Fungal cell wall disruption by alkamides from *Echinacea*

By

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“The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day.”

Albert Einstein—From the memoirs of William Miller, an editor, quoted in Life magazine, May 2, 1955; Expanded, p. 281.

Abstract

The research presented in this thesis focuses on the study of the antifungal properties, mode of action and molecular structure-antifungal activity relationship of selected alkamides.

Echinacea's antimicrobial and antioxidant activities, and its ability to modulate the immune cells activity have been demonstrated in several studies. The phytochemical composition of *Echinacea* is well established as a complex mix of caffeic acid derivatives, glycoproteins, polysaccharides and the lipophilic alkamides. The complexity of the extract composition makes it difficult to determine if the pharmacological effects of *Echinacea* preparations are caused by a specific compound, a class of compounds or are the result of the many constituents acting synergistically. However, new insights concerning the membrane permeability, bioavailability and molecular mode of action, have renewed interest in the hypothesis that alkamides are implicated in the pharmacological effects of *Echinacea*. Alkamides are secondary metabolites widely distributed in plants and the major lipophilic compounds in *Echinacea* extracts. This large group of closely related unsaturated fatty acid amides are 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. Here I propose to test the hypothesis that the antifungal mode of action of *Echinacea* extracts is through the disruption of the yeast cell wall/membrane complex by alkamides.

Dedication

In memoriam

This dissertation is dedicated to my mother Maria José Linhares Cruz, to my little sisters Artemara and Idjanira, and to a never born sibling I didn't have the chance to get to know. I'm grateful for the few years I shared their presence in this realm. I miss you!

This dissertation is especially dedicated to my father José Artero Cruz, whose life was cut short by heart disease. As a little girl he stilled in me the confidence to live my dreams, such as this one. His absence is deeply felt at this moment of accomplishment in my life.

Dad was the best example of generosity, dignity, simplicity and honesty I ever knew. In truth, everything I know that matters in life I learned from him—A man of few but very wise words. Unfortunately, the ability to listen carefully and to say just the essential is one thing I didn't take from my father. And I could have used it in some occasions! I am the most fortunate to have been my "dad's little girl" and to have had him as role model.

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In memoriam

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To the Sunshines of My Life

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This thesis is also dedicated to my lifelong partner, husband, the love of my life, and above all my very best friend Flávio Caracas Jr. Thank you for being my partner in all the ups and downs and ins and outs of life including this project. This thesis is also yours, because without your patience, understanding and endless love throughout this entire process this work would probably have not even started. You put up with a lot without ever questioning the wisdom of my dream of getting my Master's degree. And finally, I want to tell you how sorry I am that travelling and weekends have been put on hold for the last few years of our lives so I could achieve this goal in my academic pursuits. I promise that eventually, you will get your sailing boat; a canoe is still a boat right?

Aos Raios de Sol da Minha Vida

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I truly believe that our minds are like antique mosaics, exquisite and complex, made out of tiny little colorful tiles, each tile is an impression left by someone that crossed our path. Each one unique in its own way.

Having this in mind, I would like to start by paraphrasing an old song "To sir, with love", which goes like this: "But how do you thank someone who has taken you

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List of Abbreviations

5-LOX – 5-lipoxygenase

AIDS – Acquired immunodeficiency syndrome

appHC – Apparently healthy cell

BMM – Broth microdilution method

CADs – Caffeic acid derivatives

CAS – Caspofungin

CB1/CB2 – Cannabinoid receptors type 1 and 2

CFW – Calcofluor white stain

CFU – Colony forming units

clonNAT/NAT – Nourseothricin

CMM – Caco-2 cell monolayer model, an intestinal barrier model

COX-1/COX-2 – Cyclooxygenase 1 and 2

CWD – Cell wall damage

CWI – Cell wall integrity pathway

CWP – Cell wall protein

DAPI – Fluorochrome 4',6-diamidino-2-phenylindole

Delta-9-THC – Delta 9-tetrahydrocannabinol

DNA – Deoxyribonucleic acid

DHA – Docosahexaenoic acid

DPM – Drop plate method

EB – Evans blue stain

EB+ – Evans blue stained yeast cell

ECH – Echinacea extract

EtOH – Ethyl alcohol

FDA – Food and Drug Administration

GDA – Gene deletion array

GPI – Glycosylphosphatidylinositol

GPI-CWP – GPI-anchored cell wall protein

HC – Healthy cell

HIV – Human immunodeficiency virus

HPLC – High performance liquid chromatography

HYG – Hygromycin B

Log 1/C – Inverse logarithm of the molar concentration of a compound
that causes ~40% growth inhibition

Log P – logarithm of octanol-water partition coefficient

IOFIs – Invasive opportunistic fungal infections

MIC – Minimum inhibitory concentration

MW – Molecular weight

NSAIDs – Nonsteroidal anti-inflammatory drugs

Pir – Protein member of the PIR gene family

(Proteins with Internal Repeats)

Pir-CWP – A cell wall protein from the PIR family linked through
an alkali-sensitive linkage to β 1,3-glucan.

QSAR – Quantitative structure-activity relationship

r^2 – Coefficient of determination

s – Standard deviation

(-T/-S) – Untreated non Sonicated sample

(-T/+S) – Untreated and Sonicated sample

(+T/-S) – Treated non Sonicated sample

(+T/+S) – Treated and Sonicated sample

URTI – Upper respiratory tract infection

WHO – World Health Organization

1. General Introduction

Antimicrobial resistance — *The major global health challenge of the 21st century*

1.1. A need for new antimicrobials

The emergence of microbial pathogens that are resistant to existing antibiotics combined with an increasing incidence of invasive opportunistic microbial infections has become a major health concern affecting developed and developing countries alike. This microbiological-related health issue is complex, incorporating biological, environmental, geopolitical, socio-economic and demographic factors that are unique to recent times. The reality is that the constant evolution and natural selection for drug resistance is ubiquitous in nature and cannot be stopped. However, the misuse of antimicrobials both inside and outside of human medicine in many instances has facilitated the emergence of drug resistant microbial strains (WHO, 2002a).

Poor socio-economic conditions are partly accountable for the misuse of antimicrobials in human medicine. The lack of access to proper diagnostic and follow up treatment in combination with the unregulated sale of antimicrobials, a common practice in many developing countries, results in self-medication, unnecessary use of antimicrobials as prophylactic and usage of suboptimal dosage and/or substandard drugs (counterfeits, expired or improperly stored). The non-compliance with the full course of therapy is also a factor contributing to the emergence of resistant microbial strains. Non-compliance happens quite often, either because the patient cannot

afford the medication or cannot tolerate the toxicity, particularly when an extended length of treatment is required.

While the antimicrobial misuse in developing nations is for the most part due to underuse, overuse is rampant in wealthy nations. It is estimated that 50% of the global antimicrobial production is used in animal farming, including aquaculture (WHO, 2002b). Here, antimicrobial agents are extensively used not only for the treatment of infectious diseases but also as prophylactics and growth promoting agents. A consequence is that large numbers of livestock are exposed to subinhibitory concentrations of antimicrobials. Even the petroleum industry uses biocides in oil production facilities to inhibit the overgrowth of microorganisms and consequent biofilm formation, which can block pipes and produce toxic and corrosive by-products. This creates a real threat to the environment through potential leakage of these antimicrobials into the environment (Korenblum *et al.*, 2005).

The intensification of global trade and new dynamics in population movements are playing an important role in the spreading of the above mentioned problems as the results of poor antimicrobial use practices in any given country are not self-contained anymore and have become a threat to everyone everywhere (WHO, 2002b).

The rise of invasive opportunistic fungal infections

Along with the rise in antimicrobial resistance observed in the last three decades, there has been the increase in invasive opportunistic fungal infections (IOFIs). Fungal infections have jumped from the tenth to the seventh most prevalent cause of death by infectious disease in the United States from 1980 to 1997 (McNeil *et al.*, 2001). Although relatively rare in healthy individuals, currently,

IOFIs are a major cause of morbidity and mortality in immunocompromised patients where mortality rates range from 50 to 90 percent and approach 100 percent in cases of invasive aspergillosis in solid organ transplant recipients (Aronchick, 2000; McNeil *et al.*, 2001; Minari *et al.*, 2002; Franquet *et al.*, 2004). The surge observed in the frequency of these life-threatening fungal infections appears to be directly associated with an expanding population of immunocompromised individuals. A variety of sub-groups make up this population, including individuals with underlying immunocompromising conditions such as HIV/AIDS, congenital immunodeficiencies, patients under aggressive immunosuppressive treatments used in solid-organ, blood and marrow transplantation, and anti-neoplastic therapies. Another sizable sub-group comprises patients hospitalized with severe illness, including the elderly, pre-term neonates and malnourished infants, whose supportive care includes the use of invasive devices for monitoring, parenteral nutrition and assisted ventilation, just to name a few. Ironically, the successes of recent advances in healthcare technology and antifungal chemoprophylactic practices such as the prescription of prolonged course of broad spectrum antibiotics to transplant patients that have resulted in the improved survival of patients with life-threatening conditions, have likewise increased the number of individuals at high risk of contracting nosocomial infections (Singh, 2001; Franquet *et al.*, 2004; Francois *et al.*, 2005).

The emergence of new fungal pathogens

Immunocompromised patients are highly susceptible to invasive mycoses caused by fungi commonly considered to be of low virulence or non-pathogenic to mammals. The majority of IOFIs are caused by *Candida albicans*, in the case of bloodstream infections; *Aspergillus fumigatus*, in transplant recipients; and

Cryptococcus neoformans, observed in 5-10% of HIV infected patients (Walsh *et al.*, 2004; Richardson, 2005). Although advances in retroviral therapies to treat HIV have reduced its incidence, cryptococcosis is still a problem in developing nations where, due to the cost, triple combination antiretro-viral therapies are not widely available to HIV patients. Nonetheless, epidemiological data collected during the past decade indicate an upward trend in infections caused by less common fungal pathogens such as *Aspergillus* species other than *A. fumigatus*, *Candida* spp. aside from *C. albicans*, members of the Mucoraceae, and *Fusarium* species. *Fusarium* infections are not very common but are particularly severe in cancer patients, where there is a very poor prognosis; a mortality rate of 52-70% and close to 100% in non-recovered neutropenic patients. A variety of other emerging fungi has been recognized to cause IOFIs with high case fatalities rates: *Scedosporium* spp., *Acremonium* spp., *Trichoderma* spp., *Trichosporon* spp., among others (Groll and Walsh, 2001; Pfaller and Diekema, 2004; Walsh *et al.*, 2004). These life threatening fungal infections have non-specific symptoms making diagnosis difficult and many are identified only at autopsy. The related infections are also usually difficult to treat, as many emerging fungal pathogens are already resistant to the existing anti-fungals (Richardson, 2005). OIFIs typically occur in conjunction with other conditions, therefore the cost of treating these infections is difficult to calculate but it is estimated at \$2.6 billion/year in the United States alone (Wilson *et al.*, 2002).

In view of the recent epidemiological trends of an ever-growing population of immunocompromised hosts, the rise in the incidence of uncommon opportunistic mycoses and antifungal resistance, it is clear that the prevention and treatment of OIFIs will remain a major challenge that needs to be addressed urgently through

the commitment of more resources to the discovery and development of novel, more effective, safer and less toxic antifungal therapeutics. Besides the human health issue, the search for new antimicrobials is driven also by the need to ease the severe economic impact caused by fungal plant pathogens and fungal contamination and spoilage of crops, food, animal feed and raw materials that plagues agriculture, forestry, fishery and food industry in general.

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The topic of this thesis is investigation of antifungals from *Echinacea*. In the following sections I describe some necessary background on antimicrobials and then focus specifically on the yeast cell wall and antifungals from *Echinacea* that are thus relevant to the topic of this thesis.

1.2. Current antimicrobial research

When misguided perception meets reality

“Even if the pharmaceutical industry were to step up efforts to develop new replacement drugs immediately, current trends suggest that some diseases will have no effective therapies within the next ten years” – WHO, Fact Sheet #194, 2002.

It is reasonable to say that the mid-1900s was the golden era of antimicrobial discovery. Since then, a misguided ongoing perception that infectious diseases have been conquered has brought the development of novel antimicrobials to a standstill (WHO, 2002a). With the exception of the development of very expensive antiretroviral HIV drugs and a few antifungals, the pharmaceutical industry has since pursued more profitable endeavors in the treatment of chronic conditions such as cancer, diabetes, heart diseases, depression, mood disorders and erectile dysfunction. The result of decades of under investment is that researchers now have to play catch-up to microbial evolution. Needed for this is a greater understanding of the mechanisms of action of antimicrobials and novel approaches to identify unique antimicrobial drug targets.

1.2.1. Bio-prospecting for new antifungals

Searching the shelves of Nature's cupboard for answers

There was a time in our short history on this planet when nature was the only source of remedy for all our maladies. This is still the reality for the majority of the population in developing nations where access to modern medicine is a luxury. It is estimated that 80% of the population in developing countries in Africa and Asia rely

on traditional medicine for health care (WHO, 2008). Moving along the timeline of the modern era, as new knowledge of chemistry and biology and advances in synthetic organic chemistry are acquired, the development of the modern pharmaceuticals, better known as prescription drugs, became possible. Notwithstanding our advances in science, the discovery and development of novel pharmaceuticals remains a complex, time consuming and very expensive business. As a case in point, it takes about 10–15 years and US\$800 million to US\$1 billion to bring one pharmaceutical out of 10,000 potential compounds from basic pharmacological research to the market (PhRMA, 2007). Therefore, more effective strategies are required to streamline and reduce time and cost of drug development.

1.2.2. Reverse pharmacology

Studying the ethnopharmacology of natural products

A very promising and proven strategy is the reverse pharmacology approach; drug development through study of the ethnopharmacology of natural products. In this approach, traditional knowledge is used as a “search engine” to tap into generations of accumulated knowledge of indigenous peoples and healers. This approach may shorten the long list of potential drug candidates and reduce the possibility of undesirable side effects that are discovered only in final stages of clinical trials. The screening of randomly collected samples of plants and microorganisms in the hope of finding organic compounds with biological activity has also shown promising results.

The above two approaches are not original ideas. What is original is that the powerful, innovative and highly automated biotechnologies and interdisciplinary expertise that has been developed over the past decades, such as bioinformatics, cheminformatics and pharmacogenomics, can now be used to expedite the discovery of new drugs by either process. Naturally-derived compounds of interest can be more easily isolated by computerized chromatographic methods and their molecular structure can be determined by spectroscopic methods. Biological activities of molecules can be predicted by quantitative structure-activity relationship (QSAR) analysis. Molecular targets can be inferred by analysis of the activities of similar molecules with known mode of action. Synthetic analogs can be designed by sophisticated molecular modeling software that relies heavily on computer power. Entire genetic libraries of model microorganisms, such as *S. cerevisiae* and *E. coli*, with complete genomic DNA sequences at our disposal, can be used in high-throughput functional genetics technologies to elucidate the molecular mechanism of action of compounds (Galván *et al.*, 2008). Still, when it comes to the screening of natural products, researchers are faced with a multitude of compounds with potentially useful pharmacological activity. Hence the need for efficient screening assays. From initial screening of a natural product, pure extract preparation, to the isolation of potentially useful chemical structures and final molecular modification or synthetic analogs production, it remains a long way to the clinical trials phase and final regulatory approval.

1.2.3. Finding biological active compounds in nature

Hard work and serendipity in antifungal discovery

Remarkably, either by design or by serendipity, pharmaceuticals derived from natural products make up approximately 50% of all drugs in use today. Most of these natural products come from plants and microorganisms, with 25% derived from plants sources (Balandrin *et al.*, 1993). From the numerous alkaloids of the opium poppy (*Papaver somniferum*) that include morphine and codeine, to the yew tree (*Taxus brevifolia*) from where the best selling (peak sales of US\$1.6 billion in 2000) antitumor agent Taxol was derived (Stephenson, 2004), medicinal plants have proven to be a reliable source of biologically active molecules (Cowan, 1999; Ficker *et al.*, 2003). For a review of the new drugs derived from natural products in the last 25 years see Newman and Cragg (2007). In the realm of the microorganisms, the classic example of drug discovery by serendipity was in 1928, when Alexander Fleming characterized the compound considered as the first antibiotic: penicillin from *Penicillium chrysogenum* (Diggins, 2000).

Several antifungal compounds have been discovered from microbial sources as well. One example was the discovery of the antifungal griseofulvin from *Penicillium griseofulvum* in the 1960s. This is the first reported case where a secondary metabolite of a fungus is used to inhibit the growth of another fungus. Griseofulvin inhibits mitosis by blocking the assembly of microtubules, but its use is limited to the treatment of dermato-mycosis (Odds, 2003). Perhaps the most notable example of microbial bio-prospecting for antifungals lead to the discovery in 1955 of the polyene amphotericin B from a gram-positive filamentous soil bacterium,

Streptomyces nodosus. This species was isolated from a soil sample taken from the Orinoco River region in Venezuela (Trejo and Bennett, 1963). Polyenes attach preferentially to ergosterol, the principle steroid compound of the fungal cell membrane (as opposed to the sterol in human cell membranes), and create channels that result in membrane leakage and a change in osmolarity from which the cell cannot recover (Ghannoum and Rice, 1999). For nearly 30 years, amphotericin B was the only choice to control invasive mycoses until the discovery of a new synthetic class of antifungals: the azoles. Azoles, such as fluconazole and voriconazole, inhibit a late step on the synthesis of ergosterol catalyzed by a cytochrome P450 and result in a defective membrane through the accumulation of toxic sterol intermediates in the cell membrane (Odds, 2003).

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1.3. Selective cytotoxicity and the yeast cell wall

Nonetheless all the latest progress, most currently available antifungals lack selective cytotoxicity. The biochemical similarities between fungal cell and animal cell make it difficult to develop antifungals that are selectively toxic to fungi. However, the fungal cell wall and its assembly line represent a clearly defined difference to the animal cell that can be exploited in the search for antifungals with selective cytotoxicity—A very much desired trait in antifungals.

1.4. A unique antifungal target —The fungal cell wall

Representing ~30% of the dry weight of the fungal cell (Nguyen *et al.*, 1998) the cell wall is a layered structure that encases the fungal cell, but rather than being just a rigid incasing structure responsible for maintaining the cell shape and protecting it from environmental aggressions, the fungal cell wall is a complex and dynamic organelle, constantly being remodeled and regenerated to accommodate the changes that take place according to the cycle of the cell life (budding, mating, sporulation and pseudohyphal formation) or in response to environmental challenges (Cabib *et al.*, 1982; Smits, 2001). Consequently, any major disruption in the biosynthesis, assembly or function of the cell wall is lethal to the fungal cell viability, therefore the cell wall is one of the most desirable targets in the development of new antifungal drugs.

1.4.1. The yeast cell wall bilayered structure

The yeast cell wall bilayered structure (Figure 1) consists of 10–15% protein and 85–90% polysaccharides comprised of three sugars: glucose, mannose, and N-acetylglucosamine. Most of the proteins found in the cell wall are located in the outer layer and are covalently linked to mannan (a polymer of the sugar mannose), hence the classification as mannoproteins. Located in the inner layer are the glucose polysaccharides β -1,3-glucan and β -1,6-glucan, the major structural components accounting for ~60% of cell wall dry mass, and chitin, the β -1,4-linked polymer of N-acetyl-glucosamine which accounts for 1.5–6% of cell wall dry mass. (Nguyen *et al.*, 1998; Klis *et al.*, 2006).

1.4.2. The external layer of the cell wall— Mannoproteins

The external cell wall layer consists mainly of mannoproteins which accounts for 30–50% of the cell wall dry mass (the protein content is only 4–5%; the remaining mass is from highly branched carbohydrate side-chains). The majority of cell wall proteins (CWP) found in the outer cell wall layer of *S. cerevisiae* are GPI-anchored proteins (GPI-CWPs)—In eukaryotic cells many proteins are anchored to membranes by covalent linkage to glycosyl-phosphatidylinositol (GPI) thus located in the extra-cellular side of the plasma membrane or cell wall in the case of the yeast cell; these are known as GPI-anchored proteins. GPI-CWPs are in most cases indirectly linked to β -1,3-glucan through a connecting β -1,6-glucan molecule. Another group of CWPs are the Pir-CWPs; these are thought to link directly to β -1,3-glucan (Zlotnik *et al.*, 1984; Cabib *et al.*, 2001; Klis *et al.*, 2002). The outer layer of mannoproteins protects the fungal cell against enzymatic damage and limits the permeability of the cell wall

acting as a filter for large foreign macromolecules. CWPs are involved in several important cell functions such as mating, flocculation, biofilm formation and strain invasiveness. For a review of cell wall proteins see Klis *et al.* (2002) and De Groot *et al.* (2005).

1.4.3. The inner layer of the cell wall— β -glucans

The inner layer of the yeast cell wall plays a crucial role in the structural integrity of the fungal cell. This layer is an intricate three-dimensional network of glucose polymers, mostly long chains of β -1,3-glucose molecules (85%) and short chains of β -1,6-glucose molecules (15%) and a smaller amount of chitin (Figure 1). The β -1,3-glucan is the major structural component of the cell wall to which the other components are crosslinked forming a elastic scaffold-like structure that encapsulates the fungal cell providing structural support. The combination mechanical strength and elasticity protects the cell from physical damage while the scaffold like cross-linkage provide support for the external layer of mannoprotein as well as an array of cell wall proteins (non-covalent bound, covalently bond and GPI-anchored) whose functions are still to be established.

The cell wall β -1,3-glucan is synthesized by β -1,3-glucan synthase at the plasma membrane. This enzyme is thought to consist of catalytic subunits of Fks1p and its alternative subunits Gsc2p/Fks2p and Fks3p; and a regulatory subunit, a small GTPase Rho1p that plays an essential role in the control of the cell wall synthesis, as it binds and activates the Fks1p catalytic unit. Fks1p shares 88% similarity with its alternative protein Fks2p, and 55% with Fks3p. While a deletion of FKS1 causes a decrease in β -glucan and an increase in chitin and mannoprotein

levels in the cell wall, a deletion of FKS2 causes no obvious cell wall defect. However, cells lacking both FKS1 and FKS2 are not viable, an indication that Fks1p and Fks2p have overlapping functions in the synthesis of the cell wall β -glucan. As far as the role of FKS3, a *fks3* Δ mutant shows no apparent cell wall defects or genetic interactions with FKS1 or FKS2 and thus it has not been implicated in cell wall glucan synthesis (Dijkgraaf *et al.*, 2002; Klis *et al.*, 2002; Klis *et al.*, 2006).

In addition, β -1,3-glucan is synthesized as a linear polymer, but after leaving the plasma membrane, further extension by other enzymes and remodeling results in branching. A well studied enzyme thought to be involved in this remodeling is Gas1p. This 1,3- β -Glucanosyltransferase is attached to the extracellular face of the plasma membrane via a GPI-anchor and has been implicated in remodeling and cross-linking linear β -1,3-glucan chains produced by the β -glucan synthase (Mouyna *et al.*, 2000). A *GAS1* deletion results in a cell with a weakened wall and abnormal morphology. These *GAS1* mutants are larger with a more spherical shape and exhibit a higher percentage of budded and multiple budded cells that form aggregates that are difficult to disperse. This phenotype may indicate that *GAS1* is involved in the process of separation of the bud from mother cell (Popolo *et al.*, 1993). *GAS1* mutants are known to release β -1,3-glucan and β -1,6-glucosylated wall proteins into the medium (Ram *et al.*, 1998) and are observed to have an increase in chitin deposition and an alternative cross-linking of β -1,6-glucosylated wall proteins to chitin. This probably partially compensates for the defective β -1,3-glucan polymer (Popolo *et al.*, 1997). The remaining cell wall β -glucan is the highly branched β -1,6-glucan. With an average of 130 glucose monomers, the main function of this polymer in the cell wall is to act

like a glue connecting GPI-anchored cell wall proteins to the β -1,3-glucan network. In case of cell wall disruption β -1,3-glucan can also function as an acceptor site for chitin, thus interconnecting all major cell wall components (Klis *et al.*, 2006).

1.4.4. The inner layer of the cell wall— Chitin

Chitin, a β -1,4-linked linear polymer of N-acetylglucosamine, is uniformly dispersed in the lateral walls of the yeast cell and is most abundant in the chitin ring seen in and around the bud scars. The total chitin level in the yeast cell wall, including the bud scar chitin is 1–2%. The lateral wall chitin level is only 0.1–0.2%. However, the amount of chitin can increase up to 20% of the wall dry mass if a compensatory mechanism is triggered in an effort to salvage the cell from severe cell wall impairment such as cell wall mutations or exposure to drugs. Despite its small quantity, chitin is thought to have a central role in septation and it is essential to the survival of cell wall mutants such *gas1* (Klis *et al.*, 2002; Lesage *et al.*, 2005).

1.4.5. The cell wall regulatory apparatus

To maintain the complex structure of the fungal cell wall a considerable amount of energy and a tightly controlled regulatory apparatus is required, which is often referred to as the cell wall integrity pathway. De Groot *et al.* (2001), estimated that about 20% of *Saccharomyces cerevisiae* genes have a cell wall biogenesis-related function. This, together with the fact that the make-up of the fungal cell wall is unique to the fungi, make the cell wall and the cell wall integrity pathway the most desirable targets in the development of new, highly specific antifungal drugs.

Yeast cell wall diagram

Figure 1. Electron micrograph and schematic diagram of the yeast cell wall. Top panel is a transmission electron microscope image showing *S. cerevisiae* cell membrane and wall (Baba and Osumi, 1987). Bottom panel is a diagram of the section selected by a rectangle in the top panel. The outer layer of cell wall proteins (Mannoproteins, GPI anchored and others) and the inner layer comprised of a network of β -1,6-glucan, β -1,3-glucan and chitin are indicated. A schematic of the main proteins involved in the formation of the cell wall, β -1,3-glucan synthase (Fks1/Fks2) and 1,3- β -glucanosyltransferase (Gas1) are also indicated in the bottom panel. The image in the top panel was reproduced with permission of the copyright holder John Wiley & Sons, Inc., from Baba and Osumi, 1987.

1.4.6. Echinocandins and Pneumocandins

The fungal cell wall has been broken

Since the discovery that penicillin acts by disrupting the bacterial cell wall, researchers have been trying to find compounds that disrupt the fungal cell wall. The first step in this direction was taken when echinocandin B, a β -1,3-glucan synthase inhibitor, was isolated from a species of *Aspergillus* in 1974 (Denning, 1997). The breakthrough came in 1989 when MERCK researchers isolated a closely related group of compounds from the fermentation of *Glarea lozoyensis* (Schwartz *et al.*, 1989), a dematiaceous hyphomycete formerly known as *Zalerion arbuticola*, which itself was discovered in water samples from ponds in the valley of the Lozoya River, Spain in 1985 (Bills *et al.*, 1999). The compounds were named pneumocandins by MERCK to imply that *Pneumocystis carinii* and *Candida spp* were the target microorganisms, but they are in fact echinocandins (Denning, 1997).

Echinocandins and pneumocandins represent a highly specific class of antifungal drugs that act by inhibiting the enzyme β -1,3-glucan synthase; this enzyme complex is responsible for the production of β -1,3-glucan, a major component in the cell wall of many fungi as discussed above. As a result of β -1,3-glucan synthase inhibition, fungal cell wall integrity is compromised, leading to abnormal growth, reduced cell wall strength, osmotic instability, and ultimately cell lysis and cell death (Onishi *et al.*, 2000; Denning, 2003). Since its discovery, structural alterations to the lead molecule pneumocandin B0 have produced semi-synthetic derivatives with improved potency and an enhanced spectrum of activity. Caspofungin acetate (Cancidas, Merck/FDA approved 2001) was the first echinocandin approved for treatment of infections by

Candida and *Aspergillus* species that were resistant to traditional antifungals; it has also shown activity against *Pneumocystis pneumonia* (Letscher-Bru and Herbrecht, 2003). In addition to caspofungin, two new echinocandins have been approved for human use: Micafungin (Fujisawa/FDA approved 2005) and Anidulafugin (Pfizer-FDA approved 2006). These echinocandins represent important advances in the treatment of life-threatening fungal infections, as their mode of action is very different from that of any other available antifungal compound. On the other hand, they are not effective against *Cryptococcus*, nor against *Fusarium* so the search continues...

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1.5. Looking for leads in the ethnopharmacology of *Echinacea*

Figure 2. *Echinacea purpurea* (L) Moench

The genus *Echinacea* comprises four species of perennial coneflower plants in the family of Asteraceae that are native to the plains of central United States and southern Canada. *Echinacea* was named after the Greek word for hedgehog—echinos, which refers to the resemblance of its prickly conical seed head to the spines of a hedgehog. Purple varieties of this genus are known as purple coneflower. *Echinacea* taxonomy, ecology, geographic distribution, ethnobotany and history of use have been thoroughly studied and masterfully reviewed by other authors (Felter, 1898; McGregor, 1968; Shemluck, 1982; Kindscher, 1989; Borchers *et al.*, 2000; Binns, 2001; Miller and Yu, 2004; Kindscher, 2006) and, therefore, will not be discussed in depth here. The following account is a summary of these various studies.

1.6. *Echinacea*'s ethnobotany, history of use and clinical trials

1.6.1. Ethnobotany and history of use

For centuries the Great Plains tribes of Native Americans used *Echinacea* preparations by chewing the roots or topically applying a poultice of mashed roots to treat infections, toothaches, sore throats, coughs, wounds, burns, mumps, and venomous bites and stings. Also noteworthy is the fact that the use of medicinal herbs by Native Americans has a strong spiritual healing component, rather than straightforward acknowledgment of the chemical properties of the herbs used (Shemluck, 1982; Kindscher, 1989; Borchers *et al.*, 2000). Many medicinal herbs traditionally used by the Native Americans including *Echinacea* were adopted by early European settlers in North America (Kindscher, 1989). *Echinacea* was introduced to Europe where it became popular in the 18th and 19th centuries. In North America, the Eclectic practitioners (these were somewhere between homoeopaths, allopaths, and physicians) prescribed it as a remedy to treat a wide variety of conditions ranging from upper respiratory tract infections, colds and influenza, urinary tract infections, fevers (scarlet, malaria, typhoid), syphilis, sore throat, blood poisoning, diphtheria and impotence (Felter, 1898).

In the first half of the 20th century the use of antimicrobial herbal medicines, including *Echinacea*, declined when sulfonamides and other antibiotics were introduced thereafter. Later in the same century, *Echinacea* gradually regained popularity largely in Germany where, since then, the bulk of scientific research on *Echinacea* has been done. Nowadays, *Echinacea* preparations are one of the most

popular herbal remedies in North America, primarily for treating colds and the flu. The National Center for Health Statistics survey of 2007 found that the most commonly used non-vitamin, non-mineral natural product used by adults in the United States were: fish oil or omega 3 or DHA (37.4%), glucosamine (19.9%), and *Echinacea* (19.8%). In the case of children, *Echinacea* (37.2%), fish oil or omega 3 or DHA (30.5%) and combination herb pill (17.9%) are the most common natural health products used (Barnes *et al.*, 2008). Furthermore, *Echinacea* herbal preparations ranked 5th in the 20 top-selling herbal dietary supplements in the food, drug and mass market channels (health and natural food stores; food, drug and mass market retailers; warehouse and convenience stores; mail order, radio and television direct sales; internet sales; health professionals and others categories) in the United States in 2009, with \$16 million (USD) in sales and almost 7% increase from 2008 (Cavaliere *et al.*, 2010). Unfortunately, such popularity has come at a price. Although sustainably cultivated throughout the United States, Canada and Europe, over-harvesting of wild *Echinacea* for the herbal industry is creating enormous pressure on the natural population of *Echinacea*, raising a concern that the species most commonly used in the medicinal herb industry, *E. pallida* var *pallida*, *E. pallida* var *angustifolia* and *E. purpurea* may be in danger of depletion beyond recovery and will soon follow the steps of *Echinacea laevigata* and *Echinacea tennesseensis* that are now listed as endangered by the United States Fish and Wildlife Service (Kindscher, 1989, 2006).

1.6.2. *Echinacea's* clinical studies

Presently, the most common medicinal use of *Echinacea* is to prevent, shorten the duration, and treat the symptoms of upper respiratory tract infections (URTIs), such as colds and the flu. The main reason for this recent gain in popularity appears to be *Echinacea's* reported non-specific immunomodulatory properties. Several meta-analysis studies and reviews have been done in an effort to compile the results of hundreds of clinical studies on the effectiveness of *Echinacea* to reduce the incidence and duration of the common cold (Cochrane Database of Systematic Reviews, 2006; Schoop *et al.*, 2006; Shah *et al.*, 2007; Woelkart *et al.*, 2008a; Toselli *et al.*, 2009). The general consensus is that while there is evidence of *Echinacea's* efficacy in treating the common cold and of its preventive properties, there is just too much heterogeneity between clinical trials to make reliable conclusions possible. Problems with trials include: variability in the composition of the *Echinacea* preparations used in clinical trials (use of different species or species combinations, plant parts, extraction methods and combination with another herbal product), and variations in the study designs (virus type—spontaneous acquired or rhinovirus-induced, double blind placebo controlled or not, time of the initiation of treatment, age, number and immunological condition of subjects). Therefore, large-scale clinical trials with precisely standardized study designs are needed to provide definitive scientific evidence of *Echinacea's* clinical effectiveness in the prevention and treatment of common cold.

1.7. *Echinacea's* phytochemistry

The chemical make up of a best seller

Comprehensive research has been done to determine the phytochemistry of the three most medically-used species of *Echinacea*—*E. pallida* var *pallida*, *E. pallida* var *angustifolia* and *E. purpurea*. Much of that research has been done by Bauer (Bauer *et al.*, 1988a; Bauer *et al.*, 1988b; Bauer *et al.*, 1988c, 1989) and Binns (2002) and extensively reviewed in Hall and Schwarz (2002) and Barnes (2005). It is well established that the chemical composition of *Echinacea* spp is a complex mix of hydrophilic compounds, mostly caffeic acid derivatives (CADs—echinacoside, chicoric acid, caftaric acid, cynarin), glycoproteins and polysaccharides; lipophilic components, by and large alkamides and polyacetylenes; volatile essential oils, and a series of other minor constituents such as alkaloids, flavonoids, anthocyanins, and free phenolic acids. It is also well documented that the phytochemical profile of *Echinacea* extracts varies between species and within species depending on harvesting technique, geographic location, parts of the plant used and extraction methodology (Binns *et al.*, 2002; Qu *et al.*, 2005). As a case in point, all three species contain both the hydrophilic and lipophilic components, however, alkamides are unique to the roots of *E. pallida* var *angustifolia* and *E. purpurea* and for the most part nonexistent in *E. pallida* var *pallida* roots in which polyacetylenes are the major lipophilic compounds. In the same manner, composition variation also occurs in the hydrophilic fraction, for instance, while cynarin is exclusive of *E. pallida* var *angustifolia* roots, the CAD echinacoside, used to standardize *E. pallida* var *angustifolia* and *E. pallida* var *pallida* extracts is not found in *E. purpurea* aerial parts in which the CADs chicoric and

caftaric acid are found in abundance (Bauer, 1999). These variations have a practical applicability as unique constituents can be used as marker compounds to enable identification of species and standardization of *Echinacea* products.

1.8. Pharmacological findings — The purple flower power

1.8.1. *Echinacea*'s immunomodulation and anti-inflammatory proprieties

A number of studies have demonstrated a range of biological activities displayed by *Echinacea* preparations, including immunomodulation and anti-inflammatory activities, antioxidant, anti-viral and anti-fungal activities (Bauer, 2000; Hall and Schwarz, 2002; Barrett, 2003; Barnes *et al.*, 2005), but undoubtedly, the most popular therapeutic application of *Echinacea* is as immunomodulator and anti-inflammatory agent, typically taken orally in the form of ethanolic extracts and tinctures to treat upper respiratory tract infections, which are for the most part the result of a viral infection causing inflammation. In an effort to establish the validity of these claims, ethanolic, chloroformic and aqueous extracts from all three commercial species of *Echinacea* (*pallida*, *angustifolia* and *purpurea*) have been examined. These fractions have been tested *in vitro*, *ex-vivo* and *in vivo* and found to have potential to stimulate immune cells activity. Increased phagocytic activity in murine and human polymorphonuclear neutrophils (PMNs) and induction of several inflammatory cytokine secretion by macrophages are among the most reported pharmacological effects of *Echinacea* extracts (Stimpel *et al.*, 1984; Wagner *et al.*, 1988; Bauer, 1999; Rininger *et al.*, 2000; Barrett, 2003; Barnes, *et al.*, 2005).

1.8.2. *Echinacea's* antioxidant properties

The potential antioxidant activity of *Echinacea* has been the object of numerous *in vitro* studies. Facino *et al.* (1995) demonstrated that *Echinacea* protected collagen from free radical damage. In another study, Hu and Kitts (2000) found evidence of free radical scavenging activity in *Echinacea* root extracts with *E. purpurea* extracts showing the lowest antioxidant activity which led them to hypothesize that echinacoside was responsible for the antioxidant activity given that this CAD is lacking in *E. purpurea* extracts. In addition Dalby-Brown *et al.* (2005) have reported free radical scavenging activity in extracts of all three species of *Echinacea* in a study measuring protection of the human low density lipoproteins (LDL) from oxidation. Chicoric acid was considered as having the most antioxidant activity when compared with two other CADs (echinacoside and caffeic acid), and with alkamides and water-extracted polysaccharides. However, it was the mix of the three fractions (chicoric acid, alkamides and polysaccharides) that resulted in the highest percentage of antioxidant activity, indicating that the antioxidant effect of *Echinacea* extracts may be a consequence of a synergist effect of the three classes of compounds.

1.8.3. *Echinacea's* antifungal properties

Apart from numerous studies on the anti-inflammatory activity resulting from modulation of the immune system and subsequent increase in macrophage activation and phagocytosis, additional research has demonstrated that *Echinacea* extracts have direct antifungal activity. In a study by Binns *et al.* (2000), the hexane extract of commercial *Echinacea* preparations consisting of tinctures and tea of *E. purpurea*, and a mix of *E. purpurea* and *E. angustifolia*, and fresh plant parts from

E. purpurea and *E. pallida* were assayed by disk susceptibility tests. These extracts were shown to have antifungal activity against *S. cerevisiae* and several *Candida* species, with some samples showing UVA-light-enhanced antifungal activity—a phenomenon called phototoxicity (Towers *et al.*, 1997). These observations may explain the antimicrobial properties of *Echinacea* in topical preparations. These antifungal activities were corroborated by Merali *et al.* (2003) where extracts from several varieties of *E. pallida* and *E. atrorubens* were tested against clinically important human pathogenic fungi.

The findings from these previously mentioned studies have led to further investigation into the mechanism of action of *Echinacea*'s antifungal activity. In a study by Mir-Rashed and colleagues (2010), ethanolic extracts of aerial parts and roots of *E. purpurea* and *E. angustifolia* were assayed by gene deletion array (GDA) using a set of ~4600 deletion mutant strains of *S. cerevisiae*. Of the ~4600 mutants tested, twenty-three showed consistent evidence of hypersensitivity to *Echinacea* treatments. From this cluster of twenty-three, ten are known to be implicated in cell wall function, six are either related to transcription, protein or amino acid biosynthesis, and the remaining seven mutants are in genes with unknown function. The result of this study indicated that the basis for *Echinacea*'s antifungal properties is by disruption of the fungal cell wall functions. Subsequently to these observations, other experiments designed to investigate the relationship between fungal exposure to *Echinacea* extracts and disruption of fungal cell wall function were carried out. A combination of exposure to *Echinacea* extracts and sonication followed by observation using fluorescence microscopy revealed an increased frequency in fungal

cell death and cell wall damage resulted from treatment with *Echinacea* extracts. These cell wall assays supported the hypothesis, based on GDA results, that *Echinacea* extracts interfere with fungal cell wall functions.

1.9. *Echinacea*'s pharmacodynamics and mode of action studies

As a result of the above mentioned findings, three groups of phytochemicals are considered to be relevant to the pharmacological activities of *Echinacea* extracts—caffeic acid derivatives (CADs), polysaccharides, and lipophilic alkamides (Bauer, 1999). Therefore, research is now being carried out to investigate the pharmacological effects and mechanism of action of these compounds in isolation and in combination. Still, as is the case with many herbal phytotherapies, the complexity of the extract composition makes it difficult to ascertain if the pharmacological effects of *Echinacea* preparations are caused by a specific compound, a class of compounds or are the result of the many constituents acting synergistically.

1.9.1. Polysaccharides — Pharmacological findings

Stimpel (1984) and Wagner (1988) have studied polysaccharides extracted and purified from *E. purpurea* and polysaccharides obtained from *E. purpurea* cell cultures and concluded that they induced macrophage production and activation. However it is very unlikely that polysaccharides play any role in the pharmacological activities of the commonly used *Echinacea* preparations as these compounds are not present in ethanolic extractions but they can't be ruled out as having pharmacological activity in topical applications.

1.9.2. Caffeic acid derivatives (CADs) — Pharmacological findings

Of the several caffeic acid derivatives (CADs), chicoric acid—a major component of *E. purpurea*, is considered to be important to the immunomodulatory activity of *Echinacea*, given that, unlike echinacoside and other CADs, chicoric acid has been shown to stimulate phagocytosis in vitro (Bauer, 1999). However, caffeic acid conjugates have a very low bioavailability (Matthias *et al.*, 2004; Matthias *et al.*, 2005) and are therefore unlikely to be an important contributor to *Echinacea*'s *in vivo* immunomodulating activity. Another characteristic of CADs that has been studied is the antioxidant nature of these compounds, which could have potential applications for the food industry and topical preparations. As mentioned above, Dalby-Brown *et al* (2005) have reported the highest free radical scavenging activity by chicoric acid when compared with all other CADs, and other fractions of *Echinacea* extracts.

1.9.3. Alkamides — Pharmacological findings

Alkamides are secondary metabolites widely distributed in plants. This large group of ~200 closely related unsaturated fatty acid amides are found in as many as ten plant families with the highest levels found in Asteraceae, Piperaceae and Rutaceae. Alkamides from *Echinacea* 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. Alkamides are found in small amounts in the aerial parts of *E. purpurea* and *E. angustifolia* but are abundant in the roots of these two species where they are the major constituents of the lipophilic fraction. A number of alkamides have been

identified in *Echinacea* species, with the pair of isomers dodeca-2E,4E,8Z,10E/Z-tetranoic acid isobutylamides, being the main alkamides found in ethanolic *Echinacea* preparations (Bauer *et al.*, 1988a; Bauer *et al.*, 1988b; Bauer *et al.*, 1989; Binns *et al.*, 2002). This pair is usually referred as the “tetraenes” in the literature. The molecular structure of the main alkamides from *Echinacea* are shown in Figure 3 (Woelkart and Bauer, 2007).

1.9.4. Alkamide permeability to membranes and bioavailability

In contrast to CADs, alkamides are reported to have a high bioavailability and they are readily detected in human plasma after only 15 to 20 min following ingestion, and remain detectable in the blood for up to 12 hours (Matthias *et al.*, 2005; Woelkart *et al.*, 2005a; Woelkart *et al.*, 2008b). These findings make a strong case for the role of the alkamides as the main active principle in *Echinacea* phytotherapies that are taken orally. Interestingly, permeability studies *in vitro* using Caco-2 cell monolayer model (CMM), an intestinal barrier model (Matthias *et al.*, 2004) and *in vivo* comparing plasma absorption kinetics of the different *Echinacea* alkamides found a correlation between the molecular structure of alkamides and their individual bioavailability (Matthias *et al.*, 2004; Woelkart *et al.*, 2005a; Woelkart and Bauer, 2007). Generally, the alkamides from *Echinacea* can be categorized into two groups: the 2,4-dienes and the 2-ene alkamides (Figure 3). In the Caco-2 (CMM) study, pairs of 2,4-dienes and equivalent 2-ene alkamides were tested with results showing that the 2,4-dienes are more rapidly transported through the membrane compared to their equivalent 2-ene alkamides. In Matthias *et al.* (2004) study, the alkamide 2a,

which is a 2,4-diene, showed twice the permeability of the alkamide 3a—its 2-ene correspondent alkamide. Similarly, the 2,4-diene alkamide 6 showed a 75% increase in relation to the 2-ene alkamide 10 (Figure 4). In addition, the most lipophilic alkamide, the dodeca-2E,4E-dienoic acid-isobutylamide (Figure 4, alkamide 12), which does not have a terminal diene–diene moiety (double and/or triple bonds at the end of the fatty acid chain), showed the lowest permeability in the Caco-2 CMM study and was below the detectable limit in the human pharmacokinetic studies, suggesting that there is a correlation between increase in unsaturation and increase in the permeability and bioavailability of the alkamides. Moreover, methylation in either terminal of the alkamides was shown to decrease the permeability of alkamides by close to 50% in the same Caco-2 CMM study (Figure 4).

Alkamide 1. Tetradeca-2E-ene-10,12-diynoic acid isobutylamide
Alkamides 2a/2b. Undeca-2E/Z,4Z/E-diene-8,10-diynoic acid isobutylamide
Alkamides 3a/3b. Undeca-2E/Z-ene-8,10-diynoic acid isobutylamide
Alkamide 4. Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide
Alkamide 6. Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide
Alkamides 8a/8b. Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide – "Tetraenes"
Alkamide 9. Pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide
Alkamide 11. Dodeca-2E,4E,8Z-trienoic acid isobutylamide
Alkamide 13. Hexadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide
Alkamide 15. Undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide	

Figure 3. Chemical names and molecular structures of the main alkamides from *Echinacea* (Woelkart and Bauer, 2007).

<p>Alkamide 2a: Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide. Apparent permeability (Papp) twice of the 2-ene correspondent alkamide 3a.</p>	<p>Alkamide 4: Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide. Methylation on diynoic terminal decreases permeability by 45% when compared with alkamide 2a.</p>
<p>Alkamide 3a: Undeca-2E-ene-8,10-diynoic acid isobutylamide.</p>	<p>Alkamide 10: Dodeca-2E-ene-8,10-diynoic acid 2-methylbutylamide. Methylation on both ends decreases permeability by 65% when compared with alkamide 3a.</p>
<p>Alkamide 6: Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide. Apparent permeability (Papp) increased by a factor of 75% from the 2-ene correspondent alkamide 10. However, methylation on amino group decreases permeability by 57% when compared with alkamide 4.</p>	<p>Alkamide 12: Dodeca-2E,4E-dienoic acid isobutylamide. The most lipophilic alkamide tested showed the lowest permeability in the Caco-2 CMM study.</p>

Figure 4. Structures of some of the alkamides tested in the Caco-2 CMM study by Matthias *et al.* (2004), illustrating the correlation between molecular structure and ability to cross membranes.

1.9.5. Alkamides immunomodulation and anti-inflammatory activities

As is the case with CADs, early research demonstrated that purified alkamide fractions from *Echinacea* extracts have immunomodulatory and anti-inflammatory activities. Studies applying carbon clearance tests and measurement of inflammatory cytokines revealed enhanced phagocytic activity and induction of macrophage activity by alkamides from *E. purpurea* and *E. angustifolia*. Intriguingly however, the most abundant of the alkamides, the pair of isomers dodeca-2E,4E,8Z,10E/Z-tetranoic acid isobutylamides (tetraenes), showed little evidence of immunomodulatory activity in these early studies, suggesting that a minor alkamide or a combination of alkamides may be responsible for the *Echinacea* immunomodulatory effects (Bauer, 1999; Matthias *et al.*, 2007). In another study of the anti-inflammatory potential of alkamides, Cech *et al.* (2010) reported that the “tetraenes” and other alkamides show an ability to suppress the production of several inflammatory cytokines and chemokines from RAW macrophage-like cells infected with influenza A virus thus corroborating the anti-inflammatory activity of alkamides from *Echinacea*.

1.9.6. Alkamides — Mode of action studies

Alkamides have been found to inhibit cyclooxygenase (COX-1/COX-2) and 5-lipoxygenase (5-LOX). These two are key enzymes in the arachidonic acid metabolism pathway to prostaglandins, thus the search for compounds with dual inhibition activity, and more specifically COX-2/5-LOX, is a new strategy in the non-steroidal anti-inflammatory drugs (NSAIDs) research. Müller-Jakic *et al.* (1994) studied the inhibition of COX (using the sheep seminal vesicles model) and 5-LOX (using porcine leukocytes) by n-hexane extract of *E. angustifolia* roots and twenty

alkamides isolated from several varieties of *Achillea*, *Anacyclus*, *Aaronsohnia* and *E. angustifolia*. Müller-Jakic reported that while the root extract inhibited both enzymes, most alkamides inhibited only COX and just a few had an effect on 5-LOX. Confirmation of 5-LOX inhibition by root extracts of the three commercial species of *Echinacea* was reported by Merali *et al.* (2003). In another study, Clifford *et al.* (2002) investigated the potential selective inhibition of COX-1/COX-2 by alkamides isolated from *E. purpurea* roots in comparison with the following anti-inflammatory drugs: aspirin, ibuprofen, naproxen, celecoxib (Celebrex™) and rofecoxib (Vioxx™). Selective COX-2 inhibition is a desirable characteristic in anti-inflammatories as inhibition of COX-1 is known to cause more adverse reactions when compared with inhibition of COX-2. This study revealed that several alkamides showed COX-1 and COX-2 inhibition, but not a selective inhibition of COX-2, and that the “tetraenes” (the most abundant alkamides) isolated from *E. purpurea* did not show any measurable inhibitory activity for either COX-1 or COX-2. Interestingly, the same compounds isolated from *E. angustifolia* showed a 54% inhibition of COX (Muller-Jakic *et al.*, 1994). In a more recent study by Hinz *et al.* (2007), CO₂ extracts from *E. angustifolia* roots and eight isolated alkamides and were tested with human neuroglioma cells for their ability to inhibit the enzymatic activity of COX-2 and that of COX-2 mRNA and protein expression. In this study, the CO₂ root extract and three of the eight alkamides tested inhibited COX-2 activity, yet none of the alkamides inhibited COX-2 protein expression. Again, the “tetraenes” were practically inactive, showing no inhibition of the COX-2 activity. Therefore more studies are needed to determine whether or not the inhibition of COX activity by alkamides is the mechanism of action for *Echinacea* anti-inflammatory effect.

1.9.7. Alkamides and anandamine — the “bliss” molecule

Alkamides are structurally similar to the endogenous cannabinoid anandamine — The “bliss” molecule

Another proposed molecular mechanism to explain the alkamides anti-inflammatory properties is gaining prominence after the publication of several findings reporting the ability of alkamides to bind to the cannabinoid receptors CB1 and CB2 which are known to modulate inflammation (Woelkart *et al.*, 2005b; Raduner *et al.*, 2006; Matovic *et al.*, 2007; Chicca *et al.*, 2009). The CB1 receptor was first reported by Devane *et al.* (1988) and is found predominantly in the central nervous system. The CB2, on the other hand, is a peripheral subtype of cannabinoid receptor found in the spleen and immune-competent cells (Munro *et al.*, 1993). CB receptors are thought to be responsible for the wide range of effects observed when delta 9-tetrahydrocannabinol (delta 9-THC), a major constituent of *Cannabis sativa*, binds to them (for a review see Howlett *et al.*, 2004). Not much longer after the discovery of the CB1-receptor, Devane *et al.* (1992) reported the identification of a potent endogenous cannabinoid ligand: the arachidonylethanolamide (Figure 5). Termed anandamine from the Sanskrit word for “bliss” ananda, anandamine is a fatty-acid derived compound with pharmacological effects similar to delta 9-THC (Devane *et al.*, 1992).

Interestingly, alkamides are structurally similar to the anandamine (Figure 5) and as such, interact with CB-receptors as agonists mimicking the action of cannabinoid ligands such anandamine. Raduner *et al.*, (2006) and Woelkart *et al.*, (2005b) have reported the results of a radioligand displacement assays on CB1 and

CB2 receptors in which alkamides were shown to bind to CB-receptors and more selectively to CB2-receptors, in concentrations similar to that found in human plasma after *Echinacea* intake. Therefore, it is reasonable to infer that a similar *in vivo* binding of alkamides with CB2 receptors in immuno-competent cells might occur thus triggering immune and anti-inflammatory responses.

Figure 5. Molecular structure of the endocannabinoid anandamide and alkamide 3a.

Given that the enzymes COX and 5-LOX, and CB2 receptors play a significant role in the molecular pathways of the inflammation process, it is reasonable to conclude that, even though there are clear differences in the effectiveness of each alkamide, this class of compounds may be clinically useful to reduce pain and inflammation, which would be in agreement with the traditional use of *Echinacea* (Bauer, 2000; Clifford *et al.*, 2002; Hinz *et al.*, 2007).

2. The Aim of This Present Work

As a consequence of the many new insights into the pharmacodynamics and mode of action of *Echinacea* and its constituents, particularly of the alkamides, there is a renewed interest in the study of the purple coneflower that grows peacefully in backyards everywhere, oblivious to all the scientific commotion surrounding it.

Encouraged by the results of the previously referenced experiments indicating that *Echinacea* affects fungal cell wall function (Mir-Rashed *et al.*, 2010), I proposed to test the hypothesis that the antifungal mode of action of *Echinacea* extracts is through cell wall disruption by alkamides. Therefore, the aim of this research is:

1. Test if alkamides in *Echinacea* extracts have antifungal activity.
2. Develop a cell wall disruption assay to screen natural-derived compounds with potential to be further developed into new antifungal pharmaceuticals.
3. Test if alkamides have cell wall disruption activity.
4. Compare the cell wall disruption activity of different alkamides.
5. Develop a general QSAR model to study lipophilicity-antifungal activity relationships in alkamides.

3. Materials and Methods

The key procedural steps in the assay we developed to detect yeast cell wall damage are summarized in Figure 8. Experimental details for each step are given in the following sections.

3.1. Yeast strains and growth conditions

3.1.1. *S. cerevisiae* wild type S288C and cell wall mutant YMR307W

The two *Saccharomyces cerevisiae* strains used in this study were the wild type S288C (*MATa SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*) and the cell wall mutant YMR307W (*GAS1*). *GAS1* encodes 1,3- β -glucanotransferase, a GPI-anchored protein localized to the extracellular face of the plasma membrane and is involved in the remodeling of β -1,3-glucan after its synthesis by β -glucan synthase at the membrane (Mouyna *et al.*, 2000). A *gas1* null mutant has a weakened cell wall with cells abnormally round and multi-budded, with reduced viability, increased sensitivities to some cell wall-affecting drugs and elevated temperatures (Marx *et al.*, 2006; SDG project., 2008). Chitin synthesis is upregulated in *gas1* mutants to compensate for defective β -1,3-glucan (Popolo *et al.*, 1997; Ram *et al.*, 1998; Mouyna *et al.*, 2000). Moreover, strain YMR307W has been previously shown to be sensitive to *Echinacea* extracts in a study by Mir-Rashed *et al.* (2010) that showed that yeast strains with deletions of genes that have a cell wall function are highly susceptible to growth inhibition by *Echinacea* extracts.

3.1.2. Growth conditions

These strains are maintained in YPD broth (2% glucose, 2% peptone, 1% yeast extract) with glycerol (25%) at -80°C in the Biology Department at Carleton University, Ottawa ON Canada. Working cultures were inoculated from glycerol stocks to minimal synthetic complete (SC) medium [2% glucose (BDH, Toronto, Canada), 0.67% yeast nitrogen base without amino acids (Becton, Dickson and Co, Sparks, MD)] with or without 2% agar (Bioshop Canada, Burlington ON)] and supplemented with required amino acids (Sigma-Aldrich, Oakville, ON) as described in Gietz (1996).

3.2. Test compounds sources

3.2.1. *Echinacea purpurea*

Echinacea purpurea (L.) Moench plants were organically-grown in Ottawa, Ontario. *Echinacea* roots were rinsed in tap water, oven dried overnight at 50°C, and milled to <1mm (Thomas-Wiley Model 4 Mill, Arthur H. Thomas Co., Philadelphia, PA). Dried root material (40.6 g) was extracted three times with ~400 mL of 70% ethanol at room temperature for 24 h with moderate agitation. The resulting three extracts were filtered through Whatman #1 paper, pooled, and concentrated by rotary-evaporation (900–100 rpm, 850–900 mbar) and freeze-drying for 24 h. The resulting crude extract (6.68 g) was analysed by HPLC (Figure 6) and stored in the dark at -20°C for up to 2 months or until use.

3.2.2. Synthetic alkamides

Natural alkamides are present in the roots of *Echinacea purpurea* and are believed to be responsible for some of the pharmacological properties of *Echinacea* preparations (Woelkart and Bauer, 2007; Mir-Rashed *et al.*, 2010). The synthetic alkamides used in this study listed in Table 1 were synthesized as described previously (Matthias *et al.*, 2004; Matovic *et al.*, 2007) and kindly donated by Dr. R. P. Lehmann from the MediHerb Research Laboratories, Queensland, Australia. Weighed quantities of alkamides were resuspended in 70% ethanol and stored in the dark at -80°C until use.

3.2.3. Antifungal controls

Caspofungin acetate (CAS, Figure 7–A) was kindly provided by Merck & Co. (Rahway, NJ) and was used as a positive control in our experiments. CAS is the first FDA approved member of the echinocandin class of antifungals. It inhibits the synthesis of a major fungal cell wall component, β -1,3-glucan, and thus disrupts cell wall integrity (Kartsonis *et al.*, 2003). Two aminoglycosidic antibiotics, hygromycin B (HYG, Figure 7–B, Roche Applied Science, Mannheim, Germany) and nourseothricin dihydrogen sulfate (clonNAT, Figure 7–C, WERNER BioAgents, Jena, Germany), were used as negative controls as their mode of action is not related to cell wall stability but, rather, inhibition of protein synthesis.

Figure 6. HPLC profile of the *E. purpurea* extract used in the study showing UV absorbance vs. retention time. Panel A: Detection at 326nm. The major phenolic compounds are indicated. Panel B: Detection at 260nm showing the typical HPLC profile of alkamides from *Echinacea* extracts. Test conditions: Column: YMC ODS-AM (12) 100mm x 2mm ID. [YMC-9] Oven 50C; flow rate 0.5mL/min. Solvents: A= Water+ TFA 0.05%. , B= MeCN + TFA 0.05%.

Table 1. Synthetic alkamides used in this study. The seven alkamides were synthesized and kindly donated by Dr. R. P. Lehmann from the MediHerb Research Laboratories, Queensland, Australia.

Alkamides molecular structures	Code Name	Chemical Name and Molecular Weight
Alk2b	Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide MW=229.32
Alk3a	Undeca-2E-ene-8,10-diynoic acid isobutylamide MW=231.34
Alk5	Dodeca-2E-ene-8,10-diynoic acid isobutylamide MW=245.37
Alk8E	Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide MW=247.38
Alk8Z	Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide MW=247.38
Alk11	Dodeca-2E,4E,8Z-trienoic acid isobutylamide MW=249.44
Alk12	Dodeca-2E,4E-dienoic acid isobutylamide MW=252.46

Figure 7. Antifungal controls used in this study. Panel A: Biochemical structure of caspofungin (Kartsonis *et al.*, 2003). Panel B: biochemical structure of hygromycin B (from product insert). And Panel C: biochemical structure of nourseothricin dihydrogen sulfate (from manufacturer web site ("WERNER BioAgents")). Nourseothricin consists of the three homologue streptothricins: F, $n=1$ (61%), E, $n=2$ (7%) and D, $n=3$, (31%).

3.3. Antifungal susceptibility assays

Identification of optimal test compound concentration

3.3.1. Minimum inhibitory concentration (MIC) — Microdilution broth assay

All antifungal compounds used in this study were tested to determine their minimum inhibitory concentration (MIC) by a microdilution broth assay, based on methods described previously (Rex *et al.*, 1993; Rex *et al.*, 2001; Ficker *et al.*, 2003). Briefly, yeast strains were incubated overnight at 30°C, 150 rpm in SC medium and then diluted to obtain a cell density of $\sim 10^5$ CFU/mL. The culture was distributed into each microplate well (round bottom, sterile 96-well microplates, Diamed, Mississauga, ON, Canada) except for the last column, which served as a contamination control for media and test compounds. Test or control compounds were added to the first well of each row and serially diluted 1.5 X across the columns. The last column of each row was left as compound free control. The concentration of each compound used in the first well in the serial dilution was determined to be in the inhibition range by preliminary tests. Hygromycin B was used as positive antifungal control in all MIC experiments. The microplates were examined visually after stationary incubation at 30°C for 72 h. The MIC values were recorded as the lowest tested concentration that resulted in an optically clear well. The MIC assays were performed independently at least three times for each fungal strain/compound combination to derive the mean MIC. The inhibitory effect of the test compounds expressed as minimum inhibitory concentration (MIC) are summarized in Table 2.

3.3.2. MIC 40% inhibition—Drop plate method

The drop plate assay was based on the method described by Chen et al (2003) and it was used to estimate percentage of growth inhibition induced by test compound treatments and reduction in cell viability after sonication. Preliminary assays were done using 20, 40 and 60% of the MIC values determined as described in 3.3.1. Based on the results of these preliminary assays, a 40% MIC concentration was chosen as the optimal for the fluorescent microscopy assay. Briefly, yeast cells were 10 times serially diluted with fresh medium in a 96-well microplate. Using a multichannel pipette, 10 μ L drops were spotted on agar medium at concentrations of $\sim 10^4$, 10^3 , 10^2 , 10 cell/10 μ L, and colony forming units (CFU) were determined after incubation for 36–48 h at 30°C. Drop plate assays were done in triplicate. Percentage of growth inhibition was calculated as the ratio of CFU/mL of treated/non-treated samples. Based on this assay, test compound concentrations were established that caused a growth inhibition of $40 \pm 5\%$ compared to the no-inhibitor control.

3.4. Fluorescent microscopy assay

3.4.1. Treatment with test compounds

Each yeast strain tested was inoculated from a late exponential phase pre-culture in SC medium and allowed to recover to a mid-log phase by overnight incubation at 30°C and 150 rpm. Before proceeding with the assay, the cell density was adjusted to $\sim 10^7$ cell/mL using a hemocytometer, and a 10 μ L aliquot was removed for drop plate assay. The resulting CFU counting of this first aliquot was used to calculate the percentage of growth inhibition after the yeast cell exposure

to test compounds. At this point, volumes of 0.4 mL of the cell culture were transferred to 1.5 mL sterile microcentrifuge tubes, the test compounds were added at a sub-inhibitory concentration equivalent to ~40% of the MIC and the same volume of carrier control (70% EtOH) was added to a non-treated control sample. The cells were incubated at 30°C/150 rpm for about 4 h and subjected to sonication treatment as described below.

3.4.2. Ultrasound treatment – Mild sonication

The protocol used in this study to treat yeast cell with mild sonication was adapted from Ruiz *et al.* (1999). After the 4 h exposure to the test compounds, an aliquot of 90 µL was transferred from each tube to a new microcentrifuge tube and set aside as the non-sonicated control. The remaining cells were sonicated on ice for 2 min (3 mm microtip probe, 20% amplitude, pulse 15 s, interval 3 s) using a Vibra Cell sonicator (Vibra Cell VCX130. Sonic & Materials, Connecticut, USA). After sonication, all samples (non-sonicated and sonicated) were drop plate assayed, the remaining cells were pelleted by low speed centrifugation (2000 rpm, 2min), the supernatant was removed and the cells were resuspended in fresh medium and immediately observed by fluorescent microscopy as described below.

3.4.3. Fluorescence microscopy

Yeast cell suspensions were stained by applying 2 µL of a solution of 0.1% (w/v in distilled water) calcofluor white M2R (American Cyanamid, Bound Brook, NJ) and 0.1% Evans Blue (Fisher Scientific CO., Limited, Toronto, ON, Canada) to 10 µL of cell suspension on a microscope slide. A coverslip was gently applied before observation. Microscopic observations were made with an Axio Imager fluorescence

microscope (Carl Zeiss, Toronto, ON) using 40X and 100X objective lenses. Using the multidimensional acquisition feature in the Axio Imager it was possible to obtain multiple fluorescence channel images using the DAPI filter to detect calcofluor fluorescence—a blue-white will be observed staining the fungal cell walls—and Rhodamine filter to detect Evans Blue fluorescence—visualized as a white-reddish/pink fluorescence in cells with loss of metabolic activity. Images were captured with the Zeiss AxioVision software (Carl Zeiss MicroImaging GmbH, German) in the Carl Zeiss ZVI proprietary image format. The original images were saved unprocessed and copies of the original files were processed with Image J (Wayne Rasband, National Institutes of Health, USA) and transferred to Photoshop (Adobe Systems Inc., San Jose, California) for manual enumeration and further analyses.

3.4.4. Statistical analyses

The experiment was repeated at least three times for each compound 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 ~3000 cell observations per compound. Cells were evaluated for cell wall damage and viability, counted and classified in each of four groups: cell wall damaged (CWD), Evans Blue positive (EB+), budded/multi-budded (BMB) and single non-divided daughter cells (S) (Figure 9). This data set was plotted in Excel (Microsoft Corporation, Redmond, WA, USA) as a percentage of the total number of cells enumerated. Mean values and the standard error of the mean (SE) were also calculated using Excel. The statistical analysis of the experimental data was done by 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).

Figure 8. The figure above is a schematic diagram illustrating the several steps of the protocol for the fluorescent microscopy assay used in this study.

Figure 9. Fluorescent microscopy images of *S. cerevisiae* S288C cells after treatment with *Echinacea* extract followed by sonication. Image A shows the S288C cells stained with Calcofluor White/Evans Blue as seen with the DAPI filter set and image B shows the same microscopic field as seen with the Rhodamine filter set (bar = 5µm). CWD (red dots)=cell wall damaged; EB+ (white arrows)=Evans Blue positive; BMB (light blue dots)=budded/multi-budded and S (blue dots)=single non-divided daughter cells.

3.5. Alkamides QSAR modeling

A general QSAR model to study lipophilicity-antifungal activity relationships in alkamides from Echinacea

Quantitative structure-activity relationships (QSAR) models are based on the premise that the biological activity of a compound is the consequence of its chemical structure and that this relationship can be predicted through a mathematical expression (for a review of the history of QSAR, see Selassie, 2003). The QSAR paradigm can be expressed in mathematical terms as:

$$\Delta \text{ biological activity (BA)} = f (\Delta x_1 + \Delta x_2 + \dots + \Delta x_n) \quad (1)$$

Where (BA) could be LD₅₀, MICs, etc...; and x₁, ..., x_n are mathematical descriptors of key properties of molecular structures relevant to the biologic activity being studied.

A classic QSAR modeling technique is the Hansch analysis. First developed by Hansch and Fujita (Fujita and Hansch, 1967; Hansch *et al.*, 1968) more than 40 years ago. Hansch's approach correlates the logarithm of octanol-water partition coefficient (log P), a molecular descriptor of lipophilicity, with a biological activity expressed as log 1/C where C is the molar concentration of a compound producing a standard biologic response. Many QSAR linear model equations were postulated since QSAR modeling inception and after extensive evaluation of linear models Hansch and coworkers (Hansch and Clayton, 1973) showed that QSAR equations relating biologic activity (log 1/C) and lipophilicity (log P) are more often than not a parabolic equation, leading to the postulation of the parabolic model equation formally expressed as follows:

$$\log 1/C = a(\log P)^2 + b \log P + c \quad (2)$$

Where $\log P$ = the value of the lipophilicity descriptor for each molecule in the series and where each letter a, b and c , represents a coefficient calculated by fitting variations in the data by regression analysis using the least square method.

A classic Hansch QSAR modeling (Hansch *et al.*, 1968; Hansch and Clayton, 1973) was adopted to further study the role of the alkamides chemical structure and lipophilicity in influencing the antifungal activity of these molecules. The development of a QSAR model involves three key steps:

- a) Collecting a data set of experimental measurements of a biological activity for a group of analog compounds.
- b) Converting the molecular structures of the analog compounds into mathematical descriptors.
- c) Using regression analysis and statistical methods to find relationship between the two data sets.

In the QSAR model developed here, $\log P$ was calculated for each alkamide and in an Hansch-model fashion plotted against the logarithm of $1/C$ ($\log 1/C$), where C is the molar concentration of a compound producing a standard biologic response. In our case the biological activity is the antifungal properties of alkamides expressed as the molar concentration that causes ~40% growth inhibition determined as described in 3.3.2. Once the descriptors are determined and plotted the next step in QSAR modeling is to perform multiple regression analyses by the least squares method to find the best fit for the curve and the statistical parameters to evaluate the reliability of the model. A more detailed description of the methodology follows.

3.5.1. Calculation of the alkamide descriptors (log P) and biologic activity (log 1/C)

Free online tools were used to generate the data for the QSAR modeling. The SMILES (simplified molecular input line entry system) notation for the alkamides were created using the structure drawing application JME molecular editor available at Molinspiration Cheminformatics website (JME, 2011).

The log P for each alkamide was obtained by inputting the SMILES notation in another free online tool, ALOGPS 2.1 from Virtual Computational Chemistry Laboratory (VCCLAB, 2011). Several online programs are available to calculate the lipophilicity of a compound based on their structure. The decision to use ALOGPS program was based on the ability of the application to simultaneously displays log P values calculated with several algorithms (ALOGPS 2.1, Pharma Algorithms LogP, Actelion LogP , Molinspiration logP, KOWWIN logP, ALOGP, MLOGP, XLOGP2 and XLOGP3 logP calculators) and automatically calculate the average log P and standard deviation. Therefore by using ALOGPS 2.1 tool it was possible to compare the results of several algorithms with one click. As for the biologic activity measurement (log 1/C), it was calculated as the logarithm of the inverse of the alkamide molar concentration that caused a growth inhibition of $40\pm 5\%$ compared to the no-inhibitor control, the MIC_{40%} as established in 3.3.2.

3.5.2. Regression Analysis and model's goodness-of-fit evaluation

The average of alkamides log P generated by the ALOGPS 2.1 tool were plotted against the log 1/C and the curve fitting and statistical analysis of quality of the resulting model was done using GraphPad PRISM® version 5, GraphPad Software,

San Diego California USA, www.graphpad.com; and the free online curve fitting tool ZunZun Online Curve Fitting and Surface Fitting Web Site (ZunZun, 2011). The correlation coefficient r^2 was used as measure of the quality of fit of the model.

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4. Results and Discussion

4.1. Intra and inter-assay controls

4.1.1. Minimum inhibitory concentration (MIC) determination

In the first step of this study, the *S. cerevisiae* strains wild type S288C and cell wall mutant YMR307W were initially tested for their susceptibility to a range of concentrations of the following test compounds: caspofungin (CAS), hygromycin B (HYG), nourseothricin (clonNAT) (Figure 7); *Echinacea* crude extract (Figure 6); and seven synthetic alkamides from *Echinacea* (Table 1). A broth microdilution technique was used to assess the strains susceptibility to the test compounds as described in the materials and methods section. Briefly, overnight cultures (cell density of $\sim 10^5$ CFU/mL) of S288C and YMR307W strains were inoculated in 96-well microplates and incubated at 30°C with the test compounds serially diluted 1.5 X across the columns for 60 h for strain S288C and 72 h for strain YMR307W. The microplates were examined visually and MIC values were recorded as the lowest tested concentration that resulted in an optically clear well. The inhibitory effect of the test compounds expressed as minimum inhibitory concentration (MIC) are summarized in Table 2. From this the concentration was determined at which the yeast strains had 40% growth inhibition.

4.1.2. Validating 40% growth inhibition

The quantification of cell viability by the drop plate assay described in the materials and methods section was used for quality assurance purposes, as means to validate that each compound was tested at a concentration that, when compared

with the non-treated control, would produce ~40% growth inhibition during the 4h incubation (MIC 40%). Thus, each experiment was standardized to an intra and inter-assay control. The results of MIC 40% determinations are shown in Table 2.

The concentration of each compound and the associated percentage of inhibition after 4 hour incubation at 30°C with strains S288C and YMR307W is shown in Figure 10. Overall, these two yeast strains displayed similar inhibition profiles where the cell wall mutant YMR307W was generally more sensitive to inhibition by the tested compounds except for caspofungin.

Of the seven alkamides tested, the undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (henceforth referred as alk2b) required a significantly lower concentration to attain a 40% growth inhibition (Figure 10). Of note, alk2b was more effective than the two tested dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides “tetraenes”, alk8E and alk8Z. As indicated in Table 3, alk2b represents ~18% of the total alkamides in the roots of *E. purpurea* (Wills and Stuart, 1999), while the tetraenes alk8E and alk8Z are the most abundant of the root alkamides, accounting for 43–49% of the total (Kim *et al.*, 2000). Also impressive was the efficacy of the undeca-2E-ene-8,10-diynoic acid isobutylamide (henceforth alk3a) to inhibit yeast growth. Although this alkamide is almost undetectable in the roots of all *Echinacea* species tested by Binns *et al.* (2002), alk3a tended to be the second most potent inhibitor of yeast cell growth of those alkamides tested (Figure 10).

Table 2. Minimum inhibitory concentrations (MIC) results for the several test compounds used in this study. MIC40%=Minimum 40% inhibitory concentration; log 1/ C=logarithm of the inverse of MIC40% in mol/L; ECH=*Echinacea* extract; Alk2b to Alk12=alkamides (Table 1); CAS=Caspofungin; HYG=Hygromycin B; NAT= Nourseothricin, BMM= broth microdilution method; DPM=Drop plate method

Test Compound		Strain					
		S288C			YMR307W		
	UNIT	MIC BMM	MIC40% DPM	log 1/C (mol/L)	MIC BMM	MIC40% DPM	log 1/C (mol/L)
ECH	$\mu\text{g}/\mu\text{L}$	143	58		118	47.25	
	mol/L						
Alk2b	$\mu\text{g}/\mu\text{L}$	0.35	0.152		0.22	0.087	
	mol/L		0.00066	3.179		0.00038	3.421
Alk3a	$\mu\text{g}/\mu\text{L}$	0.59	0.218		0.26	0.111	
	mol/L		0.00094	3.026		0.00048	3.319
Alk5	$\mu\text{g}/\mu\text{L}$	0.81	0.310		0.52	0.210	
	mol/L		0.00126	2.898		0.00086	3.068
Alk8E	$\mu\text{g}/\mu\text{L}$	0.62	0.260		0.30	0.125	
	mol/L		0.00105	2.978		0.00051	3.296
Alk8Z	$\mu\text{g}/\mu\text{L}$	0.65	0.271		0.30	0.131	
	mol/L		0.00110	2.960		0.00053	3.276
Alk11	$\mu\text{g}/\mu\text{L}$	0.89	0.340		0.62	0.255	
	mol/L		0.00136	2.865		0.00102	2.990
Alk12	$\mu\text{g}/\mu\text{L}$	0.89	0.329		0.62	0.254	
	mol/L		0.00130	2.885		0.00101	2.997
CAS	$\mu\text{g}/\text{mL}$	0.15	0.061		0.40	0.140	
	mol/L						
HYG	$\mu\text{g}/\mu\text{L}$	0.51	0.215		0.25	0.108	
	mol/L						
NAT	$\mu\text{g}/\mu\text{L}$	0.015	0.006		0.007	0.003	
	mol/L

Figure 10. Compound concentration required during 4 hour exposure to achieve ~40% growth rate inhibition. Data points represent the means ($\pm$ SE, $n \geq 3$) for both compound concentration and percent inhibition. Panels a and b are a blow-up of the alkamides results in panels A (wild type S288C) and B (strain YMR307W). Concentrations that achieve ~40% inhibition of alkamides that are designated by the same colored digit are not significantly different (ANOVA, Tukey HSD, $\alpha = 0.05$, $P < 0.001$).

Table 3. Alkamide concentrations found in *Echinacea purpurea* roots as reported by several studies.

Molecular structure	Code name	Concn (mg/g)	Total Alk Concn (mg/g)	Source
Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	Alk2b	2.13 ± 1.08^a 0.9 ± 0.3^c	Data not available	North America wild harvested ^a New Zealand ^c
Undeca-2E-ene-8,10-diynoic acid isobutylamide	Alk3a	0.02 ± 0.02^a	Data not available	North America wild harvested ^a
Dodeca-2E-ene-8,10-diynoic acid isobutylamide	Alk5	0.00–traces ^a	Data not available	North America wild harvested ^a
Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	Alk8E	3.12 ± 0.87^a	Data not available	North America wild harvested ^a
		0.04–0.39 ^b	Data not available	Suppliers from Germany and USA ^b
Dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide	Alk8Z	0.8–3.6 ^c	6.2 ^c	New Zealand ^c
		1.34–1.46 ^d	2.78–2.92 ^d	Canadian ^d
			6.2 ± 2.4^e	Australia ^e
Dodeca-2E,4E,8Z-trienoic acid isobutylamide	Alk11	0.08 ± 0.03^a	Data not available	North America wild harvested ^a
Dodeca-2E,4E-dienoic acid isobutylamide	Alk12	0.63 ± 0.58^a	Data not available	North America wild harvested ^a

^a Binns et al., 2002^b Bauer and Reminger, 1989^c Perry et al., 1997^d Kim et al., 2000^e Wills and Stuart, 1999

4.2. Measuring yeast cell wall disruption by fluorescence microscopy

4.2.1. Yeast cell wall disruption by ultrasound-induced cavitation

Ultrasound-induced cavitation is an established method of physically disturbing yeast cell wall and membranes (Miller *et al.*, 1996; Ciccolini *et al.*, 1997; Mir-Rashed *et al.*, 2010). The premise of the method is that yeast cells that are exposed to a compound that disrupts cell wall function(s) are expected to be more sensitive to mild sonication than cells that are not exposed to the compound. In my studies, yeast cells having cell wall damage were easily identified using the light microscopy methods (Figure 11). The extent of cell wall damage observed can be related to the cell wall disrupting ability of a given test compound. In the case of Evans Blue stained cells, on the other hand, this correlation might not be so clear at first glance as the cells look intact. However, a functional intact membrane is vital to keep Evans Blue from staining the cell cytoplasm, therefore these cells are clearly struggling to keep the dye from staining the cytoplasm, which might be an indication of difficulties in the cell wall/membrane complex function(s) caused by the treatment with the test compounds.

The several types of cell wall damage observed, as well as how the yeast cells and cell wall damage appear when stained with CFW and Evans Blue under the filters (DAPI/Rhodamine) are exemplified in Figure 11. It is interesting to note how cells apparently intact (appHC) in the DAPI field can be stained by Evans Blue (Figure 11; A,B).

Figure 11. Wild type S288C yeast cells were treated with *Echinacea* extract for 4 hours prior to sonication and then simultaneously stained with CFW and Evans Blue immediately before examination with fluorescence microscopy. Micrographs were taken using the DAPI (Panel A) and Rhodamine, (Panel B) filters (100x magnification, bar=5μm). Panel C shows a superimposition of panels A and B. HC=Healthy Cell, appHC=apparently Healthy Cell, CWD=Cell Wall Damage, EB+ =Evans blue stained. Panel D (bar=5μm) displays some of the several types of cell wall damage observed in this study using CFW staining, ranging from small defects to complete collapse of cell structure.

4.3. Cell wall damage and loss of viability observations

4.3.1. Untreated Control

*Percentage of cell wall damage (CWD) and
frequency of Evans Blue stained cells (EB+)*

Untreated control cells that were not subjected to sonication (-T/-S) showed very little evidence of cell wall damage (CWD) or loss of viability (EB+). This was true for both tested strains, wild type S288C and the cell wall mutant strain YMR307W. For both strains, less than 1.5% of the yeast cells were Evans Blue-positive (EB+), a proxy for dead or non-viable cells, and less than 0.15% had noticeable cell wall damage (CWD) based on CFW staining (Figures 12, 13, 14 and Tables 2 and 3)—a good indication that the general health of the yeast cell suspension have not been compromised during the several stages of the assay.

Untreated Control — Baseline effect of sonication

Untreated control cells that were subjected to sonication (-T/+S) showed some cell wall damage (~11-12%) and some decrease in cell viability (less than 15%) (Figures 14 and 15, Tables 4 and 5). This data indicates that 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 view of this, it is reasonable to expect that the degree of cell wall damage and loss of viability on treated samples subjected to sonication (+T/+S) should be equal or greater than values observed in the (-T/+S) controls.

A) S288C CTR(-T/-S) CFW/DAPI filter

B) S288C CTR (-T/-S) EB/Rhodamine filter

Figure 12. Fluorescence microscopy images (40x magnification, bar = 5 μ m) of *S. cerevisiae* S288C untreated control cells with no sonication (-T/-S). No evidence of cell wall damage is observed (panel A: CFW/DAPI filter), nor significant loss of viability (panel B: EB/Rhodamine filter), which can be considered a good indication of the healthy state of the yeast cells.

C) S288C CTR (-T/+S) CFW/DAPI

D) S288C CTR (-T/+S) EB/Rhodamine

Figure 13. Fluorescence microscopy images of *S. cerevisiae* S288C untreated control cells subjected to sonication (-T/+S). Some cell wall damage (CWD–red dots) can be seen on panel C (CFW/DAPI filter) as well as some loss of viability (panel D: EB/Rhodamine filter), illustrating the baseline effect of the mild sonication protocols used in this study (bar =5 μ m).

4.3.2. Negative Controls

Compounds with no known direct effect on cell wall function

The compounds hygromycin B (HYG) and nourseothricin (clonNAT) are both classified as aminoglycoside antibiotics that primarily interfere with protein synthesis (Cabañas *et al.*, 1978; Brodersen *et al.*, 2000; Dembitsky, 2005; McGaha and Champney, 2007). Neither compound is reported to have direct negative effects on cell wall function(s) and were therefore selected in this study as negative control compounds for cell wall inhibition.

Percentage of cell wall damage (CWD), frequency of Evans Blue stained cells and loss of viability (EB+)

The two negative controls showed similar results to that of the untreated control cells in both strains tested. The percentage of cell wall damage (CWD) observed in the non-sonicated cells treated with either HYG (hygT/-S) or clonNAT (natT/-S) was low for both the wild type S288C ($\leq 0.02\%$) and the cell wall mutant YMR307W ($\leq 0.09\%$). These values are not statistically different from the control (-T/-S) values of 0.14% and 0.07% for S288C and YMR307W, respectively. This indicates that exposures to either HYG or clonNAT does not cause cell wall damage in the strains tested (Figure 14, Tables 4 and 5). Similarly, cell wall damage in the negative control sonicated groups (hygT/+S and natT/+S) were not significantly different from the non-treated sonicated controls; all were in the range of 11-13% (-T/+S). (Figure 15, Tables 4 and 5).

Likewise, under the conditions used in this study, treatment with either HYG or clonNAT alone or accompanied by sonication, does not result in increased

frequency of Evans Blue (EB+) stained cells in comparison with non-treated controls (Figures 14 and 15 and Tables 4 and 5). These observations validate the use of HYG and clonNAT as suitable negative controls for this study; they both inhibit cell proliferation but, under the experimental conditions used here, neither is associated with altered cell wall sensitivity to sonication.

4.3.3. Positive Control

Caspofungin, a β -1,3-glucan synthase inhibitor

Caspofungin (CAS) was the first of a new class of antifungals, the echinocandins, to be approved for clinical use to treat invasive candidiasis and aspergillosis (Kartsonis *et al.*, 2003). Recent studies with *S. cerevisiae* indicate that the products of gene *FKS1* and its paralog *FKS2* are the probable targets of caspofungin (Agarwal *et al.*, 2003; Lesage *et al.*, 2004; Markovich *et al.*, 2004). These two genes encode subunits of the β -1,3-glucan synthase complex, a key enzyme in the synthesis of β -1,3-glucan. Polymers of β -1,3-glucan and β -1,6-glucan constitute ~60% of the cell wall dry mass with β -1,3-glucan accounting for 85% of the total cell wall glucan. Moreover, β -1,3-glucan is the major structural component of the yeast cell serving as a scaffold for other molecules to attach and providing the mechanical strength of the cell wall (Klis *et al.*, 2002). As a result of caspofungin interfering with these proteins the synthesis of β -1,3-glucan is inhibited and the integrity of the yeast cell wall is compromised. Therefore, it is reasonable to anticipate that, when compared with a non-treated control, treatment of yeast cells with caspofungin will make the yeast cell wall more susceptible to damage by sonication.

As expected, the percentage of CWD in the casT/+S treatment group was significantly greater than that observed in the control groups (-T/+S) with both strains (Figure 15). The percentage of CWD observed in the wild type S288C was 20.9% and 23.4% in the YMR307W mutant in contrast with the respective (-T/+S) control values of 12.2% and 11% (Tables 4 and 5). In addition, the results for the caspofungin treated non-sonicated (casT/-S) wild type S288C show a significantly greater percentage of CWD than the non-treated control (-T/-S). This is an indication that even at the relatively low concentration used in this study, set to achieve a ~40% growth inhibition, caspofungin causes significantly more CWD (0.80%) than the -T/-S control (0.14%) and all other test compounds (Figure 15).

Contrary to the wild type S288C, the percentage of CWD in the YMR307W casT/-S group (0.24%) was not statistically different from the percentage of CWD (0.07%) observed in the -T/-S control (Figure 15) suggesting a decreased sensitivity to the effects of caspofungin by this mutant. This increased resistance to caspofungin by YMR307W when compared with the wild type S288C was also noted early on in this study using minimum inhibitory concentration (MIC) assays in which MIC (S288C) = 0.15 µg/mL and MIC (YMR307W) = 0.40 µg/ mL (Table 2).

As noted before, the cell wall mutant YMR307W is defective in the gene encoding a β -1,3-glucanosyltransferase (*GAS1*) which is involved in the cross linkage and incorporation of β -1,3-glucan into the cell wall (Ram *et al.*, 1998; Mouyna *et al.*, 2000). Deletion of *GAS1* results in several morphologic defects; cells are round and larger with higher percentage of budded and multiple budded cells forming clumps that are difficult to disperse, suggesting that the separation of the bud from mother

cell has been affected (Popolo *et al.*, 1993). In addition to morphologic defects, *GAS1* mutants have lower β -glucan content in the cell wall despite an increased β -1,3-glucan synthase activity. This is probably the result of a defective incorporation of β -1,3-glucan into the cell wall, which is reported to be released into the media (Ram *et al.*, 1998). Furthermore, an increase in mannoprotein levels and a 10-fold increase in cell wall chitin were observed in *GAS1* mutants (Popolo *et al.*, 1997). Hence it is possible that in an attempt to ensure cell wall integrity, the YMR307W mutant compensates for the defective β -1,3-glucan assembly and the resulting loss of cell wall strength by upregulation the cell wall integrity pathway (CWI) and reinforcing the cell wall with chitin (Klis, *et al.*, 2006). Therefore, it can be reasoned that the decreased sensitivity of the YMR307W mutant to caspofugin relative to the wild type S288C is the result to some extent of the adaptation of this YMR307W mutant to a defective incorporation of β -1,3-glucan in the cell wall. In any case, YMR307W serves as a strain that is partially resistant to the CWD effects of caspofungin.

Frequency of Evans Blue stained cells (EB+) and loss of viability

Adding another layer of complexity to the interpretation of the results of the non-sonicated caspofungin treated samples is the fact that casT/-S samples exhibited significantly higher percentage of Evans Blue stained cells (Figure 14) than any of the other non-sonicated samples: 3.9% in the wild type S288c and 9.58% in YMR307W mutant compared with the control values of 0.61% and 1.27% in the S288c and YMR307W, respectively (Tables 4 and 5). This marked increase in EB staining, especially in the YMR307W mutant, is in conflict with the results of the intra-assay control assay (drop plate method), which assured a growth inhibition of ~40% for

the caspofungin treated group as it did for all other treated groups (Figure 10). A plausible explanation lies in the mechanism of the Evans Blue staining assay. Evans Blue has inverse properties of a vital stain; cells with intact cell wall/membrane are able to exclude the stain from the cytoplasm whereas cells with damaged membranes can not exclude the dye from the cytoplasm. Therefore, the quantification of cell viability by the Evans Blue method is based on the assumption that incorporation of the Evans Blue dye into the cytoplasm is an indication that the cell is non-viable and incapable of repair, in other words — dead (Chen *et al.*, 2003; Jimenez *et al.*, 2009). However, quantification of cell viability by the Evans Blue uptake assay is not without its limits. Considering that given time, a cell with a temporarily non-functional cell wall/membrane complex (e.g., overtaxed or temporarily non-functional efflux pump) might be capable of repair if the cell is removed from the environment/agent causing the injury (Crutchfield *et al.*, 1999). The apparent discrepancy between the drop plate assay and the Evans Blue staining may, therefore, reflect cell wall disruption by caspofungin that does not subsequently lead to cell death.

4.3.4. Test compounds—*E. purpurea* crude extract

Echinacea crude extract—a potential cell wall disrupting agent

Echinacea has a long history of medicinal use by the Native Americans to treat infections and pain in general. The anti-inflammatory, antimicrobial and immunostimulatory properties and chemical composition of *Echinacea* have been extensively studied (for a review see Bauer, 1999; Miller and Yu, 2004). Previous experiments using gene deletion arrays indicated that yeast cell wall mutants, including the YMR307W strain used in this study, are especially sensitive to growth

inhibition with exposure to *Echinacea* extracts (Mir-Rashed *et al.*, 2010). In the present study, we further investigate the effect of mild sonication on yeast cells treated with *Echinacea* crude extract by direct fluorescence microscopy observation of cell wall damage.

*Percentage of cell wall damage (CWD), frequency
of Evans Blue stained cells and loss of viability (EB+) findings*

Our results show that the percentage of CWD in the non-sonicated samples treated with *Echinacea* (echT/-S) concentrations that result in 40% growth inhibition were not significantly different from the non-treated controls. This was true for both wild type S288C and YMR307W strains (Figure 14 and Tables 4 and 5). This is a good indication that the cells were not damaged by the treatment with *Echinacea* at the tested concentration and/or by handling during the assay. Therefore, any increase in CWD observed in the sonicated samples treated with *Echinacea* (echT/+S), in comparison to the non-treated control (-T/+S), is likely due to an effect by *Echinacea* extract on the cell wall/membrane complex. Of all the compounds tested, sonicated *Echinacea*-treated samples (echT/+S) tended to have the highest percentage of cells showing CWD (Figure 15). As shown in Tables 4 and 5, CWD for the wild type S288C and YMR307W echT/+S strains were 28.7% and 25.1% respectively, in contrast with 12.2% (S288C) and 11.0% (YMR307W) in the -T/+S controls.

Similarly, the frequency of Evans Blue stained cells in the non-sonicated *Echinacea* treated wild type group (S288C echT/-S) did not differ significantly from the non-treated control (-T/-S) (Table 4, Figure 14). A significantly higher percentage of EB+ cells (2.99%) were found in the YMR307W mutant echT/-S when compared

with the -T/-S control (1.27%). It should be noted, however, that the YMR307W echT/-S value was not significantly different from the hygT/-S (2.03%) and natT/-S (2.18%) negative controls (Table 5, Figure 14). In contrast to the non-treated controls groups (-T/+S), both strains S288C and YMR307W, when treated with *Echinacea* extract prior sonication (echT/+S), had a significantly greater frequency of Evans Blue stained cells (Tables 4 and 5, Figure 15).

Overall, these results showed that *Echinacea* treatment does increase the sensitivity of yeast to sonication as based both on increased frequencies of cells with CWD and Evans Blue staining. Thus, the experiments provide support for the hypothesis that *Echinacea* extracts contain a fungal cell wall disrupting agent (Mir-Rashed *et al.*, 2010). Given that alkamides are major constituents of the *Echinacea* extracts used in these studies, we next examined whether specific alkamides likewise disrupt the yeast cell wall/membrane complex.

4.3.5. Test compounds— Synthetic alkamides from *Echinacea*

Alkamides, the major lipophilic compounds in Echinacea

Alkamides are the major lipophilic compounds in *Echinacea* ethanol extracts and are thought to be the major active ingredient of *Echinacea* pharmaceutical preparations (Woelkart and Bauer, 2007). In this study, we investigated the effect on the yeast cell wall of seven synthetic alkamides that occur at different concentrations in *Echinacea* root extracts (Table 3). The seven alkamides tested differ from each other by the length of the carbon chain, the degree of saturation, and position of saturated carbon bonds (Table 1).

*Percentage of cell wall damage (CWD), frequency
of Evans Blue stained cells and loss of viability (EB+) findings*

None of the alkamide treated non-sonicated (alkT/-S) samples had significantly higher levels of cell wall damage (CWD) when compared with the non-treated (-T/-S) controls (Figure 14, Tables 4 and 5). In contrast to the non-sonicated samples, treatments with each of the seven alkamides followed by sonication (alkT/+S) resulted in a significantly higher level of cell wall damage (CWD) when compared with the non-treated (-T/+S) and negative (hygT/+S and natT/+S) controls. This was observed for both wild type S288C and the cell wall mutant YMR307W strains.

The percentage of cell wall damage in sonicated samples showed a clear division of the alkamides into two groups: the alkamides alk2b and alk3a together with *Echinacea* (ECH) have the highest activity, followed by the second group comprising the remaining five alkamides, including the “tetraenes”. It stands out that alk2b, alk3a and alk5 all have a 8,10 diyne moiety (two triple bonds at 8 and 10 carbon position), in contrast with only double bonds found in the 2,4,8,10 tetraenes (alk8E and alk8Z), 2,4,8 triene (alk11) and 2,4 diene (alk12) as seen in Table 1. Also, alk2b differs from alk3a and alk5 by having one extra double bond at position C4, and alk5 differs from both alk2b and alk3 in having an additional methyl group at the diyne terminal. Of the three alkamides displaying triple bonds, alk2b and alk3a were the most effective in inducing cell wall damage in both yeast strains tested. Considering that alk2b, alk3a and alk5 display such similar molecular structure, especially alk3a and alk5, it is surprising to see a statistically lower frequency of cell wall damage associated with the alk5 treatments (Tables 4 and 5).

Based on these results, it is reasonable to infer that molecular structure variations play an important role in the alkamides cell wall disrupting activity. It appears that a terminal 8,10 diyne moiety enhances the cell wall disrupting capability of alk2b and alk3a. A similar correlation was reported in a study of permeability of alkamides to Caco-2 cell monolayers—a model for the absorption of drugs by the intestinal barrier—Matthias *et al.* (2004) found a correlation between the molecular structure variations of alkamides from *Echinacea* and their permeability to the Caco-2 monolayers. The conclusion was that saturation, and methylation on either terminal of the alkamide molecule cause a decrease in alkamide permeability of the Caco-2 monolayers (Figure 4).

In Figure 15 it is also evident that when compared with the non-treated controls the treatment with alkamides by itself did not cause any significant loss of viability. As for the alkamide-treated and sonicated (alkT/+S) wild type S288C samples, the percentages of Evans Blue stained cells were significantly higher those observed for the non-treated (-T/+S) and negative (hygT/+S and natT/+S) controls but not significantly different among themselves or in comparison with either *Echinacea* or caspofungin treated samples (Figure 15). More variation was observed among the alkamide-treated samples in the cell wall mutant YMR307W (ANOVA results—Table 5). Most notable is the overlap occurring among all alkamides, with exception of alk2b and alk12, and the negative controls (hygT/+S and natT/+S). Nonetheless, the frequency of Evans Blue stained cells in all alkamide-treated samples was significantly higher when compared with the non-treated control. Overall, we can infer that treatment with any of the seven alkamides tested, with or without sonication, results in an increase in Evans blue staining, a proxy for cell death.

Figure 14. The figure illustrates the effect of treatment of the yeast cells with the test compounds with no sonication (-)Son. The top panel shows the wild type S288C results and the bottom panel shows the YMR307W cell wall mutant results. Data shows mean percentage $\pm$ SE of cell wall damaged and Evans Blue+ cells without sonication, with and without treatment ($n \geq 3000$ cells). Within a group, means linked by the same letter are not significantly different from each other (ANOVA, Tukey test $\alpha = 0.05$).

Figure 15. Effect on yeast cells of exposure to test compound followed by mild sonication. The top panel shows the wild type S288C results and the bottom panel shows the cell wall mutant YMR307W results. Data shows mean percentage $\pm$ SE of cell wall damage (CWD) and Evans Blue+ cells in sonicated (+)Son test groups with and without treatment. This data are the results of at least 3 independent experiments were $n \geq 3000$ cells. Within a group, means linked by the same letter are not significantly different from each other (ANOVA, Tukey test $\alpha = 0.05$ $P < 0.0001$).

Table 4. *S. cerevisiae* wild type S288C wall damage and cell death measurements. Data show mean percentage $\pm$ SE of cell wall damage (CWD) and Evans Blue stained cells (EB+) in two main test groups: (-)S=no sonication and (+)S=with sonication, treated with test compounds [cloNAT= Nourseothricin, HYG= Hygromycin B, CAS= Caspofungin, ECH=*E.purpurea* crude extract and the synthetic alkamides Alk2b,3a,5,8E,8Z,11 and 12 (Table 1)] or without treatment (CTR). Data shown here are the results of at least 3 independent experiments were $n \geq 3000$ cells. Within a group, means designated by the same colored and styled letter are not significantly different from each other (ANOVA, Tukey test $\alpha = 0.05$).

Test compound	Test group	CWD % $\pm$ SE	EB + % $\pm$ SE	ANOVA			
				CWD		EB+	
				(-)S	(+)S	(-)S	(+)S
CTR	(-)S	0.14 $\pm$ 0.04	0.61 $\pm$ 0.10	b		c	
	(+)S	12.2 $\pm$ 0.39	14.2 $\pm$ 0.48		c		b
cloNAT	(-)S	0.00 $\pm$ 0.00	0.51 $\pm$ 0.15	b		c	
	(+)S	12.0 $\pm$ 0.52	14.4 $\pm$ 0.73		c		b
HYG	(-)S	0.02 $\pm$ 0.02	0.52 $\pm$ 0.12	b		c	
	(+)S	13.1 $\pm$ 0.71	14.5 $\pm$ 0.70		c		b
CAS	(-)S	0.80 $\pm$ 0.21	3.90 $\pm$ 0.56	a		a	
	(+)S	20.9 $\pm$ 0.69	23.3 $\pm$ 0.86		b		a
ECH	(-)S	0.14 $\pm$ 0.06	0.48 $\pm$ 0.10	b		c	
	(+)S	28.7 $\pm$ 0.63	19.2 $\pm$ 1.16		a		a
Alk2b	(-)S	0.30 $\pm$ 0.15	0.82 $\pm$ 0.19	b		b/c	
	(+)S	28.1 $\pm$ 0.67	21.6 $\pm$ 1.03		a		a
Alk3a	(-)S	0.03 $\pm$ 0.03	0.51 $\pm$ 0.20	b		c	
	(+)S	27.4 $\pm$ 0.91	20.3 $\pm$ 0.88		a		a
Alk5	(-)S	0.04 $\pm$ 0.04	0.79 $\pm$ 0.16	b		b/c	
	(+)S	22.4 $\pm$ 0.88	21.2 $\pm$ 0.93		b		a
Alk8E	(-)S	0.10 $\pm$ 0.05	1.29 $\pm$ 0.29	b		b/c	
	(+)S	21.9 $\pm$ 0.86	19.8 $\pm$ 1.30		b		a
Alk8Z	(-)S	0.19 $\pm$ 0.06	0.70 $\pm$ 0.16	b		c	
	(+)S	20.1 $\pm$ 0.60	19.8 $\pm$ 1.01		b		a
Alk11	(-)S	0.13 $\pm$ 0.07	1.80 $\pm$ 0.22	b		b	
	(+)S	21.9 $\pm$ 1.33	20.7 $\pm$ 0.69		b		a
Alk12	(-)S	0.21 $\pm$ 0.08	1.23 $\pm$ 0.28	b		b/c	
	(+)S	20.7 $\pm$ 0.66	21.6 $\pm$ 0.78		b		a

Table 5. *S. cerevisiae* cell wall mutant YMR307W cell wall damage and cell death measurements. Data show mean percentage $\pm$ SE of cell wall damage (CWD) and Evans Blue stained cells (EB+) in two main test groups: (–)S=no sonication and (+)S=with sonication, treated with test compounds [cloNAT= Nourseothricin, HYG= Hygromycin B, CAS= Caspofungin, ECH=*E.purpurea* crude extract and the synthetic alkamides Alk2b,3a,5,8E,8Z,11 and 12 (Table 1)] or without treatment (CTR). Data shown here are the results of at least 3 independent experiments were $n \geq 3000$ cells. Within a group, means designated by the same colored and styled letter are not significantly different from each other (ANOVA, Tukey test $\alpha = 0.05$).

Test compound	Test group	CWD % $\pm$ SE	EB + % $\pm$ SE	ANOVA			
				CWD		EB+	
				(–)S	(+)S	(–)S	(+)S
CTR	(–)S	0.07 $\pm$ 0.03	1.27 $\pm$ 0.15	a		c	
	(+)S	11.0 $\pm$ 0.43	12.1 $\pm$ 0.45		c		e
cloNAT	(–)S	0.09 $\pm$ 0.05	2.18 $\pm$ 0.33	a		b/c	
	(+)S	11.3 $\pm$ 0.41	13.4 $\pm$ 0.75		c		d/e
HYG	(–)S	0.03 $\pm$ 0.03	2.03 $\pm$ 0.32	a		b/c	
	(+)S	11.8 $\pm$ 0.83	14.3 $\pm$ 0.76		c		c/d/e
CAS	(–)S	0.24 $\pm$ 0.08	9.58 $\pm$ 0.76	a		a	
	(+)S	23.4 $\pm$ 0.82	25.3 $\pm$ 1.05		a		a
ECH	(–)S	0.18 $\pm$ 0.09	2.99 $\pm$ 0.44	a		b	
	(+)S	25.1 $\pm$ 0.63	17.6 $\pm$ 0.89		a		b/c
Alk2b	(–)S	0.19 $\pm$ 0.10	2.07 $\pm$ 0.30	a		b/c	
	(+)S	23.8 $\pm$ 0.57	24.1 $\pm$ 0.84		a		a
Alk3a	(–)S	0.07 $\pm$ 0.05	2.06 $\pm$ 0.26	a		b/c	
	(+)S	24.1 $\pm$ 0.93	17.8 $\pm$ 0.92		a		b/c
Alk5	(–)S	0.04 $\pm$ 0.03	1.37 $\pm$ 0.17	a		c	
	(+)S	19.3 $\pm$ 0.38	16.7 $\pm$ 0.58		b		b/c/d
Alk8E	(–)S	0.09 $\pm$ 0.05	1.39 $\pm$ 0.28	a		c	
	(+)S	18.8 $\pm$ 0.85	17.4 $\pm$ 0.93		b		b/c
Alk8Z	(–)S	0.02 $\pm$ 0.02	1.59 $\pm$ 0.23	a		b/c	
	(+)S	18.0 $\pm$ 0.57	19.0 $\pm$ 0.82		b		b/c
Alk11	(–)S	0.05 $\pm$ 0.04	1.49 $\pm$ 0.22	a		c	
	(+)S	17.9 $\pm$ 0.86	17.6 $\pm$ 0.81		b		b/c
Alk12	(–)S	0.07 $\pm$ 0.05	1.98 $\pm$ 0.35	a		b/c	
	(+)S	17.4 $\pm$ 0.60	19.1 $\pm$ 0.84		b		b

4.4. Alkamides quantitative structure-activity relationships (QSAR)

*Quantitative structure-activity relationships (QSAR) studies of the inhibition of growth of *S. cerevisiae* by alkamides from *Echinacea**

Quantitative structure-activity relationships (QSAR) models are theoretical models that correlate the chemical structure and related physicochemical properties of a molecule to an *in vitro* or *in vivo* biological activity. The development of a QSAR model requires a data set of experimental measures of a biological activity for a group of structural analog compounds; one or more physicochemical property descriptors and statistical methods, to find the correlation between these two data sets. Once expressed as a mathematical equation, a QSAR model can be used to predict the efficacy of structurally related compounds in eliciting a biological activity. The information learned from these correlation models can then be used to tailor the chemical structure of a compound to produce more effective analog compounds.

4.4.1. QSAR non-linear relationships and optimum log P (log P₀)

Hansch and Clayton (1973), showed that the relationship between the lipophilicity of compounds (log P) and biological activities such as inhibition of growth are not linear as the biologic activity can not be infinitely increased by increasing the lipophilicity of a initial structure. In fact, after a certain point an increase in lipophilicity leads to a decrease in the biologic activity (Hansch, 1980) and more often than not these relationships are better represented by a parabolic equation. These observations led to the postulation of the parabolic model equation formally expressed as:

$$\log 1/C = a (\log P)^2 + b (\log P) + c \quad (2b)$$

Where log P is the value of the lipophilicity descriptor for each molecule in the series and where each letter *a*, *b* and *c*, represents a coefficient calculated by fitting variations in the data by regression analysis using the least square method.

Hansch findings can be explained by analyzing the journey molecules take from the site of administration to the eventual site of action. Described by Hansch as the “random-walk” process of multiple partition between biological compartments, these interactions are more complex than interactions observed in *in-vitro* studies and are heavily dependant on the molecules lipophilic character. Along this “random-walk” process a molecule crosses numerous lipid membranes and partitions in and out of aqueous and lipophilic phases, back and forth until it reaches the target site. The implication of the “random-walk” is that the biologic activity of a compound is a function of its ability to reach and bind to its target site, hence the importance of log P as a key determinant of the pharmacokinetic properties of a molecule.

As the log P approaches zero and below, hydrophilicity increases to a point that the molecules will not be able to cross lipid membranes and will remain in the first aqueous compartment they encounter never reaching the target site. Conversely, a high log P results in such poor aqueous solubility that the compound is sequestered in the first lipid phase it comes across and can not partition out of the lipid to the aqueous cellular and extracellular fluids, thus it may not reach the target site either. Therefore, an intermediate, optimum log P (log P₀) is needed to achieve maximum biologic activity. This constant represents the ideal balance between the hydrophilic-lipophilic character of the drug so that it can reach the sites of action in the minimum

time without being severely restricted by the “random-walk” process. Often a log P value between 2–4 is considered desirable for drug formulations (Crowley and Martini, 2004) and a log P of 5 is considered as the upper limit of desired lipophilicity for orally delivered drugs (Lipinski *et al.*, 2001).

Figure 16. Graphic representation of equation (2b), Hansch’s QSAR non-linear relationships between log 1/C and log P where the curve is a parabola and optimum log P₀ is the apex of the parabola.

4.4.2. QSAR modeling of the growth inhibitory activity of alkamides

I used a classic Hansch approach (Hansch and Clayton, 1973) of QSAR modelling to investigate the correlation between the lipophilic properties and the *in-vitro* antifungal activity of the seven alkamides from Echinacea listed in Table 1. Briefly, free online tools, Molinspiration Cheminformatics and ALOGPS 2.1, were used to calculate the lipophilicity descriptor log P (octanol-water partition coefficient)

for each alkamide. In a classic Hansch methodology, the alkamides log P values (Table 6) 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 MIC 40% expressed in mol/L (Table 2). Inverse logarithms for activity are used so that the most effective compounds have higher values in the chart (Figure 17). The curve fitting of the plotted data and statistical analysis of quality of the resulting model was done using GraphPad PRISM® version 5, GraphPad Software, San Diego California USA. The resulting QSAR models and statistical quality of the resulting models are discussed next.

4.4.3. Alkamides QSAR model: Regression analysis and model's goodness-of-fit

The results of the regression analysis and of the QSAR model's goodness-of-fit for each of the two strains tested, wild type S288C and the cell wall mutant YMR307W are illustrated in Figures 17, 18 and 19. The non-linear regression analysis by the method of least-squares resulted in the equations (3) and (4) below, where n is number of compounds included in the regression; r^2 is the coefficient of determination and s is the standard deviation.

Model 1—*S. cerevisiae* wild type S288C:

$$\log 1/C_{S288C} = 0.12(\log P)^2 - 1.22(\log P) + 5.97 \quad (3)$$

$$(n = 7; r^2 = 0.864; s = 0.041)$$

Model 2—*S. cerevisiae* cell wall mutant YMR307W:

$$\log 1/C_{YMR307W} = 0.023(\log P)^2 - 0.47(\log P) + 4.8 \quad (4)$$

$$(n = 7; r^2 = 0.730; s = 0.092)$$

Although both QSAR models follow the same trend, the two regression models varied in terms of goodness-of-fit/coefficient of determination (r^2). Comparing the two coefficients of determination, it is clear that the wild type strain (S288C) model is the best correlation model of the two. Model 1 has a r^2 of 0.86, which means that 86% of the total variation in y ($\log 1/C$) can be explained by variation in x ($\log P$) as described by equation (3); while in model 2 (YMR307W) r^2 is 0.73, the correlation between $\log 1/C$ and $\log P$ is not as adequately described by the regression model as in the case with model 1 (S288C). This difference can probably be attributed to the limitations of the method used to determine C (MIC40%), the colony forming unit (CFU) plate counting method (section 3.3.2. Materials and Methods). This technique assumes that each visible colony is derived from an individual cell and that all cells present were recovered during the incubation. However, when applied to a defective mutant with tendency to form aggregates that are difficult to disperse, such is the case of the YMR307W strain, it might result in an underestimation of CFUs counts and inaccurate C (MIC 40%) values that can translate into unreliable QSAR models. Therefore, it is probably wiser to develop QSAR models with the wild type yeast strain that will generate more reliable and more useful models for drug development.

In a second step the QSAR analysis was complemented by the plotting of the experimentally determined $\log 1/C$ (observed $\log 1/C$) versus the $\log 1/C$ values predicted by the QSAR model detailed in Equations 3 and 4 (Figure 17, Table 7). The results indicate a good correlation between predicted and observed alkamide antifungal activity for model 1 ($r^2 = 0.92$), and as expected by the nature of the cell wall mutant strain (YMR307W) discussed above, the correlation between predicted and observed values are not as well described by the regression model as it is in model 1 (S288C).

Therefore, even though the alkamide set used to develop the QSAR model is small, $n=7$; it provided QSAR equations that are statistically significant indicating that the models can be applied to predict the antifungal activity of this class of compounds.

4.4.4. Alkamides drug-likeness—Lipinski's rule of “5”

Besides the fact that the cell wall mutant is notably more sensitive to the antifungal activity of the alkamides, a few other interesting observations regarding the lipophilic nature of the alkamides can be derived from analyzing Figure 17.

The first main observation pertains to the drug-likeness properties of the alkamides as proposed by Lipinski's rule of 5. Developed by Lipinski *et al.* (2001), this rule-based approach to predict drug-likeness, postulates that poor absorption or permeability are more likely when there are more than “5” hydrogen-bond receptors (nitrogen or oxygen atoms with one or more hydrogen atoms— $n\text{OHNH}$), more than “10” hydrogen-bond acceptors (nitrogen or oxygen atoms— $n\text{ON}$), the molecular weight (MW) is greater than “500” and $\log P$ is greater than “5”. All numbers are multiples of 5, hence the rule of “5”. Considering that a desirable characteristic of a drug candidate is good absorption and permeability, the rule states that most “drug-like molecules” have $\log P \leq 5$, $\text{MW} \leq 500$, $n\text{ON} \leq 10$ and $n\text{OHNH} \leq 5$. Molecules violating more than one of the rules are more likely to have bioavailability issues and are not good candidates for drug development, conversely the more a compound conforms to the “rule of 5” the higher is the drug-likeness of the compound. The drug-likeness properties of the alkamides used in this study were obtained by entering the molecular structure to a free online tool (JME, 2011) and are summarized in Table 7. The properties of all seven alkamides are well within the rules but one violation was detected as alk12 has a $\log P$ value that exceeds 5.

Table 6. Calculated molecular lipophilicity descriptors (log P) and average log P $\pm$ standard deviation for the synthetic alkamides used in the present study. Log P values calculated using nine different algorithms (ALOGPS 2.1 calculator).

Calculator	Alk2b	Alk3a	Alk5	Alk8E	Alk8Z	Alk11	Alk12
ALOGPs	3.70	3.87	4.66	4.80	4.80	5.06	5.69
AC logP	3.25	3.61	4.63	4.46	4.46	4.81	5.17
AB/ LogP	3.58	3.79	4.68	4.83	4.83	5.04	5.25
miLogP	3.11	3.61	4.42	4.01	4.01	4.79	5.28
ALOGP	4.28	4.72	4.67	3.98	3.98	4.43	4.87
MLOGP	3.26	3.34	3.59	3.51	3.51	3.59	4.60
KOWWIN	3.74	3.95	4.50	4.75	4.75	4.97	5.18
XLOGP2	3.43	3.87	4.47	4.75	4.75	5.09	5.61
XLOGP3	3.20	3.64	4.16	4.21	4.21	4.55	5.48
Aver log P	3.51	3.82	4.42	4.37	4.37	4.70	5.24
	± 0.37	± 0.39	± 0.35	± 0.47	± 0.47	± 0.48	± 0.35

Table 7. Alkamides molecular properties and Lipinski's Rule of 5 for log P $\leq$ 5, MW $\leq$ 500, nON $\leq$ 10 and nOHNH $\leq$ 5. Red caption indicates a violation of Lipinski's rule of 5.

Lipinski's Rule of 5 — alkamides molecular properties					
Alkamide	log P	MW	nON	nOHNH	Violations
alk2b	3.51	229.32	2	1	0
alk3a	3.82	231.34	2	1	0
alk5	4.42	245.37	2	1	0
alk8E	4.37	247.38	2	1	0
alk8Z	4.37	247.38	2	1	0
alk11	4.70	249.40	2	1	0
alk12	5.24	251.41	2	1	1

Table 8. Predicted and experimentally observed log 1/C values and residuals for the alkamides QSAR models described by equations 3 and 4.

Wild type S288C				YMR307W		
Alkamide	Observed log 1/C	Predicted log 1/C	Residual	Observed log 1/C	Predicted log 1/C	Residual
alk2b	3.179	3.1647	0.01426349	3.421	3.428	-0.005976338
alk3a	3.026	3.0607	-0.03479016	3.319	3.336	-0.01982629
alk5	2.898	2.9254	-0.02748107	3.068	3.170	-0.107261
alk8E	2.978	2.9334	0.04456105	3.296	3.184	0.1074635
alk8Z	2.960	2.9334	0.02656117	3.276	3.184	0.1074635
alk11	2.865	2.8920	-0.02706605	2.990	3.098	-0.1121112
alk12	2.885	2.8810	0.00395157	2.997	2.968	0.03024787
n=7 $r^2=0.923$ P-value=0.0006				n=7 $r^2=0.753$ P-value=0.0113

Figure 17. Plots of predicted versus experimentally observed (Table 7) inhibitory activity of synthetic alkamides from *Echinacea* against *S. cerevisiae* wild type S288C (left panel) and cell wall mutant YMR307W (right panel) strains. Dotted lines represent 95% confidence intervals. The results showed that the QSAR model for the wild type S288C ($r^2=0.923$) can be applied to predict antifungal activity of alkamides in the interval calculated.

4.4.5. Alkamides optimal log P (log P₀) and “cut-off point”

Still analyzing Figure 17, a second observation can be made in light of the Hansch's QSAR parabola model (Figure 16). Noticeably, all seven alkamides have a log P that falls well on the more lipophilic side of the parabola, with the least active alkamides (alk11 and alk12) at the limit of desirable lipophilicity. Ideally, the model should be developed with data points covering the complete parabola, it is probable that a more complete curve could be obtained if more alkamides on the lower spectrum of log P were tested. This lack of data points precludes the detection of the optimal log P and “cut-off point”— a log P at which biological activity falls off rapidly or disappears completely as the log P increases beyond that point in a homologous series. As it is now, without data points in the most hydrophilic side of the parabola, it can not be said conclusively where alk2b is situated with respect to the optimum, we can only conclude that there is a reasonable drop in activity beyond this point, as hydrophobicity increases.

4.4.6. Alkamides log P and reported log P of antifungals and other compounds

We compared the alkamides log P to those reported for antifungals and other classes of compounds (Figure17). As discussed above, clearly the alkamides log P fall on the more lipophilic side of the Hansch's QSAR parabola model, with the most active alkamides (alk2b and alk 3a) log P values within the considered desirable range for drug formulations (Crowley and Martini, 2004) and the least active alkamides (alk11 and alk12) at the limit of desirable lipophilicity for oral formulations according to Lipinski's rule of 5 (Lipinski *et al.*, 2001). It was also noted that

a log P value between 2–4 is considered desirable for drug formulations (Crowley and Martini, 2004). Furthermore, a study by Hansch *et al.* (1968) found that log P of ~2 is ideal for drugs acting in the central nervous system (CNS)—This information is crucial for the development of antifungals to treat CNS opportunistic fungal infections, as the ability to cross the blood-brain barrier (BBB) is essential for these class of compounds. In addition, a recent survey of 670,536 combinatorial compounds, 10,968 drugs and 3,287 natural products revealed mean log P values of 4.3, 2.2 and 2.4 respectively (Vistoli *et al.*, 2008). These results confirm previous findings that for a variety of drugs acting *in vivo*, log P₀ has been found to be ~2 (Hansch and Lien, 1971) and this is a good indication of the bioavailability of natural products compared with combinatorial compounds.

While it is true that a log P of ~2 is desirable characteristic for a drug candidate, it should be noted that a large variation of log P values is observed in different class of antifungals. For instance, caspofungin—a known fungal cell wall disrupting compound and one of the controls used in this study is water-soluble and has a poor oral bioavailability attributed to a large molecular weight (MW=1093) and low log P value of –2.8, thus caspofungin is administered intravenously. Another example is Amphotericin B (MW=924), which is not water soluble at pH 6 to 7, but it is soluble in water at pH 2 or 11, however pharmaceutical formulations of Amphotericin B have a log P of 0.95. Triazoles antifungal agents fluconazole (MW=309), voriconazole (MW=349) are poorly water-soluble and have log P value of 2.17 and 2.56 respectively while itraconazole (MW=705) has a log P of 6.99. (Kethireddy and Andes, 2007). Griseofulvin (MW=353), a antifungal that inhibits mitosis, has a log P of 2.18 (Hansch and Lien, 1971).

In view of such variation observed in log P, it is not advisable to directly extrapolate *in vitro* observations of antimicrobial activity and log P relationships to *in vivo* applications without taking in consideration the complexity of the hydrophilic-lipophilic interactions a molecule endures during the “random-walk” it takes from the site of administration to the eventual site of action. Nevertheless, the log P–antifungal activity relationship observed for the most active alkamides alk2b and alk3a, the compliance with Lipinski’s “rule of 5” and reported ideal log P range of 2–4 for drug formulations (Crowley and Martini, 2004) warrants further investigation of the antifungal activity of this class of compounds.

4.4.7. Correlation between antimicrobial activity and structural substituents

To better understand the role of the various substituents in the lipophilicity and antifungal activity of the alkamide set studied here, the set was divided in two groups based on molecular structure similarities. The alkamides carrying dyienoic moieties are shown in Figure 18a while the dienoic alkamides containing two conjugated double bonds are shown in Figure 18b. The first general observation emerging from analyzing Figure 18 is the fact that the alkamides showing the most antifungal activity (alk2b/3a) have a 11-carbon chain (undeca–) as opposed to the less active remaining alkamides that have a 12-carbon chain (dodeca–). In a more detailed analysis of the dyienoic group (Figure 18a), consider alk2b as the original analog or parent compound; note how a saturation of the double bond at C4 results in the alk3a molecular structure, promotes an increase in lipophilicity and a decrease in antifungal activity. Alk3a by its turn results in the alk5 molecular structure when

a methyl group is introduced in the dyienoic terminal of the carbon chain. This also results in decreased antifungal activity.

Moving on to dienoic alkamides in Figure 18b; it is observed that when compared with alk2b, the substitution of the diynoic moiety for the dienoic moiety in conjunction with the terminal methylation has a highly negative influence on the antifungal activity of these alkamides. This fact seems to indicate that the presence of a diynoic moiety promotes antifungal activity. This is true to a certain point, as it can be deducted by analyzing the antifungal activities of the diynoic moiety carrying alk5 and the dienoic moiety carrying alk8E/Z. That Alk5 has weaker antifungal activity when compared with alk8E/Z can be explained by the lower lipophilicity of alk8E/Z as conferred by its four double-bonds. With regard to alkamides 11 and 12 (Figure 18b), while the relationship lipophilicity/antifungal activity seems to be inverted in this pair, these two differ by only one double bound on C-8, it is more likely that noise in the data is causing the inversion, but without more data points it is difficult to be certain that this is not a real inversion in the curve.

Figure 18. Alkamides log P in relation to the reported log P of several other compounds. The average log P of 10,968 Drugs=2.2; 670,536 Combinatorial Compounds CC=4.3; 3,287 Natural Products=2.4. CNS=Ideal log P for drugs to act in the Central Nervous System. Log P of various antifungals: Caspofungin (-2.8), Amphotericin B(0.95), Fluconazole & Griseofulvin (2.17 & 2.18), Voriconazole (2.56) and Itraconazole (6.99).

Figure 19. Correlation between the alkamides molecular structure and antifungal activity against *S. cerevisiae*. The two strains are picture side by side to illustrate the increased sensitivity of the cell wall mutant YMR307W to the alkamides.

5. Summary and Conclusions

5.1. Thesis Summary

The research presented in this thesis focuses on the study of the potential anti-fungal properties, mode of action and molecular structure-antifungal activity relationship of the alkamides, a large group of closely related unsaturated fatty acid amides widely distributed in plants. Abundant in *Echinacea* species, most notably in the roots of *E. purpurea* and *E. angustifolia*, these secondary metabolites are the major lipophilic compounds in *Echinacea* extracts. Alkamides are isobutylamides of unsaturated fatty acids and are 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 (Table 1).

Echinacea itself has a long history of use dating back well beyond the 1700's. *Echinacea* was used in traditional medicine in a spiritual context by American Natives to heal wounds and treat infections, and quickly adopted by North America settlers in the 1800's. *Echinacea*'s curative properties were subsequently promoted in a rather "snake-oil salesman" fashion. Reports of *Echinacea*'s healing properties reached Europe and led to its increasing popularity in the 1920's when it was used as an anti-inflammatory and antimicrobial. After the introduction of modern antibiotics, *Echinacea* and other herbal antimicrobials fell out of use until it was rescued from obscurity by scientific research conducted mostly in Germany in the last 30 years. Today, *Echinacea* is the fifth top selling herbal medicine in North America grossing \$16 million in 2009. Presently, *Echinacea* preparations are used internally to stimulate

the immune system, to prevent and treat upper respiratory and urinary tract infections, and topically for wound healing.

In vitro and *in vivo* studies have demonstrated *Echinacea*'s antifungal, antibacterial, antioxidant and anti-virus activity, and ability to modulate the immune cells activity. Efforts to determine the main active principle in *Echinacea* preparations have been hindered by the complexity of *Echinacea*'s phytochemical composition. The compounds most often recognized for the immuno-modulatory and pharmacological effects of *Echinacea* are the hydrophilic caffeic acid derivatives, mainly chicoric acid; the lipophilic alkamides and polysaccharides. Yet, if *Echinacea*'s pharmacological activities are caused by a specific compound, a class of compounds or are the result of the many constituents acting synergistically is still a matter of some debate. Nevertheless, new insights concerning membrane permeability, bioavailability and molecular mode of action, have renewed interest in the hypothesis that alkamides are implicated in the pharmacological effects of *Echinacea*.

Instigated by these observations and by preliminary results indicating that the antifungal activity of *Echinacea* is through cell wall disruption by its alkamides, this thesis aimed to further study the antifungal activity and mode of action of *Echinacea* ethanolic extracts and some alkamides therein. This was accomplished by the development of a fluorescent microscopy assay to detect and quantify yeast cell disruption triggered by exposure to a given compound and amplified by mild sonication (Figure 8). In addition, a general QSAR model was developed to study the molecular structure-antifungal activity relationship of alkamides. The insights obtained from these studies are presented and discussed in Section 4. Results and Discussion, and the conclusions are drawn in the next section .

5.2. Conclusions

5.2.1. Antifungal activity of test compounds

Echinacea extract and alkamides have antifungal activity against S. cerevisiae

The first finding from the results presented in Section 4 was the confirmation of the antifungal activity of *Echinacea* ethanolic extracts and that of the seven synthetic alkamides studied here. Consequently, it can be inferred that the alkamides in fact contribute to the *in vitro* antifungal activity of *Echinacea* against *S. cerevisiae* wild type S288C and the cell wall mutant YMR307W strains. As illustrated in Figure 10, these two yeast strains show similar profiles of growth inhibition in which the cell wall mutant YMR307W is generally more sensitive to inhibition by the tested compounds, except for the test compound caspofungin. The higher sensitivity of the cell wall mutant YMR307W to *Echinacea* extract and alkamides was somewhat expected since a higher susceptibility of YMR307W to *Echinacea* extracts was previously reported based on gene deletion array (GDA) analyses with *S. cerevisiae* viable gene deletion mutants. This previous study identified a group of 23 *Echinacea*-sensitive mutants with 16 of the mutants having a reported function; from this group a set of 10 gene deletions are reported as being involved in cell wall function, including YMR307W, suggesting that a target for *Echinacea* is the fungal cell wall (Mir-Rashed *et al.*, 2010).

The adaptations of the GAS1 mutants to a defective incorporation of β -1,3-glucan in the cell wall may explain YMR307W increased resistance to caspofungin

The cell wall mutant YMR307W is reported to have decreased resistance to a series of different class of compounds (SDG project., 2008) including hygromycin B (HYG) that along with nourseothricin (clonNAT) is used as a non-cell wall disrupting antifungal control in my research. Similarly, our results show that when compared to the wild type control S288C, YMR307W is more sensitive to both HYG and clonNAT antifungals. These observations raise the question as why does a cell wall defective yeast strain with decreased resistance to so many compounds show an increased resistance to caspofungin, an antifungal that targets the yeast cell wall by inhibiting the synthesis of a major fungal cell wall component, the β -1,3-glucan?

As discussed before in Section 4.3.3, the cell wall mutant YMR307W has a deletion of the gene *GAS1* that encodes 1,3-beta-glucanosyltransferase, a GPI-anchored protein localized to the extracellular face of the plasma membrane and involved in the remodeling of β -1,3-glucan after its syntheses by β -glucan synthase at the membrane (Mouyna *et al.* 2000). A deletion of *GAS1* results in reduced viability and several morphologic defects including cells that are abnormally round and multi-budded. This phenotype indicates that *GAS1* mutation interferes with the separation of the bud from mother cell (Popolo *et al.*, 1993). Despite an upregulated β -1,3-glucan synthase activity *GAS1* mutants are reported to have lower β -glucan content in the cell wall as a result of a defective incorporation of β -1,3-glucan with the excess being

released into the culture medium (Ram *et al.*, 1998). Furthermore, an increase in mannoprotein levels and a 10-fold increase in cell wall chitin were observed in *GAS1* mutants (Popolo *et al.*, 1997). Hence, it is hypothesized that, in an attempt to ensure cell wall integrity, the YMR307W mutant compensates for the defective β -1,3-glucan assembly and the resulting loss of cell wall strength by upregulating the cell wall integrity pathway (CWI) and reinforcing the cell wall with deposits of chitin (Klis, *et al.*, 2006). Therefore, it can be reasoned that the increased resistance of the YMR307W mutant to the inhibition of β -1,3-glucan synthesis by caspofugin is the result of the adaptation responses of the YMR307W mutant to a defective incorporation of β -1,3-glucan in the cell wall.

5.2.2. Yeast cell wall/membrane complex disrupting activity of test compounds

The fungal cell wall is a multilayered structure encapsulating the yeast cell. This dynamic interface is essential to sustain the morphology and to protect the yeast cell against adverse environmental conditions. To ensure its biogenesis and integrity at all times a considerable amount of energy and a tightly controlled regulatory apparatus, the cell wall integrity pathway, is necessary. For that, de Groot *et al.* (2001), estimated that about 20% of *S. cerevisiae* genes have a cell wall biogenesis related function. The high demand of the cell wall on the yeast gene machinery and its unique structural and chemical make-up, make the cell wall a desirable target for the development of novel selectively cytotoxic antifungals.

In this thesis, the yeast cell wall disrupting capability of the test compounds is evaluated by detecting and quantifying cell wall damage via a fluorescence microscopy assay. The basis of this assay is the assumption that a weakened yeast

cell wall/membrane complex will be more sensitive to mild ultrasound-induced cavitation (sonication). Double staining yeast cells suspensions with calcofluor white and Evans Blue makes it possible to observe and enumerate the extent of cell wall damage caused by the treatments with test compounds. Images obtained with duo fluorescence channel DAPI and Rhodamine filters will detect, respectively, calcofluor fluorescence—a blue-white staining the fungal cell walls—and Evans Blue fluorescence which is visualized as a white-reddish/pink fluorescence and indicates loss of metabolic activity in yeast cells. The key steps in this assay are summarized in Figure 8.

*Evans blue staining as indicator of cell viability
and non-CWD related cell death*

The Evans blue staining was used in this assay as a proxy for dead or non-viable cells that otherwise look intact when observed with the calcofluor white detecting DAPI filter. This staining technique allows the observation of non-CWD related death in the yeast suspensions prior and after sonication (Figure 11). With exception of the caspofungin treated groups, less than 2% of the yeast cells in the wild type S288C strain and less than 3% on the cell wall mutant YMR307W were Evans Blue-positive (EB+) prior sonication, which is a good indication of the general health of the yeast cell suspension and also an indication that the yeast cell have been exposed to a sub-lethal concentration of the test compounds before the sonication treatment.

Treatment with caspofungin at sub-lethal concentration causes significantly higher frequency of EB+ in non-sonicated samples

Considering the results of the intra-assay control assuring a growth inhibition of ~40% for the caspofungin treated group (Figure 10, Table 2), the frequency of EB+ cells observed in both strains tested was unexpectedly high. Specifically 3.9% in the wild type S288C and 9.58% in cell wall mutant YMR307W compared with the controls results of 0.61% and 1.27% in the S288c and YMR307W respectively (Tables 4 and 5). The most probable explanation for this finding is the fact that quantification of cell viability by the Evans Blue method is based on the assumption that incorporation of Evans Blue dye into the cytoplasm is an indication that the cell is permanently non-viable and incapable of repair, in other words—dead (Chen *et al.*, 2003; Jimenez *et al.*, 2009). However, it should be considered that, given time, a cell with a temporarily non-functional cell wall/membrane complex (e.g., overtaxed or temporarily non-functional efflux pump) might be capable of repair if the cell is removed from the environment/agent causing the injury (Crutchfield *et al.*, 1999). The apparent discrepancy between the drop plate assay and the Evans Blue staining may, therefore, reflect a cell wall disruption by caspofungin that does not subsequently lead to immediate cell death.

Mild sonication alone causes some loss of viability, and the combined effect of sonication and treatment with cell wall disrupting compounds causes loss of viability that goes beyond visible cell wall damage

A close examination of the statistics on EB+ cells in the non-treated and sonicated controls (-T/+S) reveals a baseline of ~14% on S288C and ~12% on YMR307W. Therefore any EB+ frequency significantly higher than these baselines can be somewhat related to the combined effects of sonication and prior treatment with test compounds.

Frequencies of EB+ in the sonicated samples treated with either aminoglycoside (protein synthesis inhibitor) antibiotics HYG or cloNAT, were not significantly different from that of the non-treated controls. Conversely, all other test compounds treated groups showed a significantly higher frequency of EB+. The nature of the disability causing this increase can only be speculated based on the fact that a functional intact membrane is vital to keep Evans blue from staining the cell cytoplasm, therefore EB+ cells are clearly struggling to keep the dye from staining the cytoplasm, which might be an indication of difficulties in the cell wall/membrane complex function(s) caused by the combined effect of sonication and the effect of a prior treatment with the test compounds that have cell wall disrupting activity, since the protein synthesis inhibitor antibiotics HYG or cloNAT did not show increased frequency of EB+.

The fluorescence microscopy assay has good specificity as demonstrated by its correct identification of negative controls with exception of caspofungin

The results of fluorescence microscopy assay show no statistically significant cell wall damage (CWD) detected in any of the non-sonicated groups with exception of the caspofungin treated wild type S288C group (Figure14). This is a good indication that the cell wall was not damaged by exposure to the test compounds and an increase in CWD frequency in the sonicated samples is likely due to an effect of the test compound on the cell wall/membrane complex. On the other hand, the CWD frequency detected in the caspofungin treated non-sonicated group was ~0.8%. This very small percentage was still singled out as significant by the ANOVA statistical analysis, which is in support of the reported very specific cell wall disrupting activity of caspofungin by inhibition of β -1,3-glucan synthase. Even at the relatively low concentration used in this study, set to achieve a ~40% growth inhibition, exclusive treatment with caspofungin without sonication causes significantly more CWD than that observed in any of the other non-sonicated test compound treated groups.

Hygromycin B and nourseothricin are suitable negative controls for this study.

As exemplified in Figure 15, the results also showed a baseline of ~12% frequency of CWD in both strains control group of non-treated sonicated cells (-T/+S) Likewise, both aminoglycoside (protein synthesis inhibitor) antibiotics HYG and cloNAT test results show a CWD frequency very close to the baseline detected for the (-T/+S) control groups and were not singled out as statistically significant by the ANOVA analysis. This indicates that exposures to either HYG or cloNAT, with

or without sonication, does not cause higher frequency of cell wall damage than that observed in the non-treated controls. This observation validates the use of these compounds as suitable negative controls for this study as they both inhibit yeast proliferation but, under the experimental conditions here, neither is associated with altered cell wall sensitivity to sonication.

Exposure to caspofungin, a β -1,3-glucan synthase inhibitor and cell wall disrupter, results in enhanced sensitivity to sonication.

As expected from a β -1,3-glucan synthase inhibitor compound, the percentage of cell wall damage in the caspofungin treated prior sonication (casT/+S) group was significantly greater than, and almost double of that observed in the control groups (-T/+S) and this was true for both wild type S288C and cell wall mutant YMR307W strains (Figure 15, Tables 4 and 5). Therefore caspofungin can be considered a suitable positive control for this assay.

Treatment with Echinacea enhances the sensitivity of yeast cells to sonication

Of all the groups tested the sonicated *Echinacea*-treated group (echT/+S) had the highest frequency of cell wall damage (Figure 15, Tables 4 and 5), supporting the hypothesis that *Echinacea* extracts contain a fungal cell wall disrupting agent (Mir-Rashed *et al.* 2010). Since alkamides are the major constituents of *Echinacea* extracts, it is reasonable to speculate that alkamides are involved in the cell wall/membrane complex disruption activity of *Echinacea* extracts. And that is what we examined next.

Alkamides are potential cell wall/membrane complex disrupting agents

Likewise the *Echinacea* extract and caspofungin, all seven synthetic alkamides showed a significantly higher frequency of cell wall damage (Figure 15) when compared with the non-treated (-T/+S) and negative (hygT/+S and natT/+S) controls. Moreover, the alkamides are clearly divided in two groups, with the more active alkamides, alk2b and alk3a, grouped together with *Echinacea* (ECH), and a second group comprised of five remaining less active alkamides. This second group includes the most abundant of the alkamides, the pair of isomeres alk8E and alk8Z, the “tetraenes” that account for ~45% of the total alkamides found in *Echinacea*.

As for the reasoning behind the higher activity of the less abundant alk2b (~18% of total alkamides) and alk3a (less than 1%) it appears to be related to the molecular structure of these alkamides. It stands out that alk2b, alk3a and alk5 have a 8,10 diyne moiety at C8 and C10 carbon position, in contrast with the double bonds found in the 2,4,8,10 tetraenes (alk8E, alk8Z), 2,4,8 triene (alk11) and 2,4 diene (alk12) (Table 1). Considering that of the three alkamides displaying diyne moieties, alk2b and alk3a were the most effective in inducing cell wall damage in both strains tested, it is intriguing that alk5 differing from alk3a only by an additional methyl group at the diyne terminal displayed such a drop in activity. Based on these observations, it is reasonable to infer that molecular structure variations in the alkamides play a key role in the yeast cell wall damage activity of these compounds. To better understand the causal relationship between these variables a proposed Quantitative Structure Activity Relationship (QSAR) model for the alkamides is discussed next.

5.2.3. Alkamides QSAR modeling

Following the paradigm that similar physicochemical properties cause similar biological activities, the goal of Quantitative Structure Activity Relationships (QSAR) modeling in drug development is to obtain a mathematical expression that best describes the relationship between physicochemical properties and biological activity and predict the biologic activity of a group of similar molecular structures. The information learned from these correlation models can then be used to tailor the chemical structure of a compound to produce more effective analog compounds. The QSAR concept was initiated by Corwin Hansch and his groups in early 1960s and has evolved since then from 2D to encompass much more complex 3D QSARs and 4D QSARs. In this thesis, a classic Hansch approach to QSAR modeling was used to study the correlation between lipophilic properties of alkamides and the *in-vitro* antifungal activity. To find this correlation, two data sets of property descriptors and statistical methods were used. As described in detail on section 4.4.2., the data set for the lipophilic properties ($\log P$) of each alkamide was established utilizing free online tools and the second data set used was the experimental values of antifungal activity $\log 1/C$, where C is the alkamide MIC40% in mol/L (Table 2). The two sets of data were plotted and QSAR model was developed using a non-linear regression analysis by the method of least-squares.

The alkamides QSAR model are reliable can be used to predict the antifungal activity of alkamides against S. cerevisiae

The two resulting equations of the alkamides QSAR modeling followed the same trend and described ~86% of the activity for the 7 alkamides in the wild type S288C group and ~73% in the cell wall mutant YMR307W (equations 3 and 4, $r^2=0.86$ and 0.73 in that order). A complementary analyses plotting the alkamides predicted and experimental antifungal activity show a good fit with $r^2=0.92$ (S288C) and 0.75 (YMR307W). Ideally, a QSAR model should be developed with data points covering most of the parabola; in our case the lack of experimental data points to the more hydrophilic side of the parabola precludes the detection of the optimal log P and “cut-off point”— a log P at which biological activity falls off rapidly or ceases completely as the log P increases beyond that point in a homologous series of molecules. Therefore it is not possible to say conclusively—even though it looks like that it is the case—that the log P of alk2b is the “cut-off point”. It can only be concluded that there is a reasonable drop in activity beyond this point. Nonetheless, the coefficients of correlation resulting from the QSAR models developed here indicate that, in particular the model developed for the wild type S288C, can be successfully used to predict alkamides in-vitro antifungal activity against *S. cerevisiae*.

Six of the seven alkamides conform to the Lipinski's “rule of 5”, and as such can be considered good candidates for drug development.

Another interesting outcome of this study pertains to the analysis of the drug-likeness properties of the alkamides as proposed by Lipinski's “rule of 5” and described in detail in Section 4.4.4. This rule-based approach predicts that molecules violating more than one of the rules are more likely to have bioavailability issues and

conversely the more a compound conforms to the “rule of 5” the higher is the compound drug-likeness and the chance it can be successfully developed into marketable drugs. As shown on Table 7, the properties of all seven alkamides are well within the rules with only one violation detected as alk12 has a log P higher than 5. Therefore it can be said that alkamides are good candidates for drug development.

Lipophilicity plays a significant role on the antifungal activity of alkamides

Of particular interest to this study were the observations drawn from Figures 18 and 19 (plots of log P versus log1/C). Figure 19 clearly illustrates the cell wall mutant YMR307W decreased resistance to the antifungal activity of alkamides when compared with the wild type S288C, and in contrast to the activity of caspofungin. This observation could be one more indication of the alkamides potential cell wall/membrane complex disruption activity.

A few other relevant observations can be derived from analyzing Figure 18 and 19. These two figures also illustrate the significant role the lipophilicity parameter has on the antifungal activity of this class of compounds. This was rather expected considering that lipophilicity is also a major factor that influences the pharmacokinetic and pharmacodynamic behavior of molecules when they take the “random-walk” from the site of administration, partitioning in and out of lipophilic membranes and hydrophilic compartments, until it can reach the target site. Noticeably, all seven alkamides have a log P positioned on the more lipophilic side of the parabola, with the most active alkamides (alk2b/alk3a) positioned towards the hydrophilic side and the least active alkamides (alk11/alk12) at the limit of desirable lipophilicity, thus

supporting the concept that there is an optimum log P and an ideal balance between lipophilicity and hydrophilicity is required to achieve maximum biologic activity.

Alkamides log P are comparable to average log P of other drugs

Still analyzing Figure 18, it is interesting to observe how the alkamides log P values compare to the log P of other antifungals and other classes of compounds reported by other studies (Figure 18). In general, a log P ~2 is considered a desirable value for a good drug candidate. While this may be true, a wide range of log P values can be observed in pharmacologically active compounds according to mode of administration and site of action. The antifungal griseofulvin has a log P of 2.18, the triazoles antifungals fluconazole and voriconazole have a log P of 2.17 and 2.56 in that order. In contrast, caspofungin has a log P of -2.8 and poor bioavailability due a high molecular weight (MW=1093), therefore it is administered intravenously. On the other side of the spectrum is itraconazole with a log P of 6.99. In the case of the alkamides studied here, the most active alk2b and alk3a are well positioned within the range of lipophilicity found in a study of 670,536 combinatorial compounds, 10,968 drugs and 3,287 natural products that found these compounds to have an average log P of 4.3, 2.2 and 2.4 respectively (Vistoli *et al.*,2008). These observations reinforce the argument that alkamides should be granted the status of good antifungal drug candidates that could be used as leads for further drug development.

5.2.4. Correlation between antimicrobial activity and structural substituents

An important issue investigated in this thesis is the role of the various substituents in the lipophilicity and consequently in the antifungal activity of the alkamide set studied. The experimental MICs showed that the alkamides alk2b and alk3a were the most active and QSAR study revealed that these two have log P in a range desirable for oral delivery drugs. To better understand how the various substituents are implicated in the antifungal activity of the alkamides, the set was divided in two groups based on molecular structure similarities as displayed in Figure 19. Alkamides carrying dyienoic moieties are shown in Figure 19a and dienioic alkamides are shown in Figure 19b.

A general observation emerging from the analysis of Figure 19, is that the alkamides showing the most antifungal activity (alk2b/3a) have a 11-carbon chain (undeca-) as opposed to the less active remaining five alkamides that have a 12-carbon chain (dodeca-). This is a first indication that the simple addition of a methyl group can greatly interfere with the antifungal activity of these compounds.

For the sake of argument, and assuming that alk2b is the original analog or parent compound, a saturation of the double bond at C4 results in the alk3a molecular structure. This single modification to the molecular structure of the original analog promotes an increase in lipophilicity and a decrease in antifungal activity. Following the same line of thought, addition of a terminal methyl group to alk3a results in alk5, a modification that results in decreased antifungal activity. Interestingly a study of permeability of alkamides to Caco-2 cell monolayers (Matthias *et al.*; 2004)— a model for the absorption of pharmaceutically active

molecules by the intestinal barrier—found a similar correlation between the molecular structure variations of alkamides from *Echinacea* and their permeability to the Caco-2 monolayers. The conclusion was that saturation, and methylation on either terminal of the alkamide molecule cause a decrease in alkamide permeability of the Caco-2 monolayers.

By the same token, when compared with alk2b, the substitution of the diynoic moiety for the dienoic moiety in conjunction with the terminal methylation have a highly negative influence on the antifungal activity of the dienoic alkamides (Figure 19b). This observation indicates that the presence of a diynoic moiety promotes antifungal activity. This is true to a certain point, as it can be deduced by analyzing the antifungal activities of the diynoic moiety carrying alk5, and the dienoic moiety carrying alk8E/Z. In this case, alk8E/Z lower lipophilicity conferred by its four double-bonds promotes the antifungal activity, while the presence of a diynoic moiety on alk5 was not sufficient to overcome the higher lipophilicity conferred by the additional methyl group in the dyienoic terminal and therefore its weaker antifungal activity. It appears that, in the case of the alkamides studied here, the resulting lipophilicity of the molecule is more relevant to the antifungal activity than the kind of molecular substitution that promoted it. Would it be a case of “it doesn’t matter how you get there, as long as you get there”?

5.3. Final conclusions

This thesis demonstrates the potential of the fluorescence microscopy assay developed here to be used as screening assay to investigate the ability of naturally-derived compound to disturb the integrity and/or interfere with function(s) of the yeast cell wall/membrane complex. Such an assay can provide valuable data to advance drug design by facilitating the exploration of the untapped pharmacological potential of lead molecules mined by bio-prospecting and reverse pharmacology/ethnopharmacology studies. Equally important, the method may lead to the development of naturally-derived novel antifungals that hopefully will reverse the global impact of antimicrobial resistance.

One of objectives of this thesis was to study the antifungal activity of *Echinacea* extracts and test the hypothesis that the antifungal mode of action of *Echinacea* extracts is through the disruption of the yeast cell wall by alkamides. In the course of this research study and thesis preparation I have come to realize that, due to the proximity of the cytoplasmic membrane to the cell wall (Figure 1), it is plausible that disruptions of membrane function(s) will somewhat affect the cell wall function(s) and vice-versa. Therefore these two cellular structures would be better served if considered as a complex, hence the expression cell wall/membrane complex used here.

The results of the research done here indicate that *Echinacea* extract and the seven synthetic alkamides tested have antifungal activity *in vitro* against *S. cerevisiae* wild type S288C and the cell wall mutant YMR307W (*GAS1*). It was noted that *Echinacea* extract and the alkamides tested are particularly more effective against

the mutant YMR307W (*GAS1*)— A mutant whose 1,3-beta-glucan is not correctly incorporated into the cell wall after its synthesis at the membrane site. This finding suggests that these compounds mode of action cause additional stress to the already overtaxed cell wall/membrane complex of this *GAS1* mutant. Moreover, yeast cells subjected to sub-lethal concentrations of *Echinacea* extract and alkamides prior treatment with mild sonication have shown a significantly higher frequency of cell wall damage when compared with the control groups, proving further support to the hypothesis that the antifungal mode of action of *Echinacea* extracts is by the disruption of the yeast cell wall/membrane complex by the alkamides.

Furthermore, preliminary unpublished studies are underway to investigate if of *Echinacea* extracts and alkamides have membrane disruptive activity. This study applies a fluorescence microplate based assay to detect leakage in liposomes encapsulating the fluorescence dye carboxyfluorescein caused by exposure of the liposomes to a membrane active compound. An impressive liposome leakage was observed when these liposomes are exposed to *Echinacea* extracts and to alk2b (data not shown), indicating a robust liposome disruption activity by these compounds. These findings suggest that these compounds could be disrupting the yeast cell wall/membrane complex by acting at the membrane level and causing cell wall damage by interfering with the biogenesis of the cell wall.

In addition to the experimental data, and of particular importance for understanding the difference in activity among the alkamides tested, are the results of the QSAR model developed here. In the process of gathering data to develop the QSAR model it was detected, based on Lipinski's "rule of 5," that alkamides have molecular properties characteristic of drug-like compounds. Noteworthy is the fact

that the log P—a measure of lipophilicity that affects membrane penetration and permeability—of the two most active alkamides alk2b and alk3a fall in the range of desirable lipophilicity for orally derived drugs while the least active alkamides fall close to the cut off point where log P higher than 5; a log P value between 2–4 is considered desirable for drug formulations. Furthermore, the QSAR studies indicate a strong lipophilicity-antifungal activity relationship in the set of alkamides tested and produced a mathematical expression that can use to predict the antifungal activity of these class of molecules.

Understanding the role of lipophilicity as a major factor in the pharmacokinetic and pharmacodynamic behavior of drugs is of paramount importance to choose a successful drug candidate. An optimum hydrophilicity-lipophilicity balance is essential for efficient drug distribution in the different compartments from the site of administration to site of action—the so called “random-walk”. This effect can be observed in Figure 19 where it is clear that an optimum log P is required to achieve maximum biologic activity and that the antifungal activity of the alkamides diminish greatly as the molecule becomes too lipophilic. It is noteworthy that the “tetraenes” are the most abundant alkamides in *Echinacea* extract (~40% of the total) but showed a relatively weak antifungal activity when compared with the less abundant alk2b (~18% of the total). Looking at Figure 19, at first glance, one is tempted to suggest that a diynoic moiety promotes antifungal activity in the alkamides, since the two most effective alk2b and alk3a carry it; while saturation of double bonds and methylation on the terminal of the alkamide molecule hinder the antifungal activity. However, a more thorough analysis reveals that, in the end, it is the lipophilicity of

the alkamide that matters, rather than the molecular substituent accountable for that lipophilicity.

To sum up, the findings of this research further document the antifungal activity of *Echinacea* extracts and alkamides from *Echinacea* as potential yeast cell wall/membrane complex disrupters. Additional unpublished data from underway studies with liposomes encapsulating a fluorescent dye suggest that these compounds could be disrupting the yeast cell wall/membrane complex by acting at the membrane level. With exception of alk12, all alkamides tested were found to have drug-like properties as defined by Lipinski's "rule of 5". The degree of lipophilicity of the alkamides ($\log P$) was found to be a key factor in determining the antifungal activity of the alkamides and the subsequently developed QSAR model may be used to predict, to a certain degree, the antifungal activity of these class of compounds. In particular alk2b, the most effective alkamide, should be granted the status lead molecule and possibly be developed in a good drug candidate.

5.4. Future directions

The outcomes obtained from this research are very encouraging to the study of naturally-derived lead molecules such as alkamides from *Echinacea*. Suggested future research directions include the examination of additional synthetic alkamides designed based on the knowledge gained here about the molecular structure and lipophilicity-antifungal activity relationship of these compounds. Likewise, further improvements and standardization of fluorescence microplate based liposome assay aiming to develop high-throughput screening methods and to further explore the potential membrane disruption activity of *Echinacea* extracts and alkamides are also advised.

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Appendix A

General observations of the effect of mild sonication on yeast cell suspensions

In addition to the cell wall damage results, a sub-lethal effect of mild sonication treatment on the samples in general was observed. These experiments were conducted at the stage in the yeast cell cycle where mother-daughter units (cells with daughter-cell buds attached) dominated the culture, ~70% (Figure 21). However, a very high incidence of viable detached single cell units was observed in sonicated samples with mother-daughter units dropping from ~70% to ~20% (Figure 21). This occurrence is even more noticeable in the YMR307W mutant, for which the abnormal spherical morphology is a clear contrast to the usual ellipsoid shape of *S. cerevisiae*. In addition, the typical budded cell aggregates characteristic of YMR307W (Popolo *et al.*, 1993; Marx *et al.*, 2006) were apparently dispersed by sonication (Figure 20). The phenomenon is better illustrated in the cell unit type distribution chart shown in Figure 8 that gives cell characteristics before and after sonication. It should be noted that this effect is significant enough to cause interference with results of drop out plate assays that rely on counting colony-forming units (CFU) before and after sonication as means of evaluating the sonication effect. The dispersion of the cell aggregates by mild sonication increases the number of CFU to a point that it surpasses the loss of viability caused by the same mild sonication, thus creating a result where more CFUs are present in the sonicated sample than in the non-sonicated control (Figure 22).

Figure 20. Fluorescence microscopy images of yeast *S. cerevisiae* YMR307W stained with CFW illustrating the effect of mild sonication in dispersing the typically budded cell aggregates characteristic of this strain (bar =5 μ m). Top image: Non-sonicated sample. Bottom image: Sonicated sample.

Figure 21. Effect of mild sonication on yeast cell aggregation. Data shows the percentage of Mother-daughter unit, single cells and cell wall damaged/Evans Blue positive in non-sonicated [(-)Son] and sonicated [(+)Son] samples, with and without treatment with each compound tested. Each data set represents the mean value from at least three determinations. The left image shows results for the wild type S288C strain while the right figure show results for the cell wall mutant YMR307W.

Figure 22. Drop out plate image illustrating how the effect of mild sonication on the cell aggregates typical of YMR307W can create results where more CFUs are present in the sonicated sample than in the non-sonicated control.