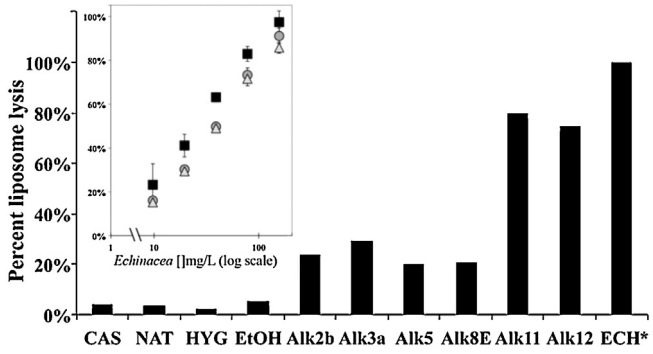


Fig. 4. Lytic activity of test compounds on liposomes encapsulating CF. *Main graph*: lytic activity of assay controls (CAS, NAT, HYG and EtOH, the carrier control), alkamides and *Echinacea* extract on yeast lipids liposomes. *Compounds were used at concentrations that resulted in 40% growth inhibition with exception of ECH, which caused 100% liposome lysis at concentrations that resulted in only 4% growth reduction. *Inset*: hierarchy of lytic effect of *Echinacea* extract on liposomes made from: yeast lipids (square); DOPC + ergosterol (circle); DOPC (triangle). Mean ± SE are based on triplicate measurements.

influence on the antifungal activity. This indicates that the presence of a diynoic moiety enhances the cell wall disrupting activity of the alkamides. However, it should be noted that alk5 has weaker antifungal activity when compared with alk8E/Z and this may be explained by the relative lower lipophilicity of alk8E/Z as conferred by its four double bonds.

A similar correlation between alkamide chemical structure and membrane permeability was reported using Caco-2 cell monolayers as a model for the absorption of drugs by the intestine system (Matthias et al., 2004). This previous study showed that alkamide saturation and methylation on either terminal of the alkamide molecule cause a decrease in permeability through Caco-2 monolayers. Moreover, as a result of the lipophilic nature of the alkamides, these molecules are expected to partition into membranes. Interestingly, we found that 100% leakage was achieved with an ECH concentration that was 20× lower than the concentration used in the cell wall disruption assay (2000–5000 mg/l). The relatively low concentration needed to obtain 100% CF leakage with ECH when compared with that needed for the alkamides individually may indicate a synergistic activity of different alkamides and/or effects of additional compounds in the *Echinacea* ethanol extract.

Intriguingly, analysis of liposome leakage caused by alkamides (Fig. 4) reveals an inverse trend to that previously discussed for the cell wall damage assay where the diynoic alkamides alk2b and alk3a were more effective than the dienoics alk11 and alk12. If alkamides are disrupting the cell wall indirectly by damaging the membrane, this result is an indication that the diynoic alkamides might have just the right hydrophilicity–hydrophobicity balance to permeate the yeast cell wall and reach the cytoplasmic membrane in the *in vivo* cell wall assay, while the more lipophilic alk11 and alk12 might not be as effective at crossing the cell wall barrier to reach the membrane. However, when direct access to membrane is granted, as is the case with our liposome assay, the dienoic alkamides are very effective at disrupting membranes.

The lytic effect of compounds on model bilayer lipid membranes is well documented and several potential mechanisms have been hypothesized, including the "Barrel" model for channel/pore formation and the "Carpet" model for membrane disruption and destabilization (Reddy et al., 2004; Shai, 1999). Amphotericin B is a "gold standard" antifungal that perturbs membrane function by binding to ergosterol and causing channel/pore formation (Esponel-Ingroff, 2008). While our results do not provide direct evidence of any particular alkamide lytic mechanism, it is unlikely that the "Barrel" model for channel/pore formation accounts for the leakage since CF is a relatively large molecule that is not likely released via small pores. We suggest the "Carpet" model for membrane disruption is a more likely model for alkamide activity. In this model, electrostatic interactions between lytic molecules and phospholipid membranes result in accumulation of the compound in the surface of the membrane causing tension between the two lipid layers and destabilization of the membrane to rupture of the liposome.

The fungal cell wall is a multilayered structure encapsulating the cell. This dynamic interface is essential to sustain the morphology and to protect fungi from adverse environmental conditions. To ensure cell wall biogenesis and integrity at all times, a considerable amount of energy and tightly controlled biochemical pathways are necessary, to the extent that about 20% of *S. cerevisiae* genes have a cell wall biogenesis-related function (de Groot et al., 2001). The high demand of the cell wall on gene machinery and its unique structural and chemical make-up, make the cell wall a desirable target for the development of antifungals. In fungi, the plasma membrane sits just inside of the periplasmic space under the cell wall. These three components of the cell wall/membrane complex form the yeast cell envelope and are structurally and functionally interconnected. With transport and assembly of cell wall components occurring throughout the membrane, it is plausible that disruptions of membrane function(s) will affect the cell wall function(s) and vice versa.

Our finding that lipophilicity of the alkamides (log *P*) is a key factor in determining antifungal activity may be used to predict, to a certain degree, the antifungal activity of this class of compounds. Therefore, the QSAR model can be used as a framework to plan specific molecular structure modifications that can lead to designer alkamide-like molecules with much improved biological activity and optimal antifungal effect. Alkamides, and diynoic alkamides in particular, may have promise as therapeutic agents and are of potential interest as a molecular model in the field of naturally derived antifungal drug design.

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