The antifungal activity of Natamycin

A novel mode of action of the polyene antibiotics

De antischimmel activiteit van natamycine

Een nieuw werkingsmechanisme van de polyeen antibiotica

(met een samenvatting in het Nederlands)

Proefschrift

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Abbreviations

ITC,	Isothermal titration calorimetry;
DOPC,	1,2-Dioleoyl-sn-glycero-3-phosphocholine;
CFDA-SE,	5-(6)-Carboxyfluorescein diacetate, succinimidyl ester;
HPTS,	8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt;
DDAO,	N,N-dimethyldodecylamine-N-oxide;
MIC,	Minimum inhibitory concentration;
CF,	Carboxyfluorescein;
LUVs,	Large unilamellar vesicles;
*p*NPP,	para-nitrophenylphosphate;
ATP_{reg},	ATP regenerating system;
IC_{50},	half maximal inhibitory concentration;
SM,	sphingomyelin;
rhodamine-DHPE,	N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-di palmitoyl-sn-glycero-3-phosphoethanolamine;
GUVs,	Giant Unilamellar Vesicles;
CFU,	colony forming units;
TM,	transmembrane;
MFS,	major facilitator superfamily;
DIPEA,	N,N-diisopropylethylamine;
NBD-Cl,	4-chloro-7-nitrobenzofurazan;
$NaCNBH_3$,	sodium cyanoborohydride;
ACES,	N-(2-Acetamido)-2-aminoethanesulfonic acid;
TLC,	Thin layer chromatography;
Abs,	absorption;
MEB,	Malt Extract Broth;
P. discolor,	*Penicillium discolor*;
S. cerevisiae,	*Saccharomyces cerevisiae*;
A. niger,	*Aspergillus niger*;
HADDOCK,	High Ambiguity Driven biomolecular DOCKing;
SSD,	sterol-sensing domains.

Chapter 1

General introduction

Antifungal therapy

In recent years, advances in the treatment of transplant recipients, patients with cancer or AIDS are accompanied by an increase in the incidence of fungal infections (1). *Candida albicans* is the most frequent species among the current fungal pathogens, followed by other *Candida* species (e.g. *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*), non-*Candida* species (e.g. *Cryptococcus neoformans*) and molds (e.g. *Aspergillus fumigatus*, *Microsporum canis*) (2). These fungi are responsible for various forms of diseases, ranging from superficial infections of the mucosal surfaces or skin to systemic infections, which, in most cases, are life threatening. Sepsis, characterized by a whole-body inflammatory state, is the tenth most common cause of death overall and the number of cases of sepsis caused by fungal organisms has doubled from 1979 to 2000 (3).

Fungal infections are a major therapeutic challenge, because the therapeutic arsenal is limited and the use of drugs is restricted due to toxicity or unfavourable pharmacokinetic profiles. Antifungals that are currently used to treat fungal infections are divided into seven major different chemical classes targeting four cellular mechanisms. A schematic overview of these antifungals and their targets is given in Figure 1. 5-Fluorocytosine is the only representative of the class of pyrimidine analogues and acts through incorporation into DNA and RNA, inhibiting protein synthesis and thus cellular function and division (4). Candins inhibit fungal cell wall synthesis by acting on β-1,3 glucan synthase, which is located in the plasma membrane (5). Antifungal agents that act on the ergosterol biosynthesis pathway are the azoles, allylamines, thiocarbamates and morpholines (5). The polyene antibiotics target the plasma membrane of fungi via an interaction with ergosterol often resulting in membrane permeabilization (6). It is interesting to note that many of the antifungals target membrane components.

Fungal resistance is a major concern with the limited number of antifungal agents available, especially if a fungus shows resistance to more than one antifungal agent (7). Resistance of fungi is mainly observed against the azoles, candins and 5-fluorocytosine and are based on changes in proteins, from point mutations to altered gene expressions in target or transport proteins (5,7). Although reports on fungal resistance to the polyene antibiotics are increasing, it is still considered a rare event (5,7). If resistance is observed to the polyenes, it is in most cases related to lower ergosterol levels or a different sterol composition in the membrane, which can be attributed to changes in the ergosterol bio-

synthesis pathway (8-11). Yeast mutants blocked in this pathway were in first instance isolated based on resistance to the polyene antibiotic nystatin, which showed less affinity for ergosterol intermediates than for ergosterol (12,13). This type of resistance was suggested not to be an adaptation of the cells to the presence of the polyene antibiotic, but to occur through the selection of naturally resistant cells that are already present in small numbers in a population (10). However, some yeast strains that acquire resistance to the azoles by changing proteins in the ergosterol biosynthesis pathway, may also be resistant to polyene antibiotics (14). Nevertheless, the low occurrence of resistant fungi to the family of polyene antibiotics, makes this a very interesting group of antifungals.

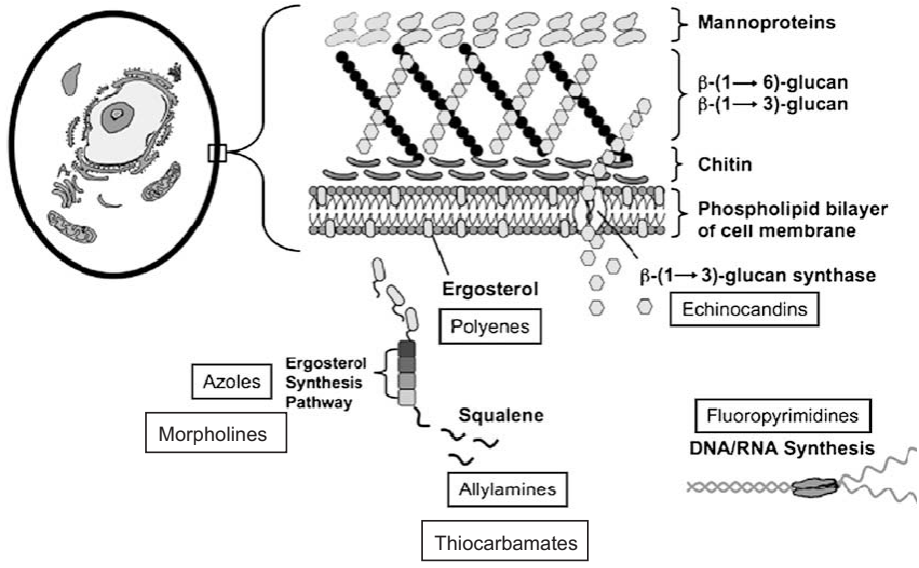

Figure 1. Targets of antifungal agents, adapted from reference (100).

Polyene antibiotics

The polyene antibiotics consist of a large family of over 200 compounds (6). Polyene macrolides are natural products synthesized by various bacterial *Actinomycetes* microorganisms (mainly *Streptomyces*) via polyketide synthase (PKS) pathways to reduce fungal organisms in their ecological niche (15). The chemical features of the polyene antibiotics are characterized by large rings that are closed by an internal ester (lactone) bond, with on one side conjugated

double bonds (hence poly*ene*) and on the other side of the ring different oxygen functions (6). The structure may contain a sugar moiety or mycosamine group, which is positively charged at neutral pH. These molecules can be amphoteric with the presence of an additional carboxyl group in the molecule. The presence of a negative charge on the carboxyl will render these antibiotics zwitterionic and thus overall neutral. Some of these antibiotics contain an aliphatic side chain, that in some occasions also contains an additional aromatic group (6). A schematic representation of the structure of the polyenes is given in Figure 2A. Amphotericin and nystatin are the main family members of the polyenes in use for the treatment of fungal infections. Both are produced in complex with co-metabolites of which amphotericin B and nystatin A1 are the main products used commercially. These polyene antibiotics have a relatively large ring structure and are amphoteric (Figure 2B - C). Although systemic treatments can lead to adverse side effects, efforts to lower the toxicity based on synthesis of different analogues or novel lipid association or encapsulation studies have shown promising results (16). The much smaller polyene antibiotic filipin is not amphoteric and is produced as a mixture of four pentaenes with filipin III (Figure 2D) as its major component. Although it is not

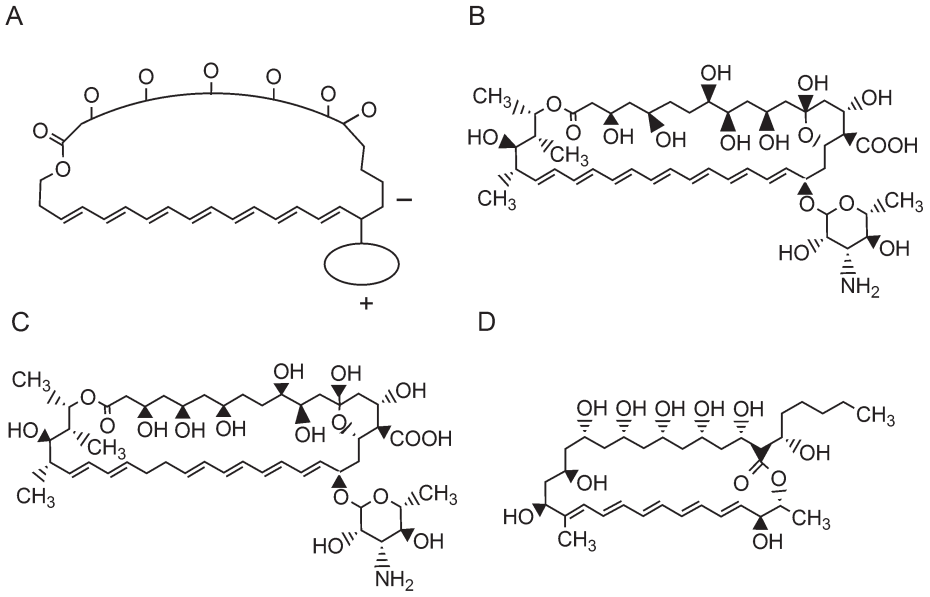

Figure 2. Molecular structures of polyene antibiotics. Shown are a schematic representation of the polyenes (A) and the structures of amphotericin B (B) nystatin A1 (C) and filipin III (D).

used clinically due to its toxicity, its fluorescent qualities have been applied to probe sterol localization and distribution in biological membranes (17,18).

Most of the polyene antibiotics are able to interact with sterols and two different categories have been proposed for their modes of action (19). Amphotericin B and nystatin belong to the first category, which have been proposed to form pores in the plasma membrane of fungi, thereby causing leakage of vital constituents, leading to cell death (6,19,20). The model for the pore structure constitutes an assembly of parallel antibiotic molecules with the hydrophobic conjugated double bonds pointing towards the hydrophobic part of the membrane, shielding the hydrophilic parts of the antibiotics that line the aqueous pore. A schematic representation of this model is given in Figure 3A. The flat ring systems of the polyenes and sterols have been proposed to interact in a 1:1 antibiotic to sterol stochiometry, through van der Waals interactions between the conjugated double bonds of the polyenes and flat sterol nucleus (19). The mycosamine group on the antibiotic is proposed to be exposed to the external medium and/or interact with the hydroxyl group of the sterol or lipid head groups (21). Although pores constituting the length of one (single length channel) and two aligned antibiotics (double length channel) are able to form, it is generally accepted that a double length channel is only possible if an antibiotic is added to both sides of the membrane, which doesn't occur *in vivo* (22-24).

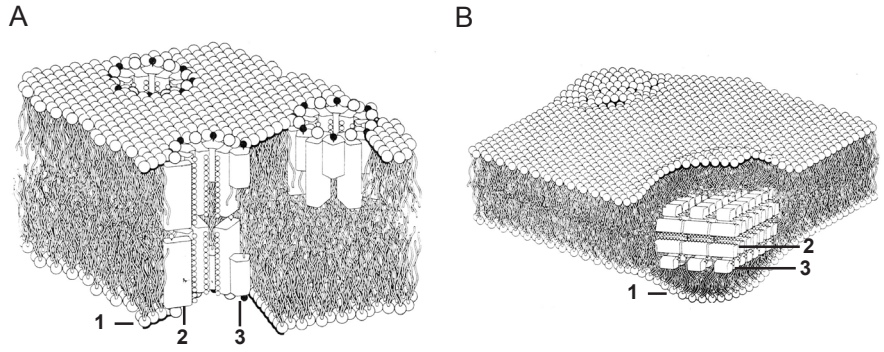

Figure 3. Different mechanisms of action of the polyene antibiotics. The schematic models represent a cross-section of a membrane and indicated are a lipid (**1**), polyene (**2**) and sterol (**3**) molecule. The mode of action of the polyenes either involves the formation of a pore (A) or the formation of large clusters in the membrane (B). Both mechanisms will cause membrane disruption and eventual death of the fungal cell. The schematic representation was adapted from reference (19).

Filipin belongs to the second category, which has been proposed to enter the membrane, but instead of forming pores, it will form large aggregates inside the hydrophobic core of the membrane, perpendicular to the plane of the bilayer (Figure 3B). These aggregates are believed to involve an interaction with sterols with the hydrophobic parts of the aggregates, while the hydrophilic parts are packed together (19). These aggregates will lead to deformations and fragmentations of the membrane leading to a general disruption of the membrane and cell death. Although the model of formation of filipin aggregates inside the bilayer and membrane disruption is accepted, the orientation of the aggregates with respect to the bilayer and importance of sterols has been disputed (25).

Natamycin

Natamycin is a member of the polyene antibiotics. Its main use is in food and solutions for the preservation of for example cheese, sausages, fruit juices, wine and yoghurt. But it is also used in medicine for the topical treatment of eyes, skin or vaginal infections. The major advantages of natamycin are its broad spectrum of activity, its long history of safe use and the fact that development of resistance has not been reported to occur. Natamycin is produced by *Streptomyces natalensis* and was first discovered in 1955 from a soil sample near Pietermaritzburg in the province of Natal, South Africa (26). It was originally called pimaricin, after Pietermaritzburg. Although natamycin has been in use since the 1960s, the exact mode of action of this antibiotic has remained elusive. Natamycin has a structure similar to amphotericin B and nystatin, although it is a much smaller tetraene and has a characteristic epoxide ring (Figure 4). Because of its relative small size, even smaller than filipin, it has been disputed if natamycin would be able to transverse the plasma membrane to form pores. (19,20) Some studies have claimed it would act in a similar fashion to filipin, causing large aggregates in the membrane and thereby causing cell death (27). But it has also been suggested that its mode of action may be purely based on the complexation of

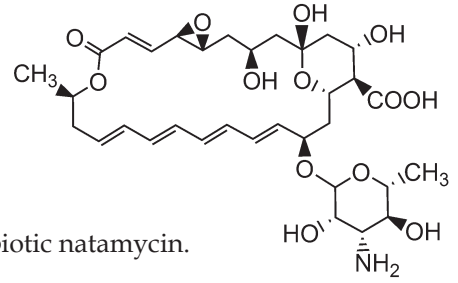

Figure 4. Structure of the polyene antibiotic natamycin.

sterols (20). Because of the relative low occurrence of resistance and to gain novel insights that may lead to more effective antifungal formulations, the studies described in this thesis will focus on unraveling the mode of action of natamycin. As a starting point, the putative interaction partner of all polyenes, ergosterol, must be examined. Below more information on this fungal sterol will be given.

Membrane sterols

Sterols are isoprenoid-derived molecules that have absolute essential functions in eukaryotes and plants (28,29). In general, there are three forms in which sterols are present in cells; as free sterols with a free 3β-hydroxyl group; steryl esters; and steryl glucosides. The major form is the free form of the sterols, which are major integral components of the membrane lipid bilayer and we will focus the attention on this form. Membrane sterols have the unique ability to increase lipid order in fluid membranes and at the same time maintain a relative fluid membrane with appreciable diffusion rates. In addition, they are believed to play a crucial role in the organization of lipid membranes in the form of specialized lipid domains (30-32). The higher order in membrane lipids leads to an increased bilayer thickness, which aid in reducing passive membrane permeability, as well as the penetration or binding of various compounds (33). Sterols are also thought to "soften" the membrane, both in terms of bending and compression.

The highest amount of sterols is found in the plasma membrane of eukaryotes, which can contain up to 20 – 40 mol % sterols to lipids, whereas it is absent in most prokaryote membranes (33). Internal membranes contain only low amounts of sterols, with a gradient of sterols from the mitochondria (3 weight %), endoplasmatic reticulum (ER, 6 weight %), to the Golgi (8 weight %) (29). Plants, algae, fungi and vertebrates synthesize sterols. Other species that depend on sterols, but take it up from the environment, termed sterol auxotrophs, are also known, for example insects (e.g. *Drosophila),* worms *(*e.g. *Caenorhabditis)* and certain types of bacteria (e.g. *Mycoplasma*) (34,35). The sterol make up of the membranes of plants, vertebrates and fungi differ markedly and their main sterols are shown in Figure 5. Sterol molecules generally contain four rings (A - D). The hydroxyl group is common to all these sterols and provides the only hydrophilic component of the molecule, allowing the proper orientation of the sterol molecule in the membrane (36). Other similarities are the double bond at C-5 in the B ring and methyl groups at C-10 and C-13. Cholesterol is the major sterol found in the membranes of vertebrates, and thus animals and

humans, and is the most extensively studied sterol (28,33). The major plant sterols, sitosterol and stigmasterol, have ring structures identical to that found in cholesterol and they differ slightly by modifications of the side chain (Figure 5). The fungal sterol, ergosterol, differs from cholesterol, by the presence of double bonds at C-7 in the B ring and at C-22 in the side chain. An additional methyl group is present at C-24 on the side chain of ergosterol, which is not there in cholesterol. Since it is the understanding that polyenes may have a preferential interaction with ergosterol for their antifungal activity, the biosynthesis, transport and functions of this sterol in fungi will be discussed (37-39).

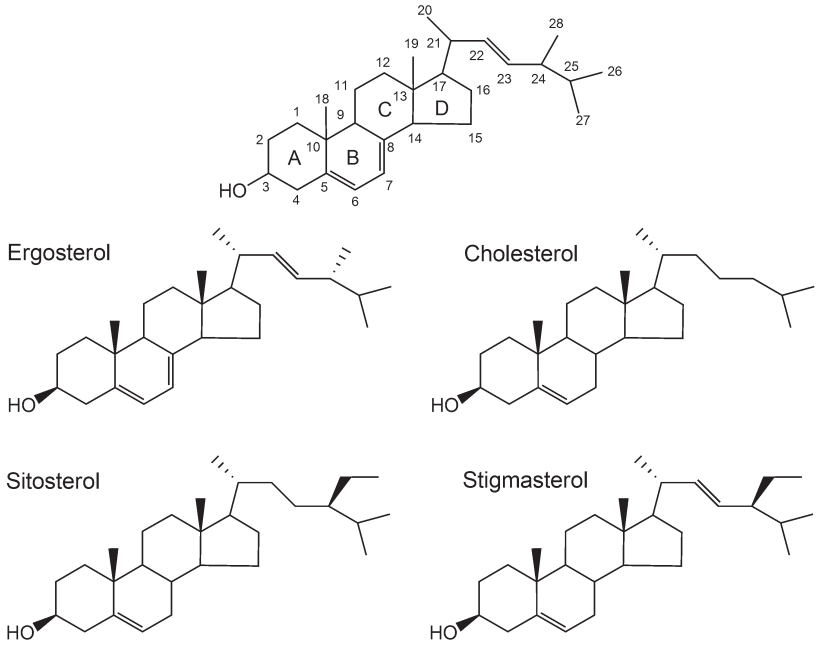

Figure 5. The main membrane sterols in fungi (ergosterol), animal cells (cholesterol), and higher plants (sitosterol and stigmasterol), together with a numbered structure of ergosterol.

Biosynthesis and transport of sterols in fungi

Sterol biosynthesis is a major metabolic commitment on the part of the cell and involves over 20 distinct reactions. The ergosterol biosynthesis pathway has been extensively characterized and is reviewed in (36). A schematic overview of the whole pathway, starting from acetyl-CoA, is given in Figure 6. The

first part of the synthesis of sterols involves the mevalonate or isoprenoid pathway (Panel A). This portion of the pathway ends with the formation of farnesyl pyrophosphate, which is the starting point for several essential synthesis pathways of for example heme, quinines, dolichols and, as pointed out, sterols (36,40-42). In addition, the farnesyl units and the related geranyl species can be the basis of hydrophobic membrane anchors for proteins, such as the RAS proteins (36). Because several essential metabolic products can be synthesized via this route, mutations in this part of the pathway are lethal (36). Starting from

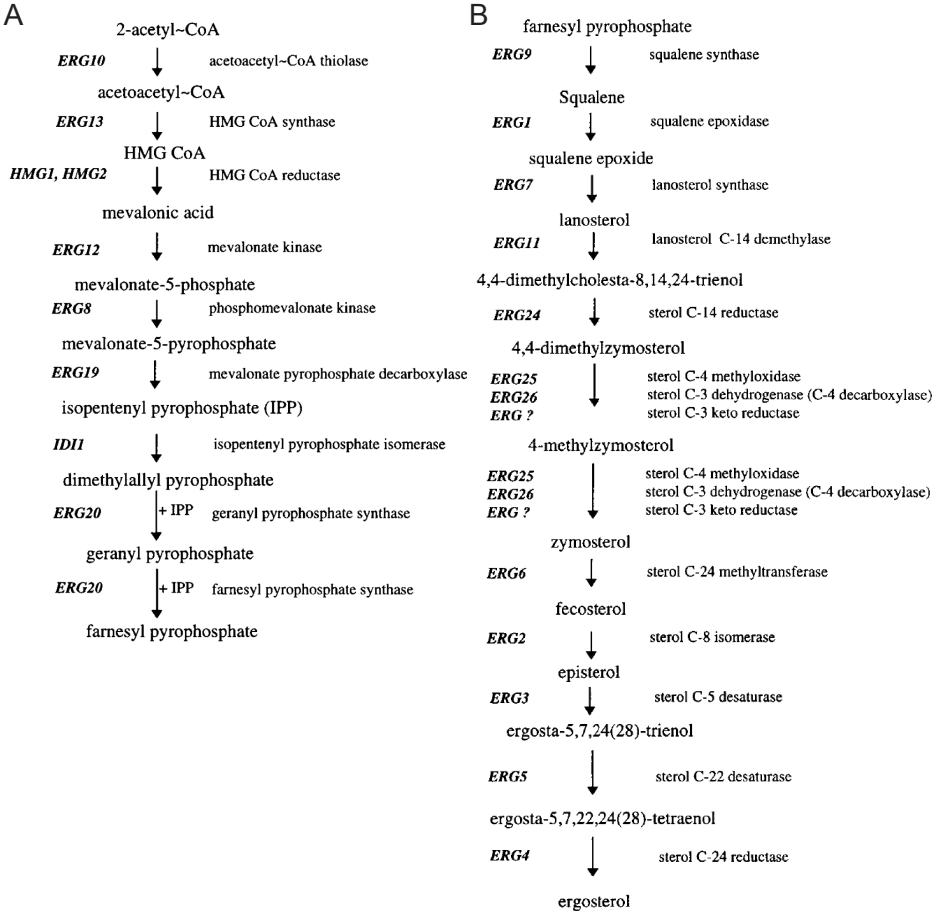

Figure 6. The ergosterol biosynthesis pathway. (A) The mevalonate pathway from acetyl-CoA to farnesyl pyrophosphate. (B) The ergosterol biosynthetic pathway from farnesyl pyrophosphate to ergosterol. The figure is adapted from reference (36), where the description of genes and gene products is given.

farnesyl pyrophosphate ergosterol is formed in twelve steps (Panel B). All the steps up to the formation of zymosterol are essential, but the final five steps in the formation of ergosterol are not essential (36). Zymosterol is not necessarily the end-product, while the enzymes converting lanosterol to ergosterol do not generally show a strict substrate preference and are not interdependent. Thus, mutations in these genes can lead to accumulation of non-physiological sterols (43,44). As described earlier, yeast strains with mutations in the enzymes for the final five steps in the formation of ergosterol can be resistant to polyene antibiotics and these strains will be assigned as ERG mutants in the text.

The biosynthesis of ergosterol in fungi occurs in the Endoplasmic Reticulum (ER) under aerobic conditions (45,46). Most enzymes in the ergosterol biosynthesis pathway are localized to the ER, but some are found in lipid particles as well. Although synthesis of sterols occurs in the ER, the concentration gradient of ergosterol increases from this organelle, across the membranes of the Golgi, until the highest concentration of ergosterol is found in the plasma membrane (Table 1) (47). Ergosterol can also be found in high levels in lipid particles, where it is stored in a steryl ester form (sterol esterified to long-chain fatty acid) (47,48). Yeast differs from most eukaryotes in that the mitochondrial ergosterol is concentrated in the inner membrane rather than in the outer membrane (47,49).

Ergosterol is the main fungal sterol, but other minor constituents formed during the biosynthesis are present in the membranes as well (Table 1). The content of sterols in fungi depends greatly on the cultivation conditions (aeration, medium composition, temperature) and growth stage (50,51). In addition, fungi existing in harsh ecological conditions may vary their ergosterol biosynthesis pathway to adjust their membrane composition (52,53). Under aerobic conditions, yeast are fully autotrophic for ergosterol and do not take up exogenous sterols from the medium (45). Under anaerobic conditions, the biosynthesis stops after the formation of squalene (Figure 6) and sterol uptake is enabled for growth (51,54). ABC transporters, like Aus1p and Pdr11p, are able to take up sterols from the extracellular environment and facilitate their movement through the cell, but the exact mechanism is still unknown (55,56). The combination of the processes that are involved in maintaining sterol homeostasis in fungi are, depending on the presence of oxygen; the sterol biosynthesis, uptake and transport (45,46). The most well-known method of transportation of sterols in fungi is through vesicular pathways going from the ER to the Golgi and secretion vesicles to the plasma membrane. Similarly, sterols are internal-

ized from the plasma membrane through endocytic vesicles to the vacuole. Less well understood are the nonvesicular cytoplasmic routes of sterol transport (57). It has been speculated that nonvesicular sterol transport in fungi can occur similar to mammalian nonvesicular transport. This was based on the seven homologues to mammalian sterol binding proteins found in yeast, termed oxysterol-binding protein homologues (OSH) (45,58). Additionally, the homologues to the human sterol transport Niemann–Pick C genes (Ncr1p, Npc2p) might facilitate sterol movement from the vacuole to other organelles in yeast (59,60).

Table 1; Membrane sterol composition of the subcellular membranes of *Saccharomyces cerevisiae* (47).

	µg of sterol / mg of organellar protein						
Subcellular fraction	Ergosterol	Zymosterol	Episterol	Fecosterol	Lanosterol	Ergosta-5,7,9,22-tetraenol	Cholesterol
Plasma membrane	400	22	ND	ND	ND	ND	ND
Secretory vesicles	384	11	52	ND	11	8,4	ND
Microsomes (40000 x g)	50	10	2,7	5,0	2,3	0,9	0,8
Microsomes (100000 x g)	8,4	1,3	0,62	0,26	0,50	0,55	ND
Outer mitochondrial membrane	6,0	0,84	ND	ND	2,8	ND	2,2
Inner mitochondrial membrane	25	0,77	ND	ND	1,4	0,91	0,40
Vacuoles	49	5,9	12	2,7	4,4	2,3	ND
Lipid particles	6.690	4.390	2.870	2.180	319	121	219

Functions of ergosterol in fungi

Ergosterol has different functions in fungi, either as bulk or more specific roles. The bulk membrane function in fungi is similar to the modulation of membrane properties in all eukaryotes as described earlier, like permeability, elasticity, lateral organization, acyl chain order and hydrophobic thickness (30,61,62). Therefore ergosterol is very important for the biophysical state of the plasma membrane of fungi to compensate for dramatic changes in environmental conditions, like temperature or osmotic pressure (54). This was shown in yeast ERG mutants that have an altered membrane rigidity, even though they

try to compensate this loss in rigidity by changing their lipid profile (63,64). A minimal amount of sterols fulfills a role as a regulatory or "sparking" function to complete the yeast cell cycle (65). This function may be non-membrane related, since the presence of only minimal amounts of ergosterol is required for cell division (66). Although not all functions of ergosterol in fungi are completely understood, ergosterol has been observed to be important for different cellular processes, such as membrane fusion and fission events and the sorting and trafficking of membrane proteins. Additionally, ergosterol can play important roles in the functioning of membrane proteins by, for example, specific ergosterol binding domains in proteins or through the biophysical effects on the membrane or putative membrane domains. These ergosterol dependent processes occur throughout the cell and will be elaborated upon in the following section.

Ergosterol importance in the fusion and fission of membranes

Fusion and fission of membranes are similar processes that rely on the central event of a merger or separation of two membranes (67). Both endocytic and exocytic pathways occur through the fusion and fission of membranes in which sterols have been shown to be important. For example, yeast ERG mutants showed impairments in the endocytic process (44,68). The effects differed per ERG mutant and it was stated that specific sterol structures were required for the internalization step and transport of both hydrophilic and hydrophobic cargo along the endocytic pathway (44,68). Not all ERG mutants displayed defects in the internalization or post internalization steps of endocytosis, but especially the double mutants erg2Δerg3Δ and erg3Δerg6Δ were shown to have the greatest defects in all aspects (44,68).

Changes in sterol structure also affected later stages of endocytosis in the cell, up to the merger with the vacuole, resulting in a fragmentation of this organelle, which was observed in erg3Δ, erg4Δ, erg5Δ and erg6Δ (44,69). *In vitro* studies showed the importance of ergosterol in vacuole-vacuole fusion in yeast (69,70). Yeast vacuoles undergo cycles of fragmentation and fusion as part of their transmission to the daughter cell (71). Further evidence for an active role in fusion has been provided by showing that an excess of sterols in vacuolar membranes caused by over-expressing ERG6 could enhance vacuolar fusion (70). In addition, an imbalance in the sterol profiles by deletion or overexpression of genes important for regulation of the ergosterol biosynthesis pathway also caused vacuolar fragmentation, underlining the importance of ergosterol in these processes (72).

Additionally, ergosterol has been shown to be important for the fusion of plasma membranes in mating yeast (73,74). During mating, pheromones are secreted and bound to specific membrane receptors, which results in the stimulation of a protein kinase signaling cascade (75,76). This will induce the mating procedure and polarized growth towards the mating partner, bringing the cells in direct contact. After the cell wall between both cells is removed, fusion factors promote fusion of the cells (76). ERG mutants (especially erg2Δ, erg3Δ and erg6Δ strains) have been shown to accumulate mating pairs with plasma membranes that are in contact, but not fused. Membrane fusion was not completely inhibited in these strains, but if a mating pair was formed the process of membrane fusion was retarded (73).

Ergosterol involvement in protein sorting and trafficking

Ergosterol was shown to be important for protein sorting and trafficking, while ERG mutants with different sterol compositions in yeast were shown to have an altered sorting and trafficking profile for different proteins, starting from the ER (77). For example, sterols have been found to play an important role in correct trafficking of the high affinity tryptophan permease, Tat2p (78). This protein is transported from the Golgi compartment to early endosomes, where it is (most likely) sorted either to the plasma membrane or the vacuole depending on the external concentration of tryptophan (78). In erg6Δ yeast, Tat2p is targeted to the vacuole regardless of the concentration of tryptophan. Similar observations have been made for the arginine transporter Can1p and a GPI anchored protease Yps1p, which are mistargeted to the vacuole in erg6Δ mutants (79,80). Fus1p, a plasma membrane protein required for yeast mating has been reported to be mislocalized to the vacuole in the erg6Δ mutant as well (81). A recent study contradicted these findings and stated that the reduced Fus1p expression in ERG mutants was because of a lower response to pheromone signaling by the reduced recruitment or formation of signaling complexes in the plasma membrane (73). The signaling complex or pheromone response pathway consists of multiple component, which include seven transmembrane domain receptors (Ste2 and 3), lipid-anchored proteins (Ste18 and Cdc42), and proteins with lipid-binding motifs (Ste5 and Far1) and illustrates the ergosterol dependency of this complex (73).

Sterol – protein interactions

As described above, ergosterol can be important for the function of a protein or protein complex, like the pheromone response pathway. The lateral organization and bilayer thickness modulated by the presence of sterols, have been proposed to influence protein function (33,82). A direct interaction between sterols and proteins is possible as well and there are several structural features of a protein that result in the protein preferentially associating with sterol containing membranes, which is mostly documented for cholesterol (83). One of the best known structural features is certain types of lipidations, like palmitoylation or glycosylphosphatidylinositol (GPI) anchors (84,85). However, there are segments of a protein that can preferentially sequester cholesterol, which are known as the cholesterol recognition/interaction amino acid consensus (CRAC) domain and the sterol-sensing domain (SSD) (83,86). Examples of proteins containing the CRAC domain are the endocytic protein caveolin, the fusion protein of HIV gp41 and the benzodiazepine or $GABA^A$ receptor (83). All proteins containing an SSD domain are involved in cholesterol homeostasis. These proteins include 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), 7-dehydrocholesterol reductase, SREBP-cleavage activating protein (SCAP) and Niemann–Pick disease type C1 related proteins (83,86). Although less well documented, ergosterol has been found to interact with proteins containing GPI anchors (87,88) and homologues of proteins containing SSD domains are found in fungi as well (59,89,90). No ergosterol recognition/interaction amino acid consensus (ERAC) domain has been reported in fungi. Ergosterol-sphingolipid structures have been indicated to be important for the functioning of the ATP-binding cassette (ABC) transporter Pdr12p. Pdr12p is known to export toxic weak organic acids, like sorbic and benzoic acids, from the cell and be responsible for the resistance of yeast to these acids (91). Deletion of ERG4 together with SUR2, responsible for the hydroxylation of the sphingoid base of sphingolipids, resulted in a loss of resistance to sorbic acid, despite its proper transportation to the cell surface (92). Sterol-sphingolipids seem to affect the activity of this drug transporting molecule in a fairly direct manner, perhaps via specialized ordered domains in the membrane of yeast.

Importance of ergosterol in ordered domains

Specialized domains, consisting of ergosterol and sphingolipids, have been suggested to exist in the membrane of yeast (75,93,94). It is speculated that these

ordered domains function via spatially enriching different proteins, making it possible to serve as platforms for many cellular events, such as signal transduction and membrane sorting and trafficking (81,93,95). In model systems, sterols can form ordered domains in the membrane via a preferential interaction with lipids containing long saturated acyl chains, like sphingolipids. These ordered domains are in a tightly packed liquid ordered (L_o) state, whereas the rest of the unsaturated phospholipid rich membrane is in a less ordered or liquid disordered (L_d) phase (32,96). The differences in packing ability lead to the phase separations. Although the methods used to study domains in living cells are still under debate, like detergent resistance or sterol depletion, microdomains are thought to exist in the membrane of fungi (31,97). In yeast, it was speculated that proteins may associate or cluster differently in the membrane and that different types of membrane domains may exist (79,98). For example, localization studies have indicated that the plasma membrane ATPase, Pma1p, was found to be localized in a different compartment, termed MCP, as compared to several amino acid transporters, termed MCC, such as the arginine permease (Can1p), tryptophan permease (Tat2p) and uracil permease (Fur4p) (99). Sur7p, a transmembrane protein that influences sphingolipid levels, is also thought to localize to the MCC compartment. These different compartments are suggested to function as a protective area within the plasma membrane to control turnover of transport proteins and may also be involved in the correct functioning of these proteins (98).

Ergosterol as a target for antibiotics

If an antifungal agent could interfere with the function of ergosterol in the membrane, this may have an effect on the general biophysical state of the membrane influencing for example the order and lateral organization or permeability and elasticity of the membrane. The process of fusion and fission events, like endocytosis, vacuolar fusion and mating of yeast, could be altered while ergosterol is implicated to be important in these processes. Similarly, influencing the specialized ordered microdomains in the membrane, could affect protein function, or their sorting and trafficking (75,79,93,94,98). Although the exact roles of ergosterol in the membrane are not completely elucidated, it is evident that it is a very important constituent of fungal membranes for membrane integrity and diverse cellular functions in yeast, which makes it an excellent target for natamycin and all polyene antibiotics.

Scope of the thesis

The aim of this thesis is to determine the antifungal mode of action of natamycin. In Chapter 2, the interaction of natamycin with sterols is examined in detail using both *in vitro* systems like model membrane systems and *in vivo* systems, such as different ERG mutants. Both isothermal titration calorimetry (ITC) and direct binding assays show that natamycin has a specific interaction with ergosterol in membranes that requires the double bonds in the B-ring of the sterol structure. In addition, the mechanism of natamycin action in relation to membrane permeabilisation was examined using leakage assays in model membrane systems and in yeast. It is shown that natamycin, unlike all polyenes of which the mode of action is known, does not act on fungi via a permeabilisation of the membrane.

In Chapter 3, the effect of natamycin on ergosterol dependent fusion processes is examined by using an *in vitro* vacuolar fusion assay. It is shown that natamycin was able to inhibit vacuole fusion *in vitro* in an ergosterol dependent manner based on the use of different ERG deletion strains. The inhibition of natamycin on vacuole fusion took place in an early stage of the fusion process, which indicated that natamycin might interfere with ergosterol dependent protein functions. This was further examined using substrate uptake assays of plasma membrane transport proteins in yeast (Chapter 4). Natamycin is shown to be able to inhibit the transport of different substrates, which was most likely not related to a reorganization of sphingolipid-sterol domains, but suggested that natamycin is able to inhibit different plasma membrane transport proteins in fungi.

In the addendum to Chapter 4, an effort is made to determine the components of a fungal cell that are accessible to natamycin, by using fluorescently labeled natamycin. The results are summarized and discussed in Chapter 5.

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Chapter 2

Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane

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Abstract

Natamycin is a polyene antibiotic that is commonly used as an antifungal agent because of its broad spectrum of activity and the lack of development of resistance. Other polyene antibiotics, like nystatin and filipin are known to interact with sterols, with some specificity for ergosterol thereby causing leakage of essential components and cell death. The mode of action of natamycin is unknown and is investigated in this study using different *in vitro* and *in vivo* approaches. Isothermal titration calorimetry and direct binding studies revealed that natamycin binds specifically to ergosterol present in model membranes. Yeast sterol biosynthetic mutants revealed the importance of the double bonds in the B-ring of ergosterol for the natamycin-ergosterol interaction and the consecutive block of fungal growth. Surprisingly, in strong contrast to nystatin and filipin, natamycin did not change the permeability of the yeast plasma membrane under conditions that growth was blocked. Also, in ergosterol containing model membranes, natamycin did not cause a change in bilayer permeability. This demonstrates that natamycin acts via a novel mode of action and blocks fungal growth by binding specifically to ergosterol.

Introduction

Fungal infections have recently become a growing threat to human health, especially in persons whose immune systems are compromised (for example by HIV, cancer chemotherapy). Only a few effective antifungal agents are currently in use; these include the polyenes, the fluorocytes and the azole derivatives. One important problem is the increase of drug resistance, particularly against azole antimyotics and fluorocytosine (1). Resistance against polyene antibiotics is still a rare event, which makes these antibiotics particularly interesting as antifungal agents. The polyene antibiotics have a ring structure in which a conjugated double bond system is located opposite to a number of hydroxyl functions. Often a mycosamine group is present in combination with a carboxyl moiety, rendering the molecule amphoteric (Figure 1). In the past convincing evidence has been presented that several members of this class of antibiotics target sterols and in particular ergosterol, the abundant and main sterol of fungal membranes (2,3). Different types of polyene antibiotics were shown to have different modes of action despite that they share a common target. The larger polyenes like amphotericin B and nystatin form pores to-

gether with ergosterol in the plasma membrane that collapse vital ion gradients, thereby killing the cells. The smaller uncharged filipin also destroys the membrane barrier, but by a completely different mechanism. Filipin forms large complexes with sterols between the leaflets of the lipid bilayer, resulting in loss of the barrier function (2). Natamycin (also called pimaricin) is a very effective member of the polyene antibiotic family with a large standing record of applications. It is produced by *Streptomyces natalensis* and used against fungal infections, but it is also widely utilized in the food industry to prevent mould contamination of cheese and other nonsterile foods (e.g. cured meats) (4). Surprisingly, the mechanism of action of this antifungal agent is still unknown and it is even unknown whether it targets ergosterol in the fungal membrane. It is relatively small while it contains a tetraene compared to a pentaene in filipin, which is already considered as a small polyene antibiotic (Figure 1). It contains a mycosamine group that renders it amphoteric, which is a feature that is also present in nystatin. While natamycin has similar features of both filipin (small) and nystatin (amphoteric), it is difficult to predict its mechanism of action.

We wanted to gain more insight into the mode of action of natamycin, which could in turn help to develop new or improved antifungal formulations or result in novel strategies to prevent fungal spoilage. To determine the interaction of natamycin with membranes in relation to its sterol composition, we tested in a comparative manner using filipin and nystatin as references,

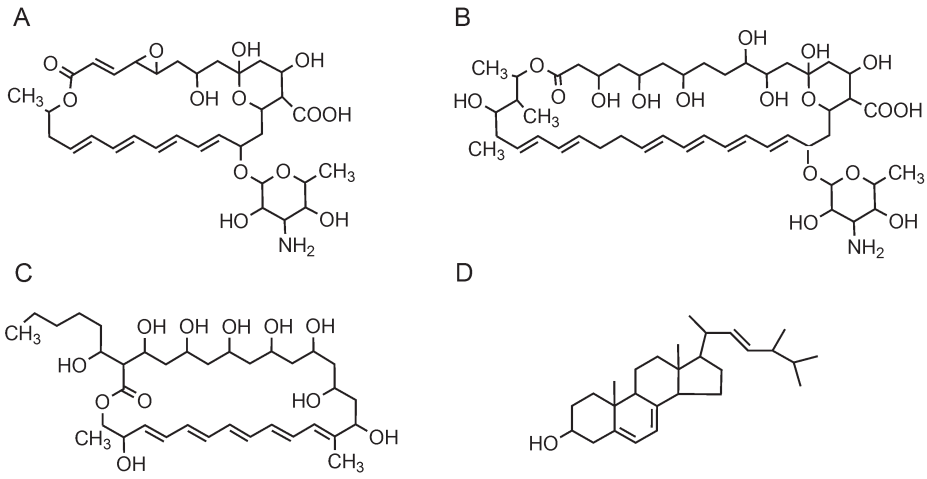


Figure 1. Structures of several polyene antibiotics and ergosterol.
(A) natamycin, (B) nystatin, (C) filipin, (D) ergosterol.

the interaction of natamycin with phosphatidylcholine model membranes of varying sterol composition using ITC and other binding studies. In addition, the ability of natamycin to permeabilize these model membranes was studied.

Parallel to the studies performed on model membranes, the effect of natamycin on yeast growth, the binding of the antibiotic with intact yeast cells and the plasma membrane integrity were determined. These studies were performed using strains that carry specific mutations in the ergosterol biosynthetic pathway (ergΔ) or that were reprogrammed to contain cholesterol as the main sterol (5). We could demonstrate that, differently from any other polyene antibiotic of which the mode of action is known, natamycin blocks fungal growth by binding specifically to ergosterol, but without permeabilizing the membrane.

Experimental procedures

Chemicals – 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Ergosterol was purchased from Larodan AB (Sweden). DOPC or sterols were dissolved in chloroform to a stock concentration of 20 mM. The phospholipid concentration of DOPC was determined by phosphate analysis according to Rouser (6). The polyene antibiotics nystatin and filipin were dissolved in DMSO, while natamycin was dissolved in 85 : 15 DMSO to H_2O (v/v); all were obtained from Sigma Chemical (St. Louis, MO). All antibiotic solutions were prepared freshly before the start of an experiment and the concentrations of the polyene antibiotics were determined by UV absorption on a Perkin Elmer UV / Vis Spectrometer (Lambda 18). The molar extinction coefficients of the polyene antibiotics were determined in methanol to be 7.6×10^4 M^{-1} cm^{-1} (318 nm), 6.7×10^4 M^{-1} cm^{-1} (318 nm) and 8.5×10^4 M^{-1} cm^{-1} (356 nm) for natamycin, nystatin and filipin respectively. The molar extinction coefficient of ergosterol was measured in methanol to be 0.97×10^4 M^{-1} cm^{-1} (262 nm).

The ionophore nigericin (dissolved in ethanol), ampicillin sodium salt, and the amino acids adenine, uracil and L-tryptophan were obtained from Sigma Chemical (St. Louis, MO). 5-(and-6)-Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (dissolved in DMSO) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) were both purchased from Invitrogen (Eugene, OR). N,N-dimethyldodecylamine-N-oxide (DDAO) was bought from Fluka Biochimica (Buchs). All other chemicals used were of analytical or reagent grade.

Strains and growth conditions – For all experiments, medium was inoculated directly from plates with colonies that were not older than 2 weeks. Unless otherwise mentioned, cells were grown overnight at 30 °C in rich medium (10 g/l yeast extract, 20 g/l bacto peptone and 20 g/l dextrose with 1 g/l adenine, 2 g/l uracil and 1 g/l tryptophan (YPUADT)) supplemented with 0.1 mg/ml ampicillin. For the strains RH6611 and RH6613 SD medium was used (1.7 g/l yeast nitrogen base without amino acids, 20 g/l glucose, 2 mg/l trace components, 5 g/l ammonium sulphate) supplemented with vitamins and the appropriate amino acids minus histidine and leucine (SD-his-leu). Yeast strains used in this study are listed with their relevant genotypes in Table 1 and the plasmids in Table 2.

MIC value determinations – Minimum inhibitory concentrations (MICs) were determined by diluting the polyene antibiotics in YPUADT (with 0.1 mg/ml ampicillin) to a concentration of 400, 350, 300 and 250 μM of which 100 μl was added to the first row of a 96-wells suspension culture plate (U-form, Greiner Bio One). This was followed by a 1 : 1 dilution series in medium. Overnight cultures were diluted back to an OD_{600} 0.0001, of which 100 μl was added to the culture plate. The total volume per well was 200 μl. The strains RH6611 and RH6613 (in SD-his-leu medium) were diluted in to an OD_{600} 0.01, because they had a very slow growth rate. The MIC value was determined to be the lowest concentration of antibiotic, which inhibits the growth of the yeast strain and could be determined by eye on the 96-wells plate after an incubation of 24 hrs at 30 °C. The experiments were performed in triplicate.

Preparation of large unilamellar vesicles (LUVs) – Large unilamellar vesicles (LUVs) with a mean diameter of 200 nm were prepared using the following protocol. Aqueous phospholipid suspensions were prepared by premixing ergosterol or cholesterol with DOPC in the desired molar ratios as solutions in chloroform and evaporating the solvent in a stream of nitrogen, followed by drying the lipid film for 20 min. under vacuum. Sterols were present in a range of 10 to 30 mol%. All following handlings were performed at 50 °C. The lipid film was hydrated and repeatedly vortexed until all lipid was removed from the walls of the test tube. Then a freeze-thaw cycle was repeated eight times using liquid nitrogen and a water bath. Subsequently, the lipid suspension was extruded 8 times through a polycarbonate membrane filter with a pore size of 0.2 μm (Whatman International, England). The size of the vesicles was determined after extrusion by using the Zetasizer 3000 (Malvern Instruments). The average of the size of the vesicles was 168 ± 3.7 nm for vesicles without

sterols, 165 ± 1.2 nm for vesicles with 10% cholesterol and 173 ± 8 nm for vesicles with 10% ergosterol. Thus no significant differences in size were observed. The resulting vesicle suspension was stored at 4 ^{o}C. The final phospholipid concentration was determined by phosphate analysis according to Rouser (6).

Table 1. Strains used in this study. The source of these strains is described in reference (5).

Description	Name	Genotype
WT	RH448	*MATα his4 leu2 ura3 lys2 bar1*
erg2Δ	RH2897	*MATα erg2(end11)-1Δ::URA3 leu2 ura3 his4 lys2 bar1*
erg2Δerg6Δ	RH3616	*MATα erg2(end11)-1Δ::URA3 erg6Δ leu2 ura3 bar1*
erg6Δ	RH3622	*MATα erg6Δ::LEU2 leu2 ura3 his4 bar1*
erg3Δ	RH4213	*MATα erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1*
erg3Δerg6Δ	RH5225	*MATα erg3Δ::LEU2 erg6Δ::LEU2 leu2 ura3 his4 lys2 bar1*
erg2Δerg3Δ	RH5228	*MATα erg2Δ (end11)-1Δ::URA3 erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1*
erg4Δerg5Δ	RH5233	*MATα erg4Δ::URA3 erg5Δ::kanMX4 leu2 ura3 his4 lys2 bar1*
WT	RH6611	*MATα his3 ura3 leu2 [pRS423] [pRS425]*
Cholesterol	RH6613	*MATα erg5Δ::TRP1 erg6::TRP1 his3 ura3 leu2 trp1 [pRS423-DHCR7] [pRS425-DHCR24]*

Table 2. Plasmids used in this study.

Plasmid	Characteristics	Reference
pRS423	Multicopy vector containing *GDP* promoter and *HIS3*	(30)
pRS423-DHCR7	pRS423 derivative vector containing *DHCR7* gene	#
pRS425	Multicopy vector containing *GDP* promoter and *LEU2*	(30)
pRS425-DHCR24	pRS425 derivative vector containing *DHCR24* gene	#

#Submitted manuscript by Souza, CM., Pichler, H., Leitner, E., Guan, X., Wenk, MR., Tornare, I. and Riezman, H.

ITC measurements – Titration experiments were carried out on a MCS titration calorimeter from Microcal Inc. LUVs were prepared as described above in 50 mM MES, 100 mM K_2SO_4 pH 6.0 or 10 mM HEPES, 100 mM NaCl pH 7.0. Similar results were obtained with the different buffers. The vesicles were injected into a sample cell (volume = 1.345 ml) containing 50 μM antibiotic in the same buffer as used for the vesicle suspension. Because the polyene antibiotics are dissolved in DMSO, an equal amount was added to the LUV suspension to compensate for any heat generated by dilution of this solvent. No more than 1 % of DMSO was present. The solutions were degassed, before the start of the titration. The experiments consisted of 44 injections, 5 μl each, of a stock solution of vesicles at 25°C (8 mM final phospholipid concentration). The results were analyzed using the ORIGIN software (version 2.9) provided by Microcal Inc. The interaction between the vesicles and the antibiotics was complex in that no clear saturation of this interaction was observed. Therefore the stoichiometry of the interaction could not be determined. An approximation of the binding constant was made using the ORIGIN software, where the value of integrated heat of the last injection was subtracted from all data and the model of one set of sites was fitted to the resulting data.

Binding assay using centrifugation of model membranes – Vesicles were prepared as described above in 10 mM MES / Tris, 15 mM K_2SO_4 at pH 7. The reduced ion strength facilitated the pelleting of the vesicles. The concentrations of antibiotics and vesicles were varied from 0 to 0.1 and 0.5 to 5 mM respectively unless indicated otherwise. Vesicles were incubated with the polyene antibiotics for one hour in an eppendorf incubator (22°C, 650 rpm), with a maximum of 1 % DMSO present. To spin down the vesicles and the bound antibiotic, 1 ml of the mixture was centrifuged in a TLA 120.2 rotor in a Beckman Ultracentrifuge (TL-100) for 1,5 hrs at 100 krpm and 20 °C.

The amount of antibiotic before centrifugation and in the supernatant and pellet was determined by UV absorption after 7 times dilution in methanol followed by centrifugation to remove any precipitated salts. The phospholipid concentrations were determined by phosphate analysis according to Rouser (6). Under these conditions less than 10 % of the phospholipids remained in the supernatant. The antibiotics were not pelleted in the absence of lipid below a concentration of 75 μM, 34 μM and 30 μM of respectively natamycin, nystatin and filipin. The binding isotherms of the interaction of natamycin with ergosterol could be described by the Langmuir adsorption model assuming that ergosterol

was the only binding site for natamycin in the DOPC vesicles and that only the ergosterol in the outer leaflet of the bilayer could have an interaction with natamycin. The Langmuir adsorption model was applied to the data of the amount of natamycin bound to the vesicles versus the amount of free natamycin in the supernatant (7). From using this model in Sigmaplot (10.0), the binding constant and the binding saturation of natamycin with ergosterol could be determined.

Binding assay using centrifugation of intact cells – Yeast were grown to the mid-logarithmic phase in 200 ml YPUADT (with 0.1 mg/ml ampicillin) or SD medium. As a negative control the *Escherichia coli* strain DH5α was used that was grown to the logarithmic phase in 100 ml Luria Broth (LB) medium at 37 °C. The cells were harvested by centrifugation at room temperature (r.t.) at 3600 g for 10 min. in a Sorvall RC 5B centrifuge (SLA 1500), washed two times in 100 ml of 10 mM MES / Tris, 15 mM K_2SO_4 at pH 7 and resuspended in a small volume of buffer. The OD_{600} of the cell suspensions was determined and a series of 1 ml cell suspensions were prepared ranging from an OD_{600} of 0 to 15. The cells were centrifuged at 3000 g for 5 min. at r.t. and resuspended in the same buffer containing 30 μM natamycin. As a control, cells were resuspended in buffer with no natamycin. The cells were incubated for one hour in an eppendorf incubator (900 rpm at r.t) and spun down for 15 min. at 3000 g. The amount of natamycin in the supernatant was determined by UV absorption as described above (spectrum from 250 to 350 nm) and used to calculate the amount of natamycin bound to the yeast cells.

Carboxyfluorescein permeability assay in large unilamellar vesicles – Carboxyfluorescein (CF) loaded vesicles were prepared as described above in 50 mM MES - KOH buffer at pH 7 (8). To remove the untrapped CF, a Sephadex G50 spincolumn equilibrated with 50 mM MES, 100 mM K_2SO_4 buffer at pH 7 was used. The CF loaded vesicles were diluted in 1200 μl of 50 mM MES, 100 mM K_2SO_4 buffer at pH 7 followed by the addition of the antibiotic. The antibiotic induced CF leakage from the vesicles was monitored by measuring the fluorescence intensity at 513 nm (excitation set at 430 nm) on a SLM AMINCO Spectrofluorometer (SPF-500). The detergent Triton X-100 was added at the end of the experiment to destroy the lipid vesicles and the resulting fluorescence was taken as the 100 % leakage value.

Proton permeability assay in large unilamellar vesicles – Proton permeability was determined in an assay with 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) loaded vesicles as performed by van Kan *et al* (2002) (9). The assay is based on the strong pH-dependence of the fluorescence of HPTS.

Vesicles were prepared as described above in a 2 mM HPTS solution in 0.2 M NaH_2PO_4 / Na_2HPO_4 buffer at pH 7. To create a lower pH at the outside and remove all the untrapped HPTS, a Sephadex G25 spincolumn was used equilibrated with 10 mM MES, 0.2 M Na_2SO_4 buffer at pH 5.5. To determine the phospholipid concentration of the resulting vesicles the lipids were first extracted according to Bligh-Dyer (10) to exclude the phosphate from the buffer in the following phosphate analysis according to Rouser (6). The effects of the polyene antibiotics on the proton permeability of the lipid vesicles was monitored by adding aliquots of antibiotic to 1200 µl of 10 mM MES and 0.2 M Na_2SO_4 buffer (pH 5.5) containing HPTS loaded vesicles (35 µM phospholipid phosphorous). The fluorescence emission was detected at 508 nm (excitation at 450 nm) on a SLM AMINCO Spectrofluorometer (SPF-500). Differing from van Kan *et al* (2002) (9), the detergent N,N-dimethyldodecylamine-N-oxide (DDAO) was used instead of Triton X-100, because DDAO did not have any effect on the fluorescence of the probe where Triton X-100 did have an effect (not shown). DDAO was added at the end to destroy the lipid vesicles and the resulting fluorescence was taken as the 100 % leakage value, while the blanc without antibiotic was used as a reference for 0 % leakage. Nigericin, a polyether ionophore known to collapse proton gradients, was used as a positive control (11).

Proton permeability assay in yeast – The assay was based on the loading of yeast cells with the probe 5-(and-6)-Carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) as described by Bracey *et al* (1998) (12,13). CFDA-SE is a non-polar molecule that spontaneously penetrates cell membranes and is converted to the anionic pH-sensitive 5-(and-6)-carboxyfluorescein succinimidyl ester (CF-SE) by intracellular esterases (9). Once the probe is internalized, amine reactive coupling of succinimidyl groups of CF-SE to aliphatic amines of intracellular proteins results in the formation of membrane impermeable pH-sensitive probe conjugates.

Wild type yeast cells from an over night culture were diluted to an OD_{600} of approximately 0.8 and then centrifuged at 3000 g for 3 min. The cells were washed and resuspended in an equal volume of 100 mM citric / phosphate buffer at pH 4 (100 mM citric acid, 50 mM NaH_2PO_4 and 50 mM KOH). CFDA-SE (100 µM) was added and the cells were incubated overnight while shaking at 37 °C. The viability of the cells was not significantly compromised by the loading conditions. Loaded cells were harvested (3000 g, 3 min), washed and resuspended in YPUADT buffered with 50 mM citric / phosphate (pH 4) to an OD_{600} of 0.4. To recover from the stress imposed by the probe loading

conditions, the cultures were left for 1 hr at 30 °C with shaking. The effects of the polyene antibiotics on the proton permeability of the yeast cells were monitored by adding aliquots of antibiotic to 5 ml of culture and measuring the OD_{600} and fluorescence at regular intervals. The OD_{600} was determined on a Helios Epsilon UNICAM spectrometer and the fluorescence emission was detected at 525 nm (excitation at 495 nm) on a SLM AMINCO Spectrofluorometer (SPF-500).

Results

Sterol specificity of natamycin binding to membranes – To test whether sterols are required for membrane affinity of natamycin we used phosphatidylcholine model membranes containing ergosterol, the main fungal sterol or cholesterol, the main sterol in mammals. The interaction between natamycin and sterols in the model membrane was first studied using Isothermal Titration Calorimetry (ITC). ITC measurements were performed where LUVs containing either

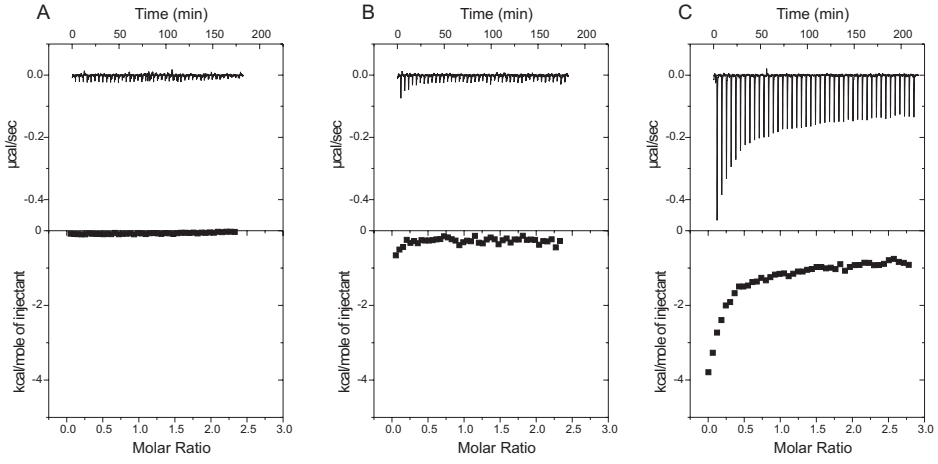


Figure 2. Calorimetric titrations of natamycin with DOPC vesicles. Vesicles contained no sterol (A), 10% cholesterol (B) and 10% ergosterol (C) and were dissolved in 50 mM MES, 100 mM K_2SO_4 pH 6.0. The top graph displays the heat peaks after consecutive injections of 5 µl vesicles with an 8 mM final phospholipid concentration into the sample cell containing 50 µM natamycin. The bottom graph shows the integrated heat per injection, which is normalized to the injected amount of moles of sterol and is displayed against the molar ratio of sterol versus natamycin. When no sterols are present, 10% of phospholipid is used to determine and display the integrated heat per injection.

no sterols, cholesterol or ergosterol were titrated into a solution of natamycin (Figure 2). Natamycin displayed no interaction with vesicles containing no sterols as the resulting heats were no different from the control (Figure 2A). LUVs containing 10 mol% cholesterol produced only minor heat effects during the first injections, which indicates that natamycin displayed only a very small interaction with cholesterol containing vesicles (Figure 2B). Interestingly, 10 mol% ergosterol containing vesicles displayed a significant amount of interaction with natamycin as evidenced by the consecutive heat effects (Figure 2C). This titration curve differs from a normal titration curve as no clear saturation of the interaction was observed. The binding constant between natamycin and ergosterol was estimated to be 5.7 x 10^4 M^{-1} (see *Experimental procedures*). Comparable large differences in effects between cholesterol and ergosterol were observed for sterol concentrations of 20 mol% (data not shown).

Furthermore, the binding of natamycin to vesicles was studied by separating the bound from the free natamycin by centrifugation. Figure 3A shows a representative graph of these results, from which can be concluded that ergosterol containing vesicles had a significant interaction with natamycin. In the absence of sterols or in the presence of cholesterol very little interaction with natamycin was observed consistent with the ITC experiments (Figure 2). A similar sterol

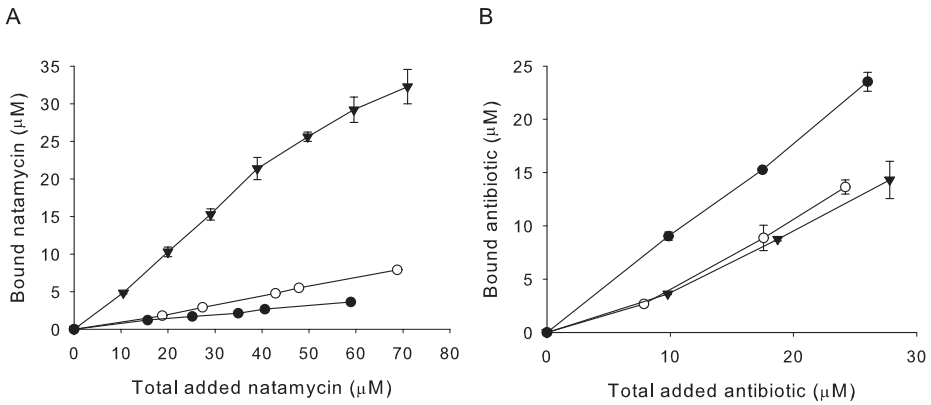


Figure 3. Interaction of polyene antibiotics with model membranes. (A) Binding of natamycin to vesicles containing 10% ergosterol (▼), 10% cholesterol (○) or no sterols (●). (B) The interaction of filipin (●), nystatin (○) and natamycin (▼) on 10% ergosterol containing vesicles was examined. The assay was performed in duplo in 10 mM MES / Tris, 15 mM K_2SO_4, pH 7.0 and the vesicles had a 2 mM final phospholipid concentration.

dependency of natamycin binding was observed when varying the concentrations of vesicles (data not shown). The binding constant was determined by the Langmuir adsorption model in SigmaPlot (10.0) to be $2.5 \pm 1.0 \times 10^4$ M^{-1}, which is in reasonable agreement with the binding constant determined in the ITC measurements. The binding saturation from the Langmuir adsorption model was determined at 72 ± 12 μM by extrapolating the data in SigmaPlot (10.0). By assuming that only the sterol in the external leaflet of the lipid vesicles could establish an interaction with the antibiotic, the sterol to antibiotic ratio was calculated to be approximately 1 : 1. If all sterols would be available for the interaction, because of sterol flip-flop, the ratio would be 2 : 1. The affinity of natamycin for ergosterol containing vesicles was compared to that of filipin and nystatin to get insight into the relative strength of this interaction. Figure 3B shows a representative graph of the results obtained with these antibiotics. Of the three polyene antibiotics filipin showed the highest affinity, followed by natamycin and nystatin.

Sterol specificity in the antibiotic action – To test if ergosterol is needed for natamycin to exert its antifungal activity *in vivo*, yeast strains carrying specific mutations in the ergosterol biosynthesis pathway (ergΔ) were used. Because of these mutations, the strains cannot synthesize ergosterol. However, they each accumulate a distinct set of sterols that, compared to ergosterol, have structural differences in the side chain and double bonds in the B or C ring (Figure 4). The availability of these strains allows us to address the sterol specificity for polyenes, in relation to their inhibitory activity.

The most prominent sterols present in the ergΔ mutants are tabulated in percentage of total sterol present, together with their minimum inhibitory concentration (MIC) values for the polyene antibiotics natamycin, nystatin and filipin in Table 3. The sterol composition of the erg strains given in Table 3 was taken from Heese-Peck, *et al.* and specifies the percentage of a listed

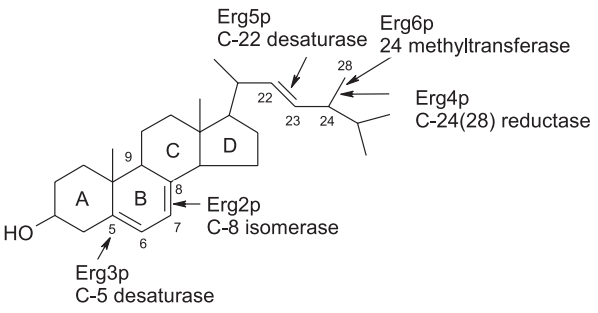


Figure 4. Ergosterol molecule with the assignment of the ring structure. Erg proteins and their functions are indicated. The corresponding genes are inactivated in ergΔ strains (5).

sterol compared to the total sterol composition of a cell (5). The most sensitive erg strain is erg4Δerg5Δ, which has a MIC value of the wild type strain. The least sensitive towards natamycin was erg2Δerg6Δ, which contained mostly zymosterol. From the strain with the highest sensitivity towards the lowest, the most striking sterol structural feature that causes the loss of activity is the loss of double bonds in ring B. For example, the sterols in erg3Δ have one double bond at position C-7,8 and it is only 3 times less sensitive to natamycin compared to the wild type, while erg2Δerg6Δ has lost both double bonds at C-5,6 and C-7,8 and is 37 times less sensitive compared to the wild type. Variations in the C_{17} side chain of the sterols did not have very large effects on the sensitivity towards natamycin, which can be observed when comparing erg4Δerg5Δ with the wild type. The yeast strain sensitivities towards nystatin were similar compared to natamycin. Filipin sensitivity seemed not to be so dependent on the sterol structure. The results demonstrate that double bonds in the B ring of the sterols are very important for natamycin to inhibit the growth of yeast, while changes of the C_{17} side chain are of less importance.

Recently a yeast strain was constructed (RH6613) which is unable to synthesize ergosterol or its related precursors, but instead was programmed to synthesize cholesterol. This enabled us to test the strong preference of natamycin for ergosterol over cholesterol as noted in the model membrane experiments. The results of growth inhibition are shown in Table 4 and show that the cholesterol producing strain was 16 fold less sensitive towards natamycin compared to the corresponding wild type. This demonstrates that also *in vivo* natamycin has a strong specificity for ergosterol over cholesterol. Moreover, given the difference in chemical structures of ergosterol and cholesterol, the importance of the double bonds of the B-ring for interaction with natamycin is further emphasized consistent with the results of the erg strains. Nystatin had the same effect on the yeast strains as natamycin, whereas filipin is apparently less specific as it was almost as effective in killing the cholesterol producing strain as the wild type strain.

To determine if the inhibition of growth was related to the amount of binding of natamycin to these yeast strains, a binding assay with the different strains was performed. All the strains were tested and in addition an *E. coli* wild type strain was taken as a negative control, because it contains no sterols in the plasma membrane. For clarity only 6 strains are depicted in Figure 5A. The highest amount of binding of natamycin was observed for the wild type (both strain RH448 and RH6611), together with erg4Δerg5Δ.

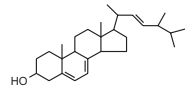
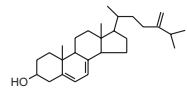
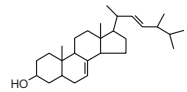
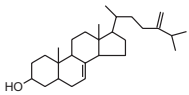
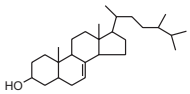
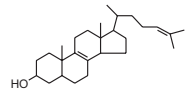
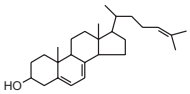
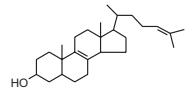
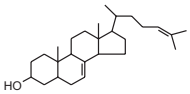
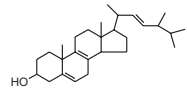
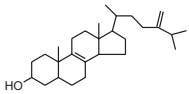
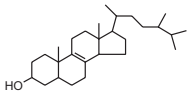
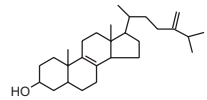
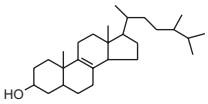
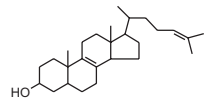
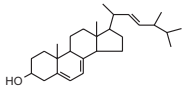
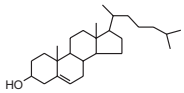
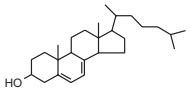
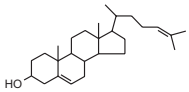
Strain	MIC_{natam} (μM)	MIC_{nyst} (μM)	MIC_{filip} (μM)
Wild Type	**1.7 ± 0.5**	**1.1 ± 0.2**	**1.2 ± 0.1**
Ergosterol (77 %) 			
Erg4Δerg5Δ	**2.1±0.6**	**2.1±0.2**	**2.5±0.9**
Ergosta-5,7,24-trienol (72 %) 			
Erg3Δ	**5.7 ± 0.8**	**3.4 ± 0.5**	**2.5 ± 0.2**
Ergosta-7,22-dienol (46 %)  Episterol (13 %)  Ergosta-7-enol (11 %) 			
Erg6Δ	**8.3 ± 0.9**	**7.8 ± 1.6**	**2.9 ± 0.2**
Zymosterol (39 %)  Cholesta-5,7,24-trienol (32 %) 			
Erg3Δerg6Δ	**18 ± 3.6**	**14 ± 4.1**	**5.2 ± 0.9**
Zymosterol (41 %)  Cholesta-7,24-dienol (40 %) 			
Erg2Δ	**22 ± 0.1**	**16 ± 1.6**	**3.4 ± 1.4**
Ergosta-5,8,22-trienol (13 %)  Fecosterol (33 %)  Ergosta-8-enol (35 %) 			
Erg2Δerg3Δ	**46 ± 14**	**21 ± 14**	**6.8 ± 2.3**
Fecosterol (39 %)  Ergosta-8-enol (40 %) 			
Erg2Δerg6Δ	**63 ± 0.1**	**52 ± 13**	**6.8 ± 0.9**
Zymosterol (86 %) 			

Table 3. The minimum concentration of the polyene antibiotics needed to inhibit the growth of different ergΔ mutants. The minimum inhibitory concentration (MIC) values for natamycin (MIC_{natam}), nystatin (MIC_{nyst}) and filipin (MIC_{filip}) are given for the different ergΔ strains, together with the structure and percentage of the most abundant sterols in an ergΔ strain, as stated in reference (5). The MIC values were determined in triplicate.

Table 4. The minimum concentration of the polyene antibiotics needed to inhibit the growth of strains RH6611 and 6613. The minimum inhibitory concentration (MIC) values for natamycin (MIC_{natam}), nystatin (MIC_{nyst}) and filipin (MIC_{filip}) are given for the different strains, together with the sterol structure and percentage of the most abundant sterols in the strain, as stated in reference[#]. The MIC values were determined in triplicate.

	MIC_{natam} (μM)	MIC_{nyst} (μM)	MIC_{filip} (μM)
Wild Type	**2.0 ± 0.4**	**0.9 ± 0.1**	**2.7 ± 0.4**
Ergosterol (78 %) 			
Cholesterol	**31 ± 6**	**13 ± 2.4**	**3.3 ± 0.6**
Cholesterol (68 %) 	Cholesta-5,7-dienol (10 %) 	Cholesta-5,24-dienol (10 %) 	

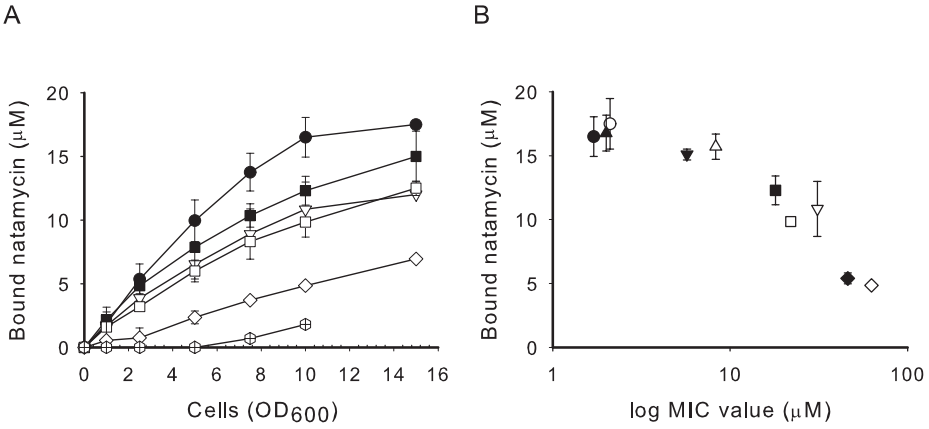


Figure 5. Binding of natamycin to different yeast strains. In figure A, the binding of natamycin with the yeast strains is depicted by addition of 30 μM natamycin to varying cell densities (OD_{600}). In B the binding of natamycin to yeast at an OD_{600} of 10 is plotted against the MIC values of the different strains. The binding was determined in duplo in 10 mM MES / Tris, 15 mM K_2SO_4, pH 7.0 and the strains examined were the wild type RH 448 (●), the wild type RH6611 (▲), erg4Δerg5Δ (○), erg3Δ (▼), erg6Δ (△), erg3Δerg6Δ (■), erg2Δ (□), cholesterol (▽), erg2Δerg3Δ (◆), erg2Δerg6Δ (◇) and the *E. coli* wild type strain (⊕).

The least amount of binding was observed for the negative control, the *E. coli* wild type strain, while strain erg2Δerg6Δ showed the least amount of binding of the yeast strains. The relation of the amount of binding of natamycin to the MIC values is depicted in Figure 5B, at a cell density corresponding to an OD_{600} of 10. The Figure shows an inverse relation between the amount of bound natamycin to the MIC value of a particular strain, strongly suggesting that the differences in MIC value towards natamycin are directly related to the difference in binding of natamycin to the yeast cells. In addition, binding studies with vesicles made from lipid extracts of plasma membrane enriched yeast membrane fractions were performed and resulted in a similar binding pattern as compared to intact yeast cells (data not shown).

Effect of polyene antibiotics on proton permeability in vitro – The binding assays as well as the MIC determinations show that there is a specific interaction of natamycin with ergosterol, which leads to an inhibition of cell growth. To test if the interaction of natamycin with ergosterol leads to changes in membrane permeability, different leakage assays were employed. Natamycin did not produce any carboxyfluorescein leakage from DOPC vesicles containing 10 mol% ergosterol in contrast to filipin which did cause carboxyfluorescein leakage (results not shown). Since nystatin, which is known to form pores, also did not cause carboxyfluorescein release from the vesicles, the pores formed by this antibiotic are apparently too small to allow passage of this dye. A similar situation could be the case for natamycin. Therefore, we tried an assay based on leakage of protons which should be small enough to pass such pores. This assay makes use of a pH dependent fluorescent probe (HPTS), which has a high fluorescent intensity at neutral pH and a low fluorescent intensity at low pH (9). An example of the effect of 5 μM of polyene antibiotics on 10% ergosterol containing vesicles is given in Figure 6A. Trace 1 was recorded by addition of the vesicles to the cuvette and following the fluorescence intensity in time (the blanc). After approximately 300 sec, the detergent DDAO is added to dissipate the vesicles and the fluorescent intensity reaches its lowest point. Nigericin (trace 2) was used as a positive control and resulted in an immediate dissipation of the proton gradient over the model membrane. Indeed, filipin (trace 3) and nystatin (trace 4) both resulted in leakage of the membrane vesicles. Strikingly, natamycin (trace 5) did not result in proton leakage at this concentration. A more quantitative analysis of the effect of the antibiotics on H^+ leakage in model membranes is given in Figures 6B-D. The results show that in strong contrast to filipin and nystatin, natamycin

did not induce any significant proton leakage in ergosterol containing vesicles even at very high concentrations (Figure 6D). This would indicate that natamycin does not act via a perturbation of the membrane barrier and thus has a completely different mode of action compared to filipin or nystatin. To test if similar effects could be observed *in vivo*, a proton leakage assay in yeast was performed.

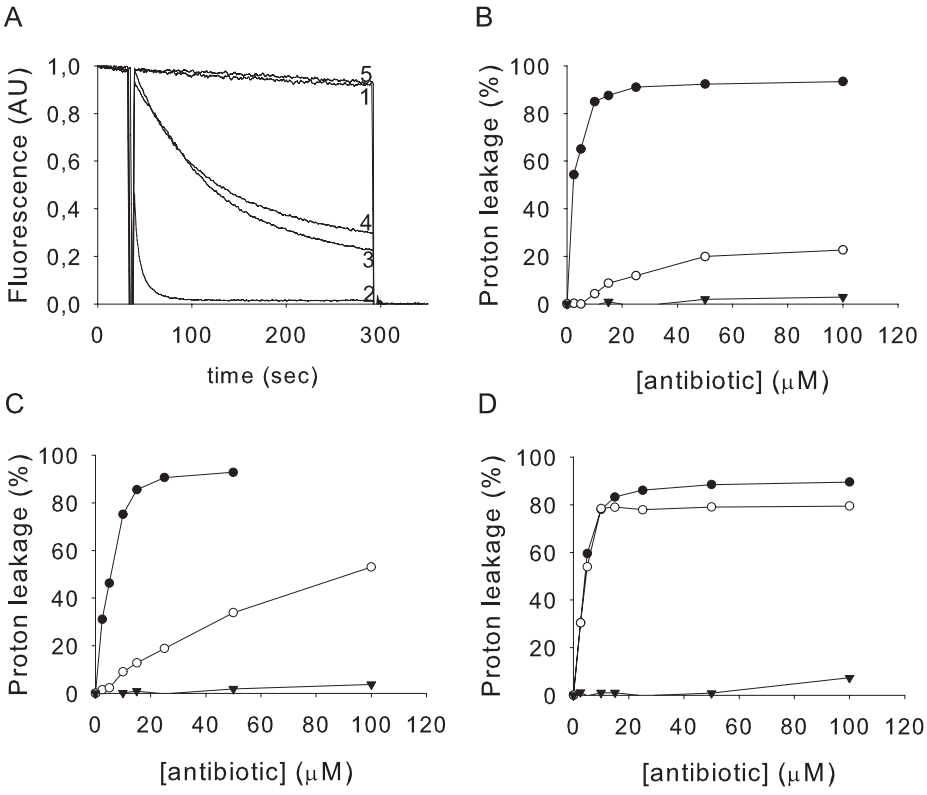


Figure 6. Effect of the polyene antibiotics on the proton permeability of membrane vesicles. (A) Time courses of HPTS fluorescence, which was influenced by (1) no addition or the addition of (2) nigericin, (3), filipin (4), nystatin and (5) natamycin (5 μM antibiotic) to 10% ergosterol containing vesicles. (B-D) The percentage of proton leakage was determined by adding various concentrations of filipin (●), nystatin (○) and natamycin (▼) to vesicles containing (B) no sterols, (C) 10% cholesterol or (D) 10% ergosterol. Measurements were performed in 10 mM MES, 0.2 M Na_2SO_4 buffer, pH 5.5 and the vesicles had a 35 μM final phospholipid concentration.

Effect of polyene antibiotics on proton permeability and growth in vivo – To correlate the results from the *in vitro* leakage assay to an *in vivo* effect, yeast cells were loaded with the pH sensitive probe CFDA-SE. The effect of the polyene antibiotics added at two fold the MIC value on the wild type yeast strain is displayed in Figure 7. The fluorescence of the loaded yeast strain was monitored after different time intervals (Figure 7A). In the absence of antibiotic, the yeast cells displayed a steady fluorescence intensity that decreased slightly in time. When natamycin was added, no further decrease in fluorescence intensity was observed. When nystatin was added to the yeast cells an immediate decrease in fluorescence intensity was observed, most likely due to the formation of pores in the plasma membrane. After the decrease of fluorescence, a gradual increase of the fluorescence intensity was observed, which indicates that the yeast cells try to restore the ion gradient over the plasma membrane. Figure 7B shows that with the same conditions used to study the antibiotic induced release of protons, growth was inhibited by both natamycin and nystatin further emphasizing the difference in mode of action between these polyene antibiotics. In conclusion, natamycin does not kill yeast cells by permeabilizing the plasma membrane.

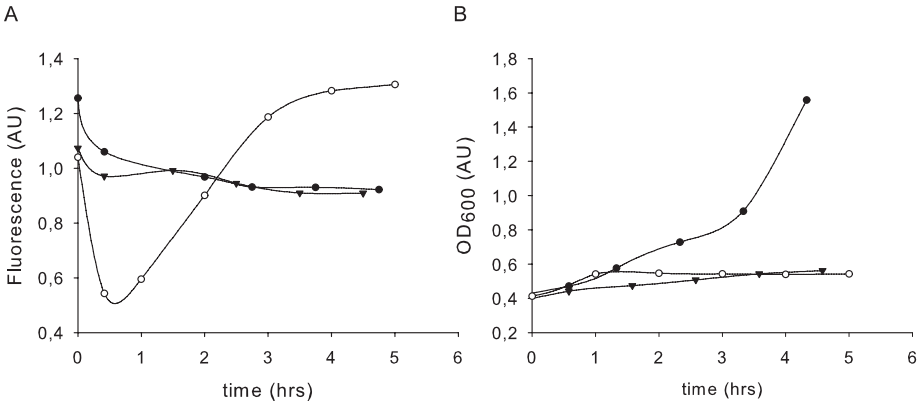


Figure 7. Effect of the polyene antibiotics on CFDA-SE loaded wild type yeast cells. Yeast cells in YPUADT medium buffered with 50 mM citric / phosphate (pH 4) were followed in time after no addition of antibiotic (●) or the addition of nystatin (○) or natamycin (▼) (2.5 μM). (A) The fluorescent intensities and (B) the optical densities were monitored at regular time intervals.

Discussion

In this study we have demonstrated that natamycin kills yeast by specifically binding to ergosterol but without permeabilizing the plasma membrane. This novel mechanism sets natamycin apart from other polyene antibiotics studied so far. We included two of these as a reference in this study.

The ITC and direct binding studies in both model and yeast membrane systems demonstrated that natamycin binds with an apparent affinity of approximately 100 μM specifically to ergosterol with a stoichiometry of approximately 1 to 1 or 1 to 2 depending whether the sterol is available for interaction only in the outer leaflet or in both leaflets of the membrane. This stoichiometry range is in good agreement with the stoichiometry reported before for other polyene antibiotic-sterol interactions (14). However, given the complexity of the binding data and the unknown nature of the natamycin-ergosterol complex a more quantitative discussion of the binding data is not possible.

Both the results from the model system and the yeast mutants gave a clear picture of the requirements within the sterol structure for the binding to natamycin, where only variations in the double bonds of the B-ring resulted in large differences in interaction, especially the sp^2 hybridization of C-7. The packing of the sterol molecule together with natamycin is probably related to this structural requirement. The conformation of ring B in ergosterol differs from the conformation of this ring in cholesterol, which is clearly illustrated in Figure 8. The sp^2 hybridization at C-7 in ergosterol (indicated with an arrow, Figure 8A) results in a 1,3-diplanar chair conformation, which is lacking in cholesterol giving a half-chair conformation (Figure 8B) (15). Natamycin has a tightly constrained molecular topology which gives a very high apparent structural order (16). Therefore it is very likely that the diplanar chair conformation of the B-ring in ergosterol will result in a more efficient interaction. For amphotericin B, similar results were observed, where the sp^2 hybridization at C-7 was of critical importance for the interaction of this antibiotic with sterols in model membranes, while the double bond at C-5,6 was not essential (17).

The sterol specificity of natamycin in model and biomembranes was more comparable to nystatin than to filipin. This can also be observed from the additional ITC experiments that are given as supplemental data. The observed order of binding for filipin in the ITC experiment was 10% ergosterol > 10% cholesterol > 0% sterol leading to the values of 41.3, 20.4 and 17.4 x 10^4 M^{-1} respectively. Filipin

did not seem to be as dependent on sterol structure nor the presence of sterols as the apparent K values to different membranes did not vary much (in agreement with literature) (18-20). The binding of nystatin seemed more similar to natamycin and the K value is slightly lower compared to natamycin; 2.72 to 5.7 x 10^4 M^{-1}.

We have shown that the interaction between natamycin and ergosterol leads to an inhibition of yeast growth and cell death, but, this is not via a permeabilisation of the membrane as is exhibited by nystatin. The structure of the natamycin-ergosterol complex is unknown, but assuming that it is similar to nystatin-ergosterol complexes, two possible explanations can account for the difference in mode of action. One is that the formed complex of natamycin and ergosterol might be too tight to pass even an ion as small as a proton. Secondly, the formed complex could be too small to span the complete bilayer. If the mode of action of natamycin does not involve permeabilisation, then how does it act? In this light it is worth recalling that for the polyene antibiotics that are known to permeabilize the membrane, also other modes of actions have been proposed such as oxidative damage of membrane structures (21-23). The mode of action of natamycin must be related to an important function of ergosterol in the yeast cells. For example, sterols are known to have an ordering effect on the membrane, it is thought that they reside in specific sterol-rich domains in membranes and they are also known to be involved in endocytosis, exocytosis and vacuolar fusion (24-27). Natamycin might inhibit these important processes by binding to ergosterol such that the sterol cannot perform its functional effects.

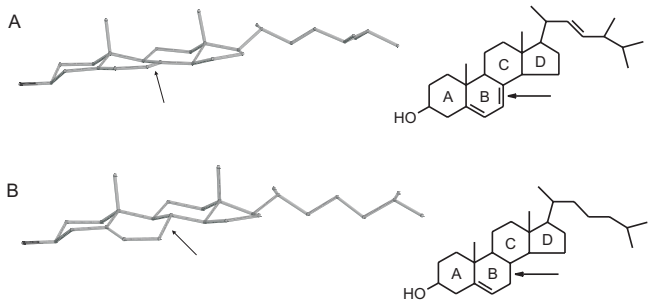


Figure 8. The conformation of the ring structures in (A) ergosterol and (B) cholesterol viewed from the side at approximately the same angle, together with the flat structures. The arrow indicates the C-7, 8 bond, resulting in different B-ring conformations; a 1,3-diplanar chair conformation in ergosterol and a half-chair in cholesterol. Structures were taken from crystal structures given in refs (28,29).

Acknowledgements

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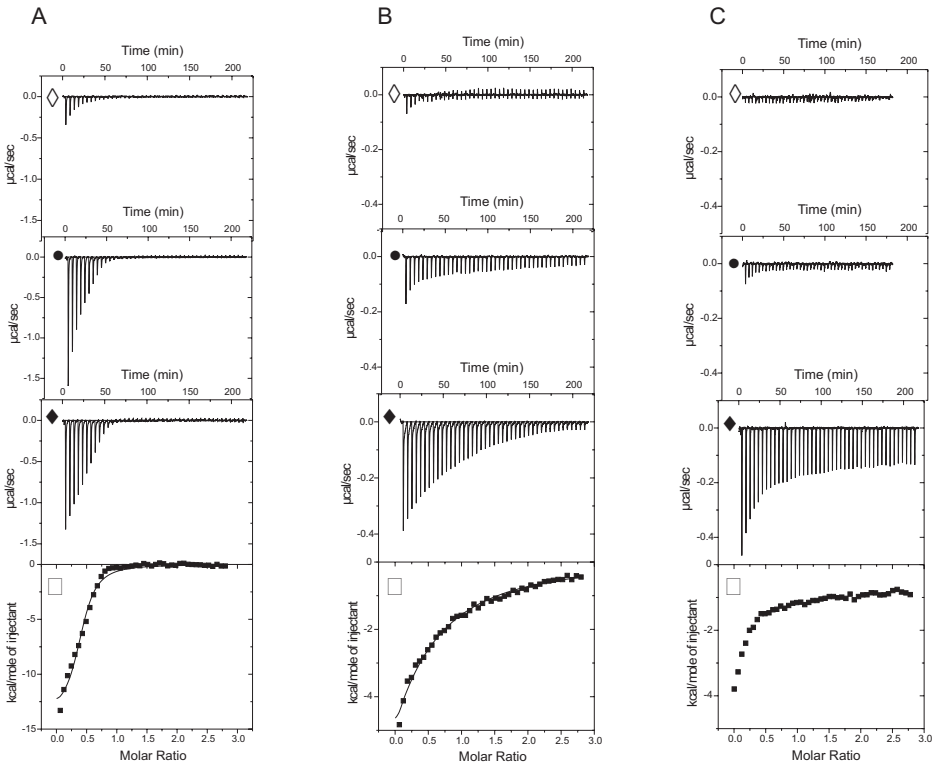
Supplemental Data

Supplementary Figure 1. Calorimetric titrations of the polyene antibiotics with vesicles. Filipin (A), nystatin (B) and natamycin (C) were dissolved in 50 mM MES, 100 mM K_2SO_4 pH 6.0. The top pannels display the heat peaks after consecutive injections of vesicles with no sterol (◊), 10% cholesterol (•) or 10% ergosterol (♦) (8 mM final phospholipid concentration) into the sample cell containing 50 µM antibiotic. The bottom graphs (□) show the integrated heat per injection for 10% ergosterol containing vesicles, which is normalized to the injected amount of moles of sterol and is displayed against the molar ratio of sterol versus antibiotic.



Chapter 3

Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol

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Abstract

The antifungal antibiotic natamycin belongs to the family of polyene antibiotics. Its antifungal activity arises via a specific interaction with ergosterol in the plasma membrane (te Welscher et al. (2008) J. Biol. Chem 283: 6393). However, this activity does not involve disruption of the membrane barrier function, a well known property of other members of the polyene antibiotic family, such as filipin and nystatin. Here we tested the effect of natamycin on vacuole membrane fusion, which is known to be ergosterol dependent. Natamycin blocked the fusion of isolated vacuoles without compromising the barrier function of the vacuolar membrane. Sublethal doses of natamycin perturbed cellular vacuole morphology, causing the formation of many more small vacuolar structures in yeast cells. Using vacuoles isolated from yeast strains deficient in the ergosterol biosynthesis pathway, we showed that the inhibitory activity of natamycin was dependent on the presence of specific chemical features in the structure of ergosterol that allow the binding of natamycin. We found that natamycin inhibited the priming stage of vacuole fusion. Similar results were obtained with nystatin. These results suggest a novel mode of action of natamycin and perhaps all polyene antibiotics, which involves the impairment of membrane fusion via perturbation of ergosterol dependent priming reactions that precede membrane fusion and may point to an effect of natamycin on ergosterol dependent protein function in general.

Introduction

A growing threat to human health is the increase of invasive fungal infections, especially in persons whose immune systems are compromised. Only a few anti- fungal agents have proven to be effective; including the polyenes, the fluorocytes, and the azole derivatives, but an increase of resistance has been observed for several members (1). Polyene antibiotic resistance is still a rare occurrence, which makes these antibiotics particularly useful as antifungal agents. In the past, convincing evidence has been presented that this class of antibiotics target sterols, in particular ergosterol the abundant and main sterol of fungal membranes. The interaction of these antibiotics with ergosterol leads to changes in the membrane that ultimately cause the destruction of the membrane barrier (2-4). Natamycin (also called pimaricin) is a very effective member of the polyene antibiotic family with a large record of applications. Natamycin is produced by

Streptomyces natalensis and is used for the topical treatment of fungal infections, and it is also widely utilized in the food industry. For many years people have believed that the polyene antibiotic natamycin would kill fungi by permeabilising the plasma membrane. Only recently have we discovered that in marked contrast to amphotericin B, filipin or nystatin, the polyene antibiotic natamycin does not act via membrane permeabilisation (5). And yet, its activity is strongly ergosterol dependent and requires a specific sterol structure (5). We aim to elucidate the mode of action of natamycin and, through the detailed understanding of its mechanism, new and improved antifungal formulations may be developed. Because of the specific interaction with ergosterol, natamycin may act via excluding ergosterol from performing important functions in the membrane.

Besides important rules in modulating membrane fluidity, regulatory processes and domain formation, sterols also have been shown to be important during membrane fusion and fission events (6-8). Both fusion and fission are similar processes that rely on the central event of a merger or separation of two membranes. This requires a transient reorganization of membrane lipids into highly curved fusion intermediates (9). Both endocytic and exocytic pathways are dependent on the fusion and fission of membranes in which sterols have been shown to be important (10,11). For example, by deleting different *ERG* genes in *Saccharomyces cerevisiae,* strains are formed with altered sterol compositions (12,13). These strains show deficiencies in the endocytic process, as well as plasma membrane fusion (12-14). This implies that these processes are dependent on ergosterol and have specific structural requirements for the sterols present.

The fusion reaction of isolated vacuoles from yeast can be studied via a content mixing assay and has been used as a model system to examine membrane fusion reactions in general, particularly because it uses much of the same mechanisms as other fusion reactions (15,16). Ergosterol has been shown to be required for the fusion of vacuoles, indicating the importance of ergosterol in vacuolar fusion in yeast (17,18). Here, we have used this model system to decipher the mode of action of natamycin. Natamycin was able to inhibit the vacuolar homotypic fusion. Like the overall inhibitory effect of natamycin on yeast cells, the inhibition on vacuolar fusion was not due to membrane permeabilisation. Natamycin acted at an early stage of the fusion process, even before membrane contact. This activity was dependent on the presence of specific chemical features in the structure of ergosterol and may involve an effect on protein functions that are ergosterol dependent.

Materials and Methods

Chemicals – The polyene antibiotics nystatin and filipin were dissolved in pure DMSO and natamycin was dissolved in 85 : 15 DMSO to H_2O (v/v); all were obtained from Sigma Chemical (St. Louis, MO). The concentrations of the polyene antibiotics were determined spectrophotometrically on a Perkin Elmer UV / Vis Spectrometer (Lambda 18). The molar extinction coefficients and corresponding wavelengths of the polyene antibiotics in methanol were $7.6 \times 10^4\ M^{-1}\ cm^{-1}$ (318 nm), $6.7 \times 10^4\ M^{-1}\ cm^{-1}$ (318 nm) and $8.5 \times 10^4\ M^{-1}\ cm^{-1}$ (356 nm) for natamycin, nystatin and filipin respectively. Poly-l-lysine, ACES, DEAE-dextran, ficoll (MW 400.000), para-nitrophenylphosphate (*p*NPP), quinacrine, neomycin, ATP, creatine kinase, creatine phosphate, leupeptin, pepstatin, *o*-phenanthroline, Pefabloc SC, apyrase (VI and VII) were obtained from Sigma Chemical (St. Louis, MO). MDY-64 was purchased from Molecular Probes (Eugene, OR). Antibodies against Sec18p or Vam3p were purified as IgG fractions from rabbit sera as previously described (19). All protein concentrations were measured using Bio-Rad protein assay reagents from Bio-Rad Laboratories (Richmond, CA) using bovine serum albumin as a standard.

Strains, growth conditions and genetic modifications – Strains used for vacuole staining and isolation are listed in Table 1. Yeast cells were grown at 30°C in 10 g/l yeast extract, 20 g/l bacto peptone and 20 g/l dextrose without (YPD) or with (YPUADT) supplementation of 2 g/l uracil, 1 g/l adenine, and 1 g/l tryptophan. *ERG* gene deletions were performed in strains KTY1 and KTY2 by homologues recombination of PCR products using primers with ~ 40 nucleotides of homology to the 5′ and 3′ ends of the gene of interest and 20 nucleotides homology to the pRS 403 vector as the template (Table 2) (20).

Vacuole isolation and fusion reactions – Vacuoles were isolated and their fusion tested as previously described (16). Standard fusion reactions contained vacuoles isolated from two strains (3 μg protein each) with either alkaline phosphatase deleted (*pho8Δ* KTY2 parental strains), or with proteinase A and proteinase B deleted (*pep4Δ, prb1Δ* KTY1 parental strains), 30 μl of PSS buffer [20 mM piperazine-*N,N*′-bis(2-ethanesulfonic acid (PIPES-KOH), pH 6.8, 0.2 M sorbitol, 125 mM KCl, 5 mM $MgCl_2$] supplemented with 10 μM coenzyme A, a protease inhibitor cocktail (6.6 ng/ml leupeptin, 16.6 ng/ml pepstatin, 16.6 μM *o*-phenanthroline, 3.3 μM Pefabloc SC) and an ATP regenerating system (ATP_{reg}; 1 mM Mg-ATP, 0.5 mg/ml creatine kinase, 40 mM creatine phosphate) and 1 mg/ml cytosol (isolated as described previously (21)).

Table 1. Strains used in this study.

Description	Name	Genotype
Cytosol strain	K91[a]	*pho8::URA3, pho4::LEU2*
Strain used in vacuole staining	RH448[b]	*MATa his4 leu2 ura3 lys2 bar1*
WT	KTY1[c]	*pep4::kanMX, prb1::LEU2*
	KTY2[c]	*pho8::kanMX*
erg4Δ	YWY1-4[d]	KTY1; *erg4::HIS3*
	YWY2-4[d]	KTY2; *erg4::HIS3*
erg3Δ	YWY1-3[d]	KTY1; *erg3::HIS3*
	YWY2-3[d]	KTY2; *erg3::HIS3*
erg2Δ	YWY1-2[d]	KTY1; *erg2::HIS3*
	YWY2-2[d]	KTY2; *erg2::HIS3*

[a] Obtained from G. Eitzen.

[b] Obtained from H. Riezman.

[c] Based on BY4742 (*his3, leu2, ura3, lys2)* and obtained from G. Eitzen (18).

[d] This study.

Table 2. Primers used in this study.

5′ERG4-KO	5′GATACGGATA TTTACGTAGT GTACATAGAT TAGCATCGCT AGATTGTACT GAGAGTGCAC 3′
3′ERG4-KO	5′TG TAAAATAAGT TAATGAAGTG GATAGAAAAA GAAAATAA CTGTGCGGTA TTTCACACCG 3′
5′ERG3-KO	5′AAAAAAGATA ATAAGAAAAA TATTCGTCTA GATTTGAGAT AGATTGTACT GAGAGTGCAC 3′
3′ERG3-KO	5′TCGACCTCCT TGATGAAATG TTCAACTTCC TTAACTTGAG CTGTGCGGTA TTTCACACCG 3′
5′ERG2-KO	5′TCGCTCAATC AAACTAAGAC TAGCCCAGAC CATTATAGCC AGATTGTACT GAGAGTGCAC 3′
3′ERG2-KO	5′TAATGGACTA CCGCATGACT GATTTCGTGA GGTCGGGCAG CCTGTGCGGTA TTTCACACCG 3′

Reactions were incubated for 90 min at 27°C and then assayed for alkaline phosphate activity. For this, 470 µl of developer solution (250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM $MgCl_2$, 1.5 mM *p*NPP) was added to the reactions and incubated for 5 min at 30°C. The reaction was stopped by addition of 500 µl 1 M glycine-KOH, pH 11.5, and the absorption at 400 nm was determined. Fusion reactions with the strains containing *erg*Δ that are based on KTY1 and KTY2 (Table 1) were performed in a similar manner, with one exception, that no cytosol was added to these reactions.

Quinacrine uptake assay – Reactions contained vacuoles (60 µg) freshly isolated from strain KTY2 in 150 µl of PSS buffer. To have an active H^+-pumping system that allows for acidification of the vacuoles the reactions were supplemented with ATP_{reg}. Quinacrine (200 µM) was added from a 10 mM stock in water and polyene antibiotics were added in a concentration range from 0 to 400 µM. The reactions were incubated for 20 min at 27°C, after which the reaction mixtures were placed on ice, 1 ml of PSS buffer was added and the vacuoles were spun down for 4 min, 14000 rpm at 4°C (22). The pellet was resuspended in 150 µl of 0.4% Triton X-100 and the fluorescence of the quinacrine was determined (ex 421 nm/em 496 nm) using a QM-4SE spectrofluorometer with a four-position sample holder (Photon Technologies Inc., London, United Kingdom). The percentage of uptake of quinacrine at a given polyene antibiotic concentration was determined by comparing the fluorescence of vacuoles supplemented with ATP_{reg} (100%) to vacuoles without ATP_{reg} (0%).

Staging assay – Staging was performed as described (22,23). Standard fusion reactions were started at 27°C as described above. At different time points (0, 10, 20, 30, 45 or 60 min), inhibitors of the specific stages or PS buffer as a control (20 mM PIPES-KOH, pH 6.8, 0.2 M sorbitol) were added. The inhibitors used were 4 µl (250 µg/ml) αSec18 (priming), 4 µl (150 µg/ml) αVam3 (docking) or placing on ice (fusion) at the indicated times. The inhibitor effects were compared with the effects of the polyene antibiotics natamycin (200 µM), nystatin (200 µM) or filipin (100 µM). After 90 min the amount of fusion was determined by measuring the alkaline phosphatase activity.

Vacuole staining and observation of live yeast cells – A small single colony of strain RH448 (Table 1) was grown aerobically for 16 h at 30°C in 50 ml YPUADT. Cultures were diluted to an OD_{600} of 0.3 and after 1.5 h of growth, the cells were inoculated on poly-l-lysine coated cover slips as described in reference (24).

Concentrations of 0, 0.5, 1.0 and 1.5 μM natamycin were used and incubated for 5 h at 25°C. Yeast cells were stained with 10 μM MDY-64 dissolved in ACES buffer (10 mM ACES, 0.02% Tween-80, pH 6.8), incubated for 2 min, followed by an ACES buffer wash step. After removal of ACES buffer, the glass cover slides with the immobilized cells were put upside-down on top of a thin layer (< 0.5 mm) of 2% agar. Images were acquired by automatic exposure at a magnification of 100 x 2.0 with a Zeiss Axioplan II microscope equipped with a Plan-ApoChromat 100x/1.4 oil objective, an additional 2x slider and Zeiss filter set 09. Images were captured with a Zeiss AxioCam MRc digital camera and a shutter system (Ludl Electronic Products Ltd., USA) run by Zeiss AxioVision 4 software.

Results

Effect of natamycin on fusion of isolated vacuoles – Vacuole fusion can be assayed using a content mixing assay. Vacuoles are isolated from two strains; one strain contains normal vacuole proteases, but is deleted for alkaline phosphatase (ALP, the *PHO8* gene product), and the other strain is deleted for vacuolar proteases and hence bears catalytically-inactive pro-ALP (Table 1). Neither population of purified vacuoles has phosphatase activity. Vacuole-to-vacuole fusion allows the proteases to gain access to the pro-ALP and convert it to the catalytically active form, which can be assayed by a colorimetric enzyme assay (16,25). The effect of natamycin on vacuolar fusion was compared to that of two other polyene antibiotics, filipin and nystatin and the chemical structures are given in Figure 1A. Different concentrations of these antibiotics were added to standard fusion reactions and the amount of fusion signal was compared to controls with no antibiotics (100% fusion) and incubation on ice (0% fusion) (Figure 1B). Filipin was most efficient in inhibiting vacuole fusion, with a half maximal inhibitory concentration (IC_{50}) of 14 μM. This was followed by nystatin with an IC_{50} value of 36 μM. The inhibition profiles of filipin and nystatin are in accordance to the profiles observed by Kato *et al.* (17). Natamycin was also able to inhibit the fusion of vacuoles, with an IC_{50} of 56 μM. The maximal amount of inhibition caused by natamycin (71% ± 2.0) is lower compared to nystatin (96% ± 0.5) and filipin (90% ± 7.5).

Effect of the polyene antibiotics on vacuole permeability – The permeabilisation of the vacuolar membrane could potentially explain the observed inhibition on fusion by the polyene antibiotics. Vacuolar fusion requires an intact membrane to maintain a required electrochemical potential as well as the ability to

release calcium (23,26). Although natamycin is unable to permeabilise model membranes or the plasma membrane of yeast cells (5), this does not rule out the possibility that natamycin may permeabilise the vacuolar membrane. To determine the effect of the polyene antibiotics on the permeability of the vacuole, a quinacrine assay was performed (27). Quinacrine is a fluorescent compound, known to accumulate in acidic compartments like vacuoles. If the pH gradient of the vacuole is compromised (e.g. by permeabilisation of the membrane), quinacrine will be unable to accumulate in the vacuole, resulting in a reduced fluorescence. Vacuole acidification is maintained by the vacuolar type H^+-ATPase (V-ATPase), which requires ATP (28). In Figure 2A the accumulation of quinacrine in purified vacuoles with or without an active H^+-pumping system are compared, $+ATP_{reg}$ and $-ATP_{reg}$ respectively. Extended incubation with the ATP degrading enzyme apyrase results in no uptake of quinacrine due to inhibition of V-ATPase function (Figure 2A). Both filipin and nystatin used at 100 µM inhibited quinacrine accumulation. The quinacrine uptake is even less compared to vacuoles incubated without ATP_{reg} or added apyrase, indicating that intact vacuoles are still able to accumulate some quinacrine, while vacuoles

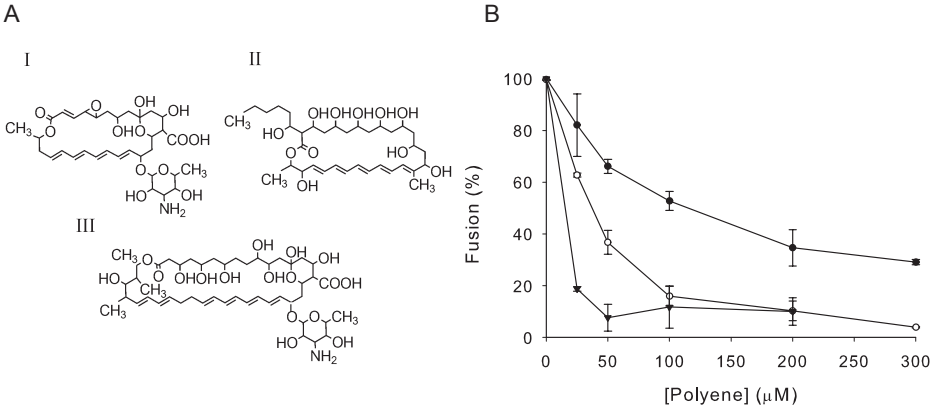

Figure 1. Effect of the polyene antibiotics natamycin, nystatin and filipin on the fusion of isolated vacuoles. (A) Structures of natamycin (I), filipin (II) and nystatin (III). (B) Vacuoles isolated from yeasts with a wild type sterol composition (3 mg protein of KTY1 and KTY2 each) were incubated with different concentrations of natamycin (●), nystatin (○) and filipin (▼) in a standard fusion reaction. After 90 min the amount of fusion was determined by measuring the alkaline phosphate activity. The experiment was performed with freshly isolated vacuoles in triplicate and each measurement was performed in duplicate.

treated with filipin and nystatin do not. This is likely the result of a total loss of the membrane barrier function caused by these polyene antibiotics. Natamycin had no effect on the quinacrine accumulation at this concentration. A similar picture emerged when a broader range of polyene concentrations was used. The percentage of uptake of quinacrine in treated vacuoles was determined via normalization to the quinacrine uptake in vacuoles with or without supplementation of ATP_{reg} (Figure 2B). The results clearly show that both filipin and nystatin were able to cause membrane permeabilisation at the same concentrations that inhibit fusion. Natamycin, however, did not disrupt the vacuolar membrane at any of the concentrations used. This is in agreement with the results found previously that natamycin does not permeabilise model membranes nor the yeast plasma membrane (5). These results suggest that the mode of inhibition of vacuolar fusion for natamycin is fundamentally different from the other polyenes.

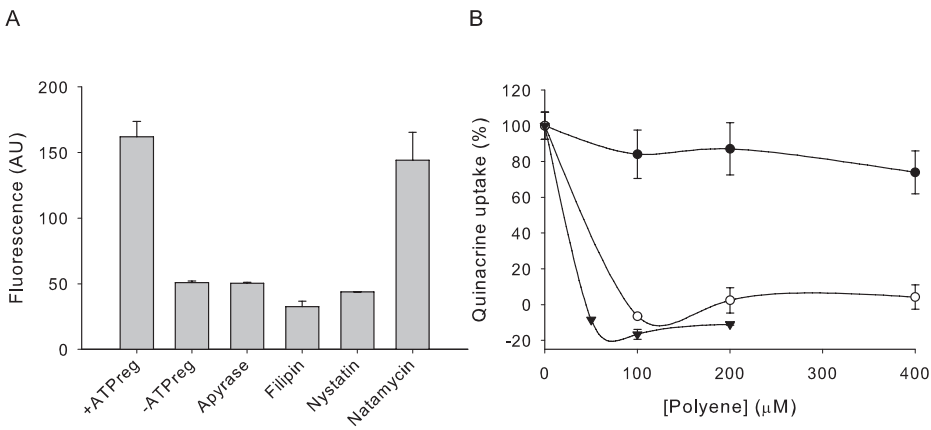

Figure 2. Quinacrine uptake of isolated vacuoles after incubation with the polyene antibiotics. Vacuoles from the KTY2 strain (60 µg protein) were incubated with quinacrine (200 µM) and different antibiotics. After 20 min of incubation, vacuoles were reisolated and the accumulation of quinacrine was determined by the amount of fluorescence (Ex 421 nm/Em 496 nm). (A) The fluorescent quinacrine uptake of vacuoles incubated with or without ATP_{reg}, apyrase (VI and VII, 5U each) or polyene antibiotics (100 µM). (B) The percentage of quinacrine uptake of vacuoles incubated with different concentrations of natamycin (●), nystatin (○) or filipin (▼). The experiment was performed with freshly isolated vacuoles in duplicate.

Sterol dependency of inhibition of fusion by polyene antibiotics – Binding of natamycin to membranes is highly dependent on the presence and chemical structure of sterol molecules (5). To test whether the inhibition of vacuolar fusion by natamycin is related to the chemical structure of ergosterol, we studied the effect of natamycin on the vacuole fusion of different *ERG* mutant strains. This was achieved by deleting the genes of specific sterol biosynthesis proteins in the parental strains KTY1 and KTY2 (Table 1) resulting in different sterol profiles.

From these strains, the vacuoles were isolated and their fusion ability was tested in the content mixing assay, as described above. The biosynthesis proteins and their function, together with the structure of ergosterol are shown in Figure 3A. Deletion of *ERG4* leads to changes in the tail part of the sterol, while deletions of *ERG3* or *ERG2* cause a loss of double bonds in the B-ring. We found that vacuoles isolated from the *erg4*Δ strain-pairs fuse at levels comparable to WT, however, vacuoles isolated from the *erg3*Δ and *erg2*Δ strain-pairs showed a significant reduction in fusion (Figure 3B). These results confirm that the fusion of vacuoles is dependent on the chemical structure of sterols present in the vacuolar membrane (17). In addition, the isolated vacuole fusion results correlate well with the extent of vacuole fragmentation previously shown by morphological analyses in the intact yeast strains (13,17). The relative small, but reproducible amount of fusion of the isolated vacuoles from the *erg3*Δ and *erg2*Δ strain-pairs allowed us to determine the sterol dependency of the polyene antibiotic fusion inhibiting activity. The results are presented in percentage of fusion relative to the specific amount of fusion obtained in the absence of polyene antibiotics of that particular erg deletion strain-pair (Figure 3C). Filipin did not show any dependence on sterol structure for its inhibition of fusion. This is in accordance with the lack of dependence on sterol structure for its binding to membranes or inhibition of yeast growth (5). Natamycin and nystatin show similar inhibition patterns. Loss of the double bonds in the B-ring by deletions of *ERG3* (5,6-position) and especially *ERG2* (7,8-position) showed a loss of inhibition caused by natamycin and nystatin (Figure 3C). Changes to the lipid embedded tail by deleting *ERG4* did not have a significant effect on natamycin or nystatin inhibition. Together, these results indicate that the presence of double bonds in the B-ring of the sterol, specifically at the 7,8-position, are essential for the ability of natamycin and nystatin to inhibit vacuole fusion. Similar structural requirements for sterols have been observed for natamycin and nystatin in their membrane binding and inhibition activity towards yeast cells (5).

Therefore, the inhibition of vacuole fusion caused by natamycin and nystatin is most likely directly related to their binding of sterols in the membrane.

Stage specific inhibition of fusion by the polyene antibiotics - To determine how natamycin inhibits fusion, we examined its effect on the different stages in the fusion process (23,25). Homotypic yeast vacuole fusion occurs in three different stages: priming, docking, and fusion (25). Because different stages are dependent on different proteins, it is possible to examine fusion reactions of isolated vacuoles by using specific inhibitors. For example, the priming and docking stages

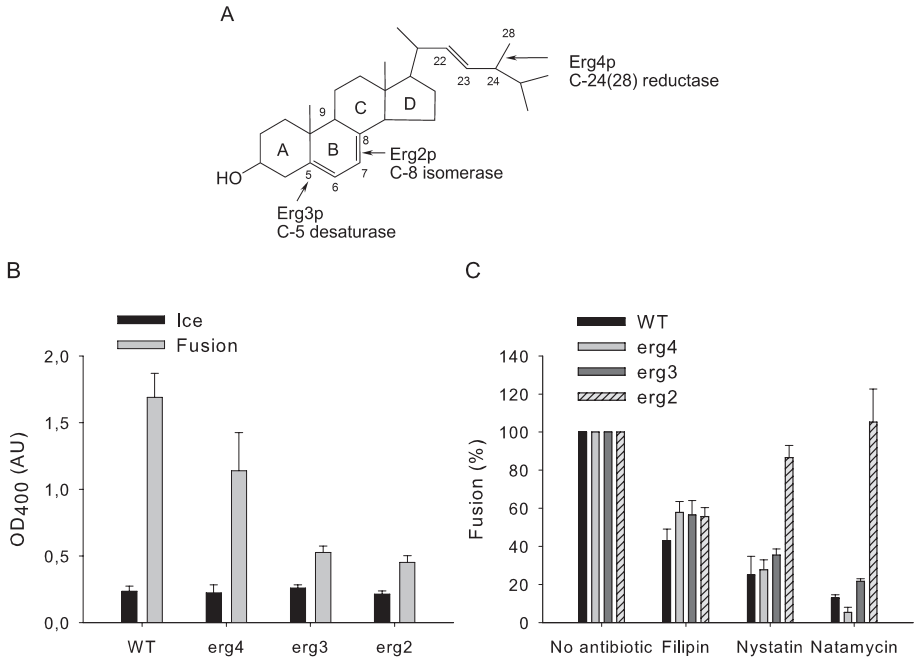

Figure 3. Ergosterol dependency of the polyene antibiotics for vacuole fusion. (A) The structure of ergosterol together with different proteins in the ergosterol biosynthesis pathway and their functions as indicated (17). (B-C) The effect of the polyene antibiotics on the fusion of vacuoles isolated from yeast strains with different sterol compositions. (B) The fusion of vacuoles isolated from yeasts with different *ERG* deletions and *PHO8* or *PEP4* (3 µg protein each) are compared to a reaction on ice. (C) The fusion of isolated vacuoles without antibiotic corrected for ice was normalized to a 100% and compared to the effect on fusion caused by the polyene antibiotics filipin (20 µM), nystatin (100 µM) and natamycin (100 µM). The experiment was performed in triplicate with freshly isolated vacuoles. No cytosol was added to these reactions.

can be inhibited by antibodies against Sec18p and Vam3p, respectively, proteins that are essential for these steps of the fusion process (19,23). To inhibit the fusion stage, a vacuole fusion reaction is placed on ice. In this manner a staging assay can be performed, where the specific inhibition profiles of the polyene antibiotics can be compared to the inhibition profiles of the known inhibitors. Figure 4A shows the inhibition profiles of the controls αSec18 (priming), αVam3 (docking) and ice (fusion). Vacuole priming occurs within the first 30 min. of fusion reactions. Thus, compounds that inhibit this stage of the fusion process only show inhibition within this time span. Indeed, the inhibition profile of αSec18 fits this criterion. Conversely, compounds that inhibit the final stage, membrane bilayer mixing, will show inhibition throughout the whole time-span of the reaction. Such an inhibition profile shows the least amount of fusion (*ice*, Figure 4A). Inhibitors of the docking stage will display their activity between the profiles of the priming and the fusion *(αVam3*, Figure 4A).

For clarity, the inhibition profiles of the polyene antibiotics are compared separately to the controls in for filipin, nystatin and natamycin (Figure 4B-D, respectively). The inhibition profile of filipin lies in between the docking and the fusion profiles, indicating it most likely inhibits between these stages (Figure 4B). This fits with the membrane permeabilising activity of filipin, because a pH-gradient is necessary for the docking stage (23,27). The profile of nystatin inhibition overlaps with the control for the priming, αSec18, which indicates it acts on the priming (Figure 4C). Natamycin also showed a similar inhibition profile as the profile of priming (Figure 4D). Given its inability to cause membrane permeabilisation and the specific interaction of natamycin with ergosterol, this indicates that the effect of natamycin is related to an ergosterol-dependent function in the priming stage of vacuole fusion.

The effect of natamycin on the cellular morphology of the vacuoles – To determine if natamycin also has an effect on vacuole fusion in intact yeast cells, cells were incubated with different concentrations of natamycin for five hours and stained with the vacuolar membrane marker, MDY-64 (Figure 5). Filipin and nystatin were not included in this assay, because their permeabilizing effect on the yeast membrane may cause a free entry of the dye into the cell (29). Most control cells, untreated with natamycin, have more than one vacuole per cell and treatment with natamycin resulted in a fragmentation of the vacuoles, already visible after treatment with 0.5 μM, which is about 30% of the minimum inhibitory concentration (MIC) of this strain for natamycin (Figure 5A) (5). A quantification of the

number of vacuoles per yeast cell shows that approximately 98% of untreated cells had maximally two vacuoles (Figure 5B). However, after incubation with natamycin, this number dropped to 35% owing to an increase of the number of vacuoles per cell to a maximum of 6 in 5% of the cases. These results show that natamycin treatment of whole yeast cells results in the fragmentation of the vacuoles.

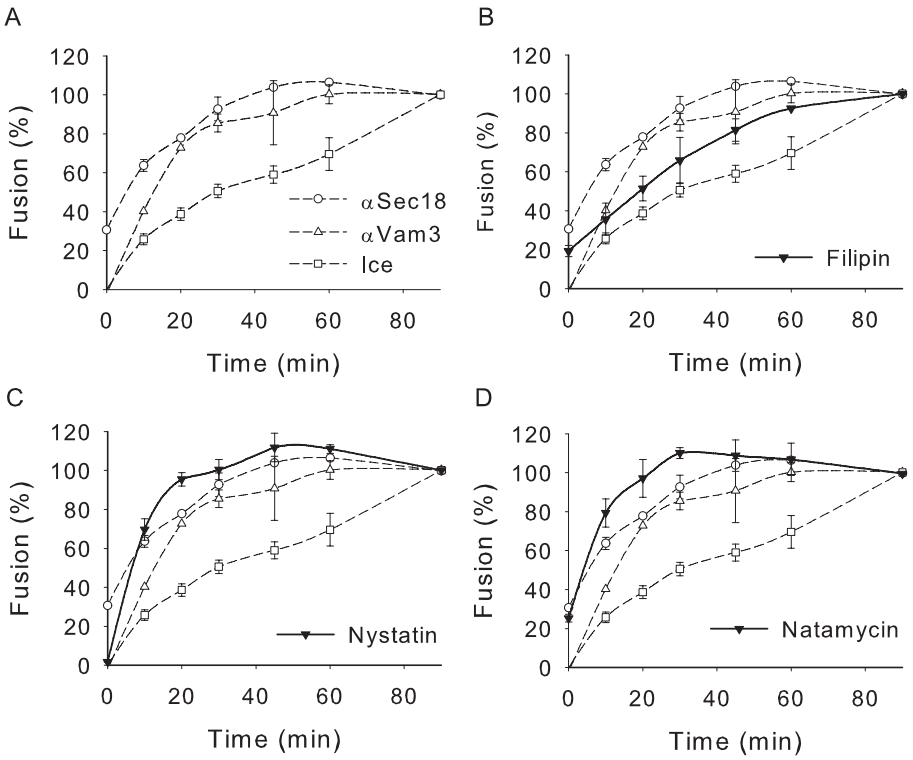

Figure 4. Inhibitory staging assay of vacuole fusion with the polyene antibiotics filipin, nystatin and natamycin. Vacuoles isolated from yeasts with a wild-type sterol composition (3 µg protein of KTY1 and KTY2 each) were incubated with different inhibitors for different time periods. (A) Profiles of the staging inhibitors used as controls for priming (αSec18, ○), docking (αVam3, Δ) and fusion (ice, □). (B-D) The inhibition patterns of the polyene antibiotics (▼) are given together with the controls for filipin at 100 µM (B), nystatin at 200 µM (C) and natamycin at 200 µM (D) respectively. The experiment was performed in triplicate with freshly isolated vacuoles. See *Materials and Methods* for details.

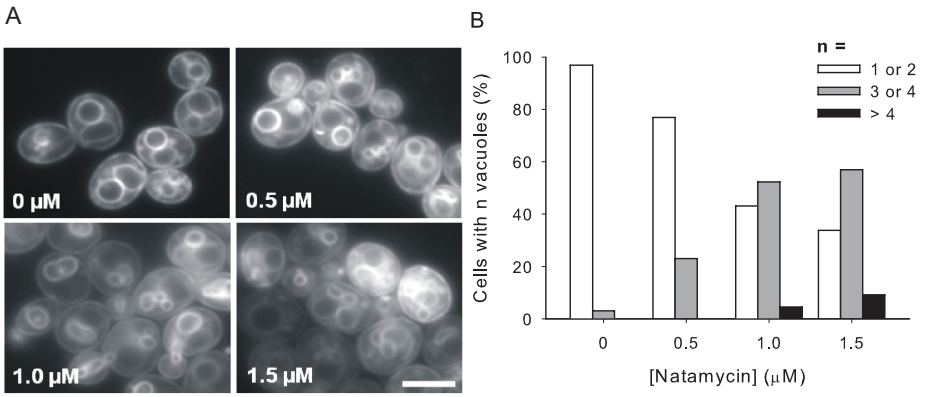

Figure 5. The effect of natamycin on the cellular morphology of vacuoles in yeast. (A) Yeast cells were incubated with 0, 0.5, 1.0 or 1.5 µM natamycin for 5 hours at 25°C. This was followed by staining with MDY-64 and fluorescence microscopy. Bar = 5 µm. (B) The number of vacuoles per yeast cell was determined for a total of 65 cells. White bars represent 1 or 2 vacuole lobes, grey bars 3 or 4 vacuole lobes and black bars are more than 4 vacuole lobes per cell.

Discussion

Although the biological consequences of the action of the polyene anti-fungal compound natamycin are not known, the mode of action is thought to arise via a specific interaction with ergosterol, but does not involve membrane permeabilisation. In this study, we have demonstrated that natamycin is able to interfere in the process of vacuole fusion in a sterol dependent manner. This inhibition also did not involve membrane permeabilisation and seemed to take place early in the fusion mechanism, even before any membrane contact had occurred.

Ergosterol is known to be important during fusion and fission processes, including vacuole fusion (11,14,18). To determine if natamycin was able to act on these processes via its specific interaction with ergosterol, the effects of this antibiotic on the fusion of isolated yeast vacuoles was studied using a content mixing assay (15,16). Indeed, natamycin was shown to inhibit the fusion process of isolated vacuoles. In addition, this inhibition was not related to a permeabilising effect, similar to natamycin's inability to permeabilise model membranes or yeast cells (5). The sterol structure-dependency of the vacuolar fusion inhibition by natamycin was almost identical to the sterol structure-dependency for its activity towards yeast cells as well as its binding to sterols in model membranes (5).

All were dependent on the presence of sterols containing double bonds in the B-ring, most importantly at the 7,8-position (5). Therefore we conclude that natamycin inhibits vacuolar fusion through the specific interaction with ergosterol.

Treatment of yeast cells with natamycin led to a fragmented vacuolar morphology that is characteristic for a defect in vacuole fusion (30,31). A similar vacuolar morphology has been observed in conidia of *Penicillium discolor* (*P. discolor*) upon natamycin treatment (M.R. van Leeuwen and J. Dijksterhuis, unpublished observations). We therefore conclude that natamycin is able to inhibit vacuole fusion both in purified vacuoles, as well as in intact yeast cells. Besides this inhibition of vacuolar fusion, natamycin may act on more ergosterol dependent membrane fusion and fission processes through its interaction with ergosterol (15,32). Indeed, natamycin has been shown to inhibit the early stages of endocytosis in the fungus *P. discolor* (29), an ergosterol dependent fission process (12,13). This suggests that the basis of the toxicity of natamycin could be the inhibition of fusion and fission processes. To act on vacuole fusion in an intact yeast cell, most likely requires natamycin to enter this cell. This could be either via permeation across the plasma membrane or in an early stage, via endocytosis. Currently, we have no information whether natamycin enters the cell and if so via which mechanism this occurs.

The polyene antibiotics nystatin and filipin were shown to be more efficient in inhibiting the fusion of isolated vacuoles. These differences are probably directly related to the relative affinity of the polyenes for ergosterol and their differences in membrane permeabilizing activity. Nystatin and natamycin had similar binding affinities to ergosterol (5), yet nystatin is more efficient in its inhibition of vacuole fusion. This is best explained by the ability of nystatin to permeabilise the vacuole membrane, thereby increasing its efficacy of vacuole fusion inhibition. Filipin displayed the highest affinity for ergosterol and severely damages the membrane-barrier (4,5). Altogether this likely explains why filipin was the most efficient inhibitor of vacuole fusion in our assays.

What would be the mechanism behind the inhibition of fusion caused by natamycin? We have observed that through the specific interaction with ergosterol, natamycin was able to act on the early priming stage of fusion. During this phase, no actual contact between the vacuolar membranes has taken place (33), making it unlikely that natamycin will act on lipid reorganization. The priming phase consists solely of the rearrangements of different

protein complexes (for reviews see (33) and (25)). Thus, the most straightforward conclusion is that natamycin is able to disturb these rearrangements as a result from its binding to ergosterol and suggest a more general mode of action namely to disturb ergosterol dependent protein functions.

This immediately poses the question whether the other members of the family of polyene antibiotics, which all bind to ergosterol (2,3,5,34), are also able to act on the priming stage through their interaction with ergosterol. Indeed, we have shown that nystatin is able to act on the priming stage, as was observed previously as well and the same is true for amphotericin B (17). The effect of filipin is less clear, because we found it to act in between the docking and fusion stage, while in a different study filipin was shown to act on the priming stage (17). The differences in results are likely best explained by different assay conditions. These findings points to a dual mode of action for some members of the polyene antibiotic family, where all members have the basic ability to act through the inhibition of ergosterol dependent protein functions, while the additional ability is to permeabilise the membrane. This relates to a freeze-fracture electron microscopy study, where natamycin, nystatin and filipin all produced distinct morphological effects on the fungal membrane, indicating the different end-results by the mechanisms involved in polyene-sterol interactions (35). Because natamycin *only* has the basic ability to bind ergosterol, it is the ideal candidate to study the basic mode of action of the polyenes. In addition, it makes natamycin an interesting tool for cell biology, when analyzing ergosterol dependent protein functions.

Interestingly, there is another naturally produced family of antibiotics where several members are known to have a dual mode of action. This is the anti-bacterial lantibiotic family, a group of small antimicrobial peptides, a large part of which are known to bind the bacterial cell wall component lipid II and through this interaction block cell wall synthesis (36). In the group of lantibiotics that are able to bind lipid II, several members are long enough to span the lipid bilayer and have an additional ability to form pores (36,37). This striking parallel shows how nature has repeatedly used dual modes of action for membrane active antibiotics and this might be applicable to other families of membrane active antibiotics as well.

Acknowledgments

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Chapter 4

Natamycin exerts its antifungal activity by impairing membrane protein function

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Abstract

Natamycin is a polyene antibiotic which has been used for years in the treatment of fungal infections. Its antifungal activity arises from the specific binding to ergosterol, but in contrast to other polyene antibiotics, this does not result in a loss of barrier function. The observation that natamycin blocks the early priming phase of vacuole fusion (Chapter 3 of this thesis) resulted in the hypothesis that natamycin might impair protein function in an ergosterol dependent way. This hypothesis was tested in this study by determining the effect of natamycin on different plasma membrane transport proteins using uptake assays. Natamycin was shown to inhibit the transport of three different substrates, without causing leakage of these substrates and suggests that natamycin is able to inhibit different plasma membrane transport proteins in fungi. Transport inhibition was found to be reversible. Inhibition of transport by natamycin was most likely not related to a reorganisation of sphingolipid-sterol domains, but most likely is a result of the disturbance of ergosterol dependent protein functions. These findings suggest that all members of the polyene antibiotics have the basic ability to act through the inhibition of ergosterol dependent protein functions, while some have the additional ability to permeabilise the membrane.

Introduction

The polyene antibiotics are the only group of anti-fungal antibiotics that directly target the plasma membrane via a specific interaction with the main fungal sterol, ergosterol. Natamycin is a member of the polyene antibiotic family and is produced by the bacterium *Streptomyces natalensis.* Natamycin is widely utilised in the food industry and in pharmacotherapy for topical treatment. It has been the understanding that this antibiotic would act similar to other polyenes by permeabilizing the plasma membrane. However, unlike the well studied amphotericin B, nystatin and filipin, natamycin is unable to cause leakage in model membranes nor in yeast cells (1). Natamycin does inhibit yeast cells effectively via a specific interaction with ergosterol and the antifungal activity was shown to be dependent on this interaction. Natamycin was shown to be able to inhibit the fusion of yeast vacuoles in an ergosterol dependent manner, suggesting that impairment of this important process is involved in the antifungal activity of natamycin (Chapter 3 of this thesis). Inhibition of endocytosis

was also suggested to be part of the mode of action of natamycin (2). Since both processes involve many different proteins, the possibility has to be considered that natamycin acts on these proteins. In agreement with this hypothesis, inhibition of vacuole fusion was found to take place in an early stage of the fusion process most likely via ergosterol dependent protein functions (Chapter 3).

In this study we investigated whether natamycin is able to specifically inhibit membrane proteins in the ergosterol rich plasma membrane of yeast. The plasma membrane of fungi was chosen as test system, since it contains the highest amount of ergosterol in fungi and it is the first membrane encountered by natamycin (3,4). The effect of natamycin on the uptake of arginine, proline or glucose over the plasma membrane was examined. Natamycin completely blocked the uptake of all the substrates studied, which suggested an inhibition of different plasma membrane transport proteins, without causing leakage of these substrates. The inhibition of transport proteins by natamycin was reversible. Inhibition of transport by natamycin was most likely not related to a reorganisation of sphingolipid-sterol domains, but seems to be due to an ergosterol dependent effect on membrane protein function.

Experimental procedures

Chemicals - 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), egg (chicken)-sphingomyelin (SM) and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Ergosterol was obtained from Larodam AB (Sweden). N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-DHPE) and 5-6,carboxyfluorescein were from Molecular Probes (the Netherlands) and methyl-β-cyclodextrin from Sigma Chemical (St. Louis, MO). DOPC, rhodamine-DHPE or sterols were dissolved in chloroform and SM was dissolved in chloroform / methanol 2:1. Phospholipid concentrations were determined by phosphate analysis according to Rouser (5).

1-[U-^{14}C] arginine monohydrochloride (11.8 GBq/mmol), D-[U-^{14}C] glucose (9.98 GBq/mmol) and L-[U-^{14}C] proline (9.47 GBq/mmol) were from GE Healthcare Europe Gmbh, formerly known as Amersham Biosciences. The polyene antibiotics nystatin and natamycin were obtained from Sigma Chemical (St. Louis, MO) and dissolved in pure DMSO and 85 : 15 DMSO to H_2O (v/v) respectively. The concentrations of the polyene antibiotics were determined by UV absorption on a Perkin Elmer UV / Vis Spectrometer (Lambda 18). The molar extinction

coefficients and corresponding wavelengths in methanol were 7.6 x 10^4 M^{-1} cm^{-1} (318 nm), 6.7 x 10^4 M^{-1} cm^{-1} (318 nm) for natamycin and nystatin respectively.

Strains and growth conditions – The *Saccharomyces cerevisiae* strain used in this study was D273-10B. The cells were grown at 30°C on rich [10 g/l yeast extract, 20 g/l Bacto-peptone and 20 g/l dextrose (YPD)] or minimal [Yeast Nitrogen Base (YNB) without amino acids] media (Difco, USA), containing 20 g/l dextrose, unless indicated otherwise. YNB media were enriched by a "drop-out" mixture containing all amino acids with the exception of arginine or proline where indicated.

Uptake and release of arginine – For arginine uptake experiments, a yeast culture was grown overnight in YPD. Cells were washed, resuspended in YNB without arginine and grown until an OD_{600} between 0.3 and 0.8. At the start of the assay, cells were incubated with natamycin at indicated concentrations for 5 min. and at each time point the amount of colony forming units (CFU) was determined. To a portion of the cell suspension (between 200 and 600 µl), a mixture of radioactive and non-radioactive arginine (ratio of 1 : 5) was added at a final concentration of 30 µM. Samples of 100 µl were withdrawn at different time intervals, diluted in 1 ml of ice-cold water and placed on ice. To determine background radioactivity, a similar amount of radioactive arginine was diluted in 1 ml of ice-cold water and treated similar to the other samples. The samples were filtered on membrane filters (CA, 0.45 µm pore size, 47mm diameter) from Nalgene (Nunc, International), after which the filter was washed with 4 ml of ice-cold water. The filters were dissolved in 4 ml Insta-Gel Plus Scintillation cocktail (Packard Bioscience) and the radioactivity was counted using Tri-Carb 2300TR liquid scintillation analyzer (Packard Bioscience). Signals were corrected for background radioactivity and differences in cell density. To determine the release of arginine, the same protocol was followed as for the uptake, but instead of adding natamycin (20 µM) at the start of the assay, it was added 8 min. after the addition of arginine to the cell suspension. The effect of natamycin on cell growth (OD_{600}) was determined after three hours.

Reversibility of the inhibition of arginine uptake by natamycin – To determine if the yeast cells are able to recover from an incubation with natamycin, an overnight grown yeast culture was diluted in YNB (-arginine) medium and grown until and OD_{600} between 0.3 and 0.5. At the start of the assay, cells were incubated without or with 20 µM of natamycin for 5 min. Cells were washed and resuspended in YNB (-arginine) medium. All cultures were placed in

an incubator at 30°C. At each subsequent step, namely incubation with natamycin, and 1 and 2 hours of growth after washing the cells, the uptake of arginine (followed for 10 min.) and the OD_{600} and CFU were determined.

Transport assay of glucose or proline – The transport assays for glucose and proline were performed essentially as described for the transport assay of arginine with the differences described below that were based on reference (6). At the start of the glucose uptake assay, cells were resuspended to an OD_{600} of approximately 5 in YNB, but without glucose. To monitor uptake of proline, the cells were adjusted to 50 mM potassium phosphate buffer, pH 6.6, 2 mM $MgSO_4$ and 1% glucose by incubating them for 1hr at an OD_{600} of 5, after which the assay was started. The final concentrations and ratio's of radioactive and non-radioactive compounds added to the cell suspensions were 1 mM glucose (ratio of 1 : 80) and 20 µM proline (ratio of 1 : 2).

Preparation of GUVs - Giant Unilamellar Vesicles (GUVs) were prepared by electroformation (7,8). The effects of the polyene antibiotics or methyl-β-cyclodextrin were studied using GUVs of DOPC/SM/ergosterol at a 45:45:10 molar ratio, because both the yield of the vesicles as well as the percentage of vesicles showing phase separations was higher in comparison to other tested lipid mixtures. In addition, an identical sterol composition had been previously used in permeability and binding studies, which would aid the comparison of the results (1). Rhodamine-DHPE (0.05 mol % of total lipids) effectively partitions into liquid disordered (L_d) domains. Typically, 4 µl of a ternary mixture of lipids (final concentration of 5 mM in chloroform / methanol 2:1) was applied to platinum electrodes and dried using a nitrogen stream. To remove any residues of solvents, the GUV cells were placed in a vacuum desiccator for 20 min. GUVs were prepared in 600 µl of a 12 mM sucrose solution and a voltage of 1.7 Volts with a frequency of 10 Hz was applied for 2 hours, after which the suspension containing vesicles was transferred to an eppendorf vial. The phospholipid concentration was determined after study of the samples.

Confocal fluorescence microscopy – 200 µl of the vesicle suspension was transferred into a well of an 8-well coverglass chamber (Lab-Tek chambered coverglass, Nunc). The membrane-impermeable dye 5-6,carboxyfluorescein could be added to the outside of the GUVs to aid in the detection of the vesicles. Multiple samples were examined with different amounts of antibiotic and in general, a particular GUV was examined before and after the

addition of natamycin and followed in time. Images were taken with a Nikon Eclipse TE2000U inverted microscope, equipped with C1 confocal laser scanning unit and CFI Plan Fluor 60x objective (Nikon N.A 1,4). The green and red fluorescence signals were acquired using double excitation (488 nm line from an Argon – Ion laser and 543 nm line from a He-Ne laser) and detection (emission bandpass filters 515/30 and 585/30). Images were generated and analyzed using software of EZ-C1 (Nikon) and Image J (NIH, USA).

Preparation of large unilamellar vesicles (LUVs) – Large unilamellar vesicles (LUVs) with a mean diameter of 200 nm of DOPC/SM/ergosterol (45:45:10), DOPC/ergosterol (90:10), DOPC/SM (50:50) or pure DOPC were prepared as described before (1). Briefly, lipids suspensions were premixed in the desired molar ratios as solutions in chloroform and methanol, whereafter the solvents were removed in a stream of nitrogen, followed by drying the lipid film under vacuum for 20 min. The lipid film was hydrated in 10 mM MES/Tris, 15 mM K_2SO_4 at pH 7, followed by eight freeze-thaw cycles. Subsequently, the lipid suspension was extruded 8 times through a polycarbonate membrane filter with a pore size of 0.2 μm (Whatman International, England).

Binding assay using model membranes – The binding of natamycin to model membranes was studied using a centrifugation assay as described in (1). In short, LUVs at 1.5 mM final phospholipid concentration were incubated with natamycin (varying from 0 to 0.1 mM concentrations) for 1 h in an Eppendorf incubator (22°C, 650 rpm), with a maximum of 4 % DMSO present. 1 ml of the mixture was centrifuged in a TLA 120.2 rotor in a Beckman Ultracentrifuge (TL-100) for 1,5 hrs at 100 krpm and 20 °C. The amount of antibiotic before centrifugation and in the supernatant and pellet were determined by diluting the samples 7 times in methanol followed by centrifugation to remove any precipitated salts and measurement of the UV absorption. Under these conditions less than 10% of the phospholipids remained in the supernatant. Natamycin was not pelleted in the absence of lipid below a concentration of 75 μM.

Results

The effect of natamycin on arginine uptake – To determine if natamycin is able to affect proteins in the ergosterol rich plasma membrane of yeast, the plasma membrane transporter Can1p was studied. Can1p is reported to reside in ergosterol enriched domains in the plasma membrane of yeast (9). Can1p is the

specific transport protein for arginine and acts as a proton / arginine symporter (6). The **G**eneral **A**mino acid **P**ermease (Gap1p) is able to transport arginine as well, but the expression is low in cells grown in the presence of ammonium due to transcriptional repression (6,10). Therefore, the transport activity of Can1p was studied in cells grown in the presence of ammonium (9). In this assay, yeast cells were incubated with different concentrations of natamycin and the uptake of arginine was determined. Figure 1A shows that natamycin causes a dose dependent decrease in the uptake of arginine. This can be due to either natamycin dependent inhibition of the transport process or due to natamycin induced leakage of arginine. To test this, cells were allowed to take up arginine for 8 min, whereafter natamycin was added. Natamycin addition blocked uptake immediately without causing release of the arginine already taken up (Figure 1B).

Next we tested the effect of natamycin on the growth and the viability of the yeast cells under identical conditions to the arginine uptake assay. Natamycin caused a concentration dependent reduction of growth of 60 to 80% for 2 to 20 µM respectively (Figure 1C). A 5 min. incubation of natamycin with the yeast cultures had no effect on the colony forming units (CFU) and thus cell viability (Figure 1C). These results show that natamycin inhibits arginine uptake at concentrations that inhibit cell growth, but without killing the cells.

The regrowth of natamycin treated cells on the plates reported above indicates that the inhibition of natamycin on the arginine uptake may be reversible. To test this directly, a culture was washed after incubation with natamycin and subsequently resuspended in fresh medium and the arginine uptake was monitored in time. The control culture which had not been incubated with natamycin showed an increase in the arginine uptake, which stabilised after 1 hour (Figure 2A). A control culture that had been incubated with natamycin, without washing afterwards, is blocked in the uptake of arginine from the start to the end of the assay. The culture which had been washed after natamycin incubation was blocked in the arginine uptake at the start of the assay and showed a steady increase of uptake in time, reaching the same level of uptake after 2 hrs as the control without natamycin (Figure 2A). These results show that a natamycin induced block of arginine uptake in yeast cells can be reversed.

To determine if a reversed block in arginine uptake also corresponds to a reversed inhibition of growth, cell viability was monitored as CFU in parallel to the arginine uptake as described above (Figure 2B). As expected, the

control culture without natamycin shows an increase in cell viability, while the natamycin control culture shows a decrease in cell viability in time. The culture which had been washed after natamycin treatment showed an initial decrease of 50% in cell viability which did not change, meaning that the cells did not significantly regain their ability to grow in this time period (Figure 2B).

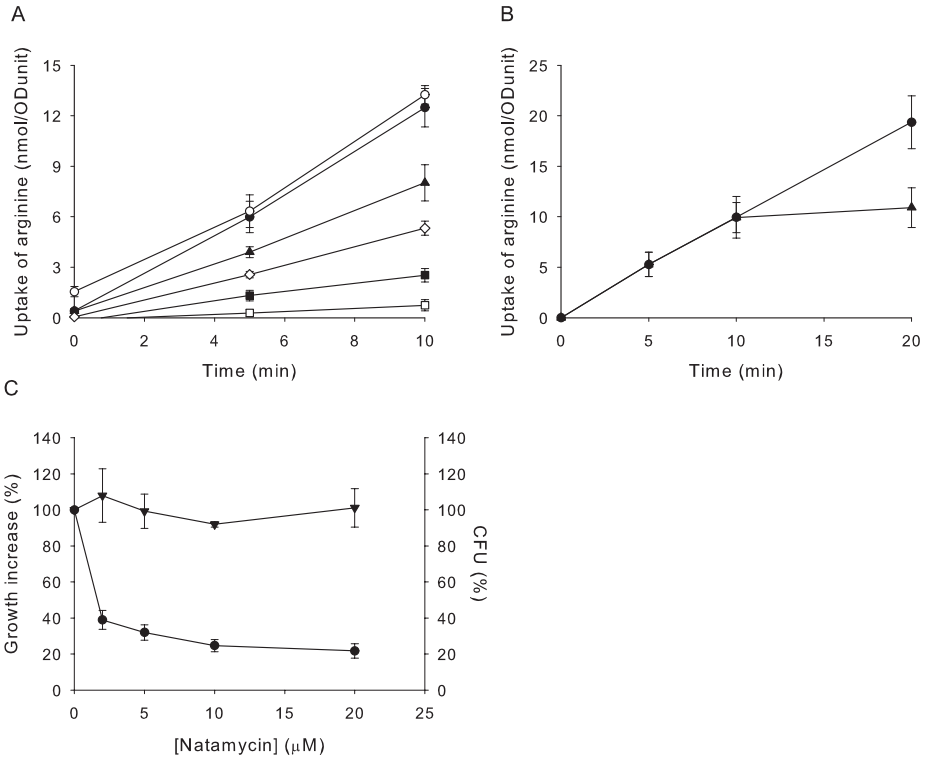

Figure 1. The effect of natamycin on the uptake of arginine. At time 0, ^{14}C-arginine (30 μM) was added to the cells. The uptake of arginine was followed in time and corrected for the amount of cells (ODunit). (A) Cells were incubated 5 min. before the addition of arginine with natamycin from 0 μM (●), 2 μM (▲), 5 μM (◊), 10 μM (■), 20 μM (□) and DMSO (○) was added as a control. (B) Release of arginine from yeast cells was studied by adding 20 μM natamycin (▲) or no natamycin (●) at 8 min. after the addition of arginine and following the uptake of arginine in time. (C) Growth over 3 hours (●) and the colony forming units (CFU, ▼) of the yeast cultures were determined after the addition of different concentrations of natamycin and are given in percentage to the value observed in the absence of natamycin (100%). The results shown are the averages of three separately performed experiments with standard deviation.

These results show that a reversed block in arginine uptake does not correspond to a reversed inhibition of growth, which indicates that the inhibition of arginine uptake by natamycin alone cannot be responsible for the growth inhibition of yeast suggesting that there might be additional targets for natamycin.

Effect of natamycin on other membrane transport proteins – To search for these targets in the plasma membrane, the effect of natamycin on the transport of two other substrates, i.e. glucose and proline, was tested. Glucose is transported in yeast via hexose transporters and of the 20 genes that encode these proteins, seven are known to encode functional glucose transporters. All of them transport their substrates by passive, energy-independent facilitated diffusion, with glucose moving down the concentration gradient (11). Proline can be taken up by a specific high-affinity permease Put4p or the general amino-acid permease Gap1p, which are nitrogen regulated (6,10). Other low-affinity systems are also present, like Agp1p and Gnp1p (10).

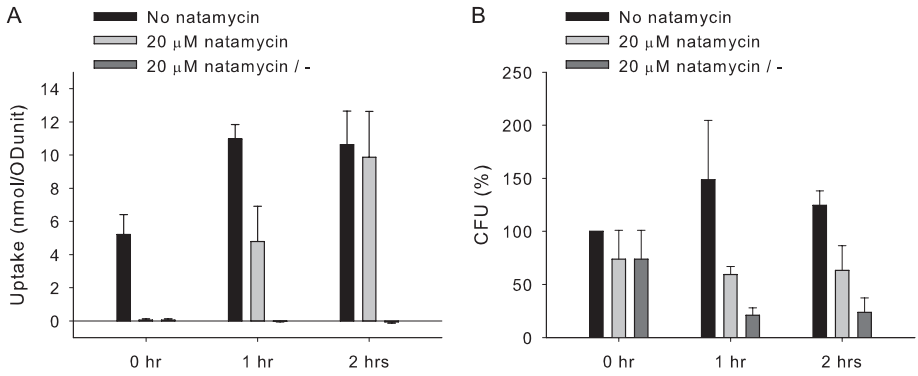

Figure 2. Recovery of yeast cells after natamycin treatment. The uptake of arginine (A) and the colony forming units (CFU) (B) were determined after washing the cells that were incubated with natamycin. Cultures were incubated with or without 20 μM natamycin for 5 min. and a control sample without removal of natamycin containing medium was used (20 μM natamycin / -). Samples were taken in time from cultures starting with a 5 min incubation of natamycin (0 hr). Cultures were washed and resuspended in fresh medium and samples were taken after 1 hr or 2 hrs. The uptake of arginine was determined at 10 min., and corrected for the amount of cells. CFU are expressed in percentage to the culture without natamycin at the start of the assay (100%). The results shown are the averages of two separately performed experiments with the spread of the data.

Cells untreated with natamycin reached an uptake of 9 or 1.2 nmol / OD units after 10 min for respectively glucose and proline. These values were normalised to 100%, to allow comparison of the effect of natamycin on both compounds. Both the uptake of glucose and proline are completely blocked by the addition of natamycin (Figure 3) at similar antibiotic to cell ratios that blocked arginine uptake. These results show that natamycin is able to inhibit the uptake of different important substrates of yeast which demonstrates that it can inhibit different membrane transport proteins thereby causing a block in cell growth.

The effect of natamycin on membrane domains – An obvious possibility is that natamycin affects ergosterol enriched domains in the membrane that might be important for membrane protein function (12,13). An *in vitro* model membrane study was used to unambiguously determine the effect of natamycin on ergosterol enriched domains. In model membrane systems sphingolipid-sterol domains can be readily prepared and studied (14,15). GUV systems are particularly suited because the domains are large enough to be visualised by fluorescence microscopy (16,17). Figure 4 is a representative example of the results obtained with GUVs prepared with a lipid mixture of DOPC/SM/ergosterol of 45:45:10 molar ratio and 0.05 mol % rhodamine-DHPE. The fluorescent lipid rhodamine-DHPE was chosen because it selectively partitions into liquid disordered (L_d)

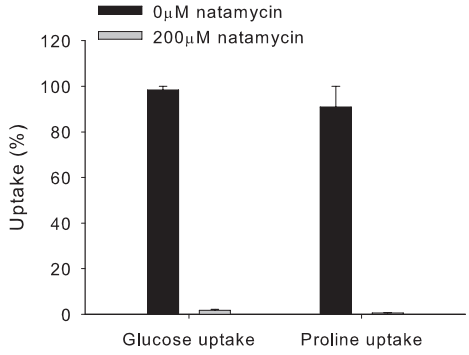

Figure 3. Effect of natamycin on uptake of glucose and proline by yeast cells. Cells were incubated with 0 or 200 μM of natamycin after which the uptake of ^{14}C-glucose or ^{14}C-proline were assayed. The uptake of the different compounds by yeast cells is expressed in percentage to the uptake of a compound by cells untreated with natamycin at 10 min. For further details see *Experimental procedures*. The results shown are the averages of two separately performed experiments with the spread of the data.

domains allowing detection of the coexisting phases. Sphingomyelin was used as a model for yeast sphingolipids. Before addition of natamycin (Figure 4A), the GUV shows liquid ordered (black areas) as well as liquid disordered phases (red areas). No apparent changes were observed in the sterol enriched domains after addition of natamycin in an excess of four to eight times the amount of ergosterol (Figure 4B-D). GUVs became unstable and ruptured at a higher ratio of natamycin to ergosterol (Figure 4E). Never could we observe an intermediate phase between dissipation of liquid ordered domains and rupture of the vesicles.

As a positive control the effect of methyl-β-cyclodextrin on the domains was investigated. This cyclic oligosaccharide is known to bind and extract sterols from membranes and is able to disturb cholesterol enriched membrane domains in GUVs (18-20). Figure 5 shows that indeed the ordered sphingolipid-ergosterol domains are disrupted by methyl-β-cyclodextrin. These results show that the polyene antibiotic natamycin is unable to dissipate ergosterol enriched domains in a model membrane system.

The lack of effect of natamycin on the domains might be due to the inability of natamycin to bind to the tightly packed sphingolipid-ergosterol domains. To test this we compared the binding of natamycin to vesicles composed of DOPC/SM/ergosterol (45:45:10 molar ratio) to DOPC/ergosterol (90:10 molar ratio). Natamycin was able to bind efficiently vesicles containing sphingolipid-ergosterol domains (Figure 6). The binding of natamycin to vesicles of DOPC/SM/ergosterol (45:45:10 molar ratio) was somewhat lower compared to the more disordered DOPC/ergosterol (90:10 molar ratio) system. This is largely due to the low level of aspecific binding to pure DOPC (dashed line in Figure 6). These results demonstrate that natamycin binds to sphingomyelin–ergosterol domains without disrupting them and suggests that the inhibitory effect of natamycin on the different plasma membrane uptake systems is not due to a disruption of ergosterol enriched domains.

Figure 4

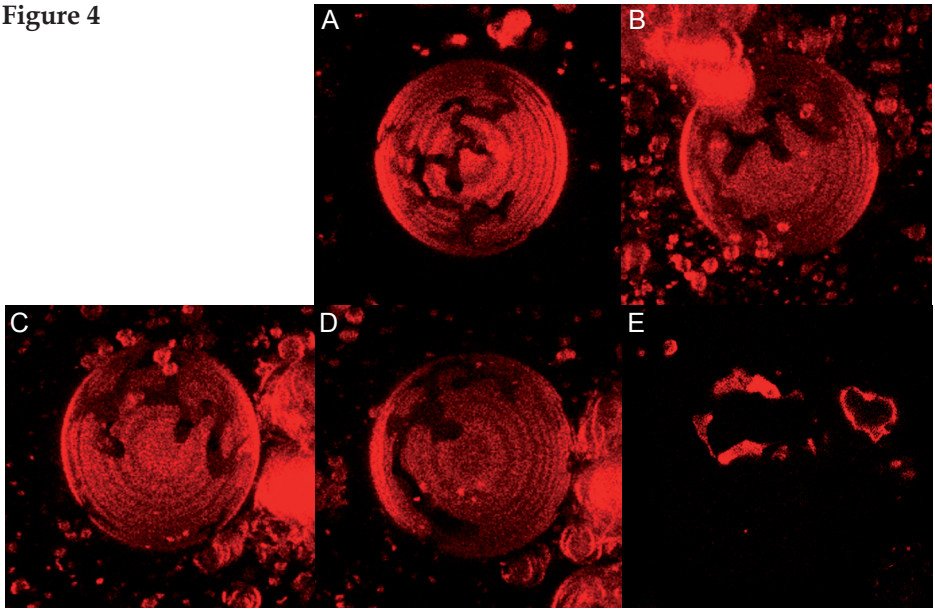

Figure 5

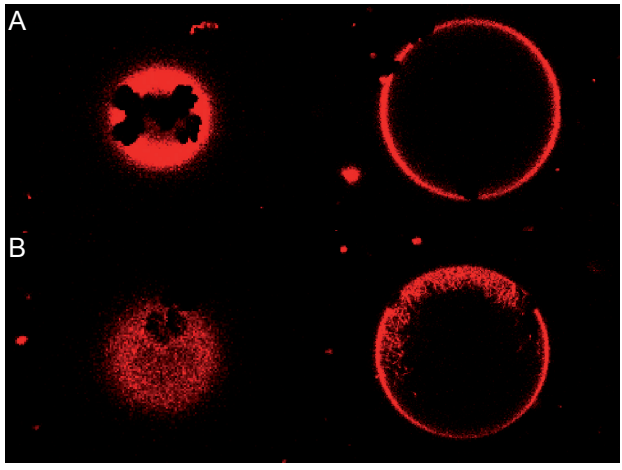

Figure 6

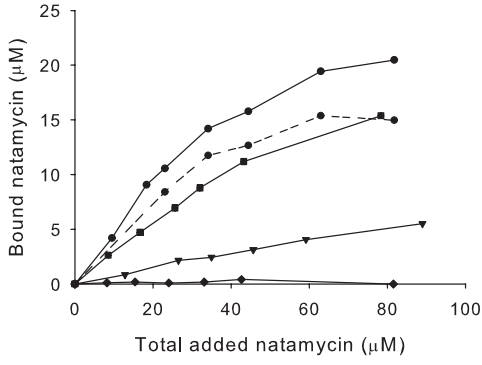

Figure 4. Fluorescence imaging of the effect of natamycin on sphingolipid-ergosterol domains in a GUV. The confocal fluorescent images were formed by the stacking of images to form an overview of the GUV before (A) and after addition of 2 nmol (B), 3 nmol (C) and 4 nmol (D) natamycin. Equatorial image of the GUV after addition of 5 nmol natamycin (E). The GUV was composed of DOPC/SM/ergosterol (45:45:10 molar ratio) and 0.05% rhodamine-DHPE (red), with approximately 0.5 nmol ergosterol present in the sample.

Figure 5. Fluorescence imaging of the effect of methyl-β-cyclodextrin on sphingolipid-ergosterol domains in a GUV. Images display one confocal plane of the bottom (left) and equatorial (right) sections of a GUV before (A) and after (B) addition of 16.5 nmol methyl-β-cyclodextrin. The GUVs were prepared with a ratio of 45:45:10 DOPC/SM/ergosterol and 0.05% rhodamine-DHPE (red), with a maximum of 0.67 nmol of ergosterol present in the sample.

Figure 6. The interaction of natamycin with spingolipid-ergosterol domains in LUVs. Binding of natamycin to vesicles containing DOPC, SM or ergosterol in a ratio of 90:0:10 (●), 45:45:10 (■), 100:0:0 (▼) and 50:50:0 (♦). In dashed (…●), the binding of natamycin to vesicles of DOPC/ergosterol (ratio of 90:10) was corrected for binding to the control (ratio of 100:0). The assay was performed in 10 mM MES/Tris, 15 mM K_2SO_4, pH 7.0 and the vesicles had a 1.5 mM final phospholipid concentration.

Discussion

This study shows that natamycin inhibits the uptake of different important substrates over the plasma membrane of yeast, without causing leakage of these substrates. The inhibition of substrate uptake occurs at similar concentrations of natamycin as inhibition of growth and was found to be reversible suggesting that inhibition of the transport proteins involved is the key cytotoxic activity of natamycin.

The amino acid substrates studied were arginine, proline and glucose, which can be transported into yeast cells via different transporters. Under the conditions tested arginine is taken up by the specific arginine transport protein, Can1p (6). Proline can be taken up by four different proteins, Put4p, Gap1p, Agp1p and Gnp1p, which are all nitrogen regulated and should be active under the conditions tested (6). The uptake of proline by the low-affinity systems of Agp1p and Gnp1p is probably negligible in comparison to the uptake by the high affinity systems of the specific proline transporter Put4p and general amino-acid permease Gap1p (6,10,21). For glucose there are 20 genes in yeast that encode proteins similar to glucose (hexose) transporters (HXT1 to HXT17, GAL2, SNF3 and RGT2) (11). Yeast cells only express the glucose transporters appropriate for the amount of extracellular glucose available and the most metabolically relevant glucose transporters are HXT1 to HXT7 (11). While the HXT genes are transcriptionally regulated in response to glucose, the Hxt2p, Hxt6p and Hxt7p are probably responsible for glucose uptake in cells growing on low concentrations of glucose, while Hxt1p and Hxt3p take up glucose when glucose is abundant (11). Yeast grown at 2% glucose (w/v) express Hxt1p – 3p and Hxt6p/7p and these proteins are most likely responsible for most of the glucose uptake in the assay conditions (22,23). Following this line of reasoning, it is most likely that natamycin inhibited the uptake of arginine, proline and glucose in yeast through the inhibition of the transport proteins Can1p, Put4p, Gap1p, Hxt1p, Hxt2p, Hxt3p, Hxt6p and Hxt7p.

The recuperation of arginine uptake did not lead to a recovery of growth in the same time span, which was an indication that more severe functions then arginine uptake were disturbed by natamycin. The amino acid transporters in yeast belong to a unique family of permeases, while the Hxt proteins belong to the major facilitator superfamily (MFS) of transporters (11,21,24). There is a high similarity within the families of either the amino acid permeases or MFS

and therefore it is likely that natamycin may affect more family members or even more membrane transport proteins. Because natamycin had no effect on vacuolar acidification, it is unlikely that natamycin can inhibit all membrane transporters in yeast (Chapter 3). Vacuolar acidification is maintained through the V-ATPase, which is considered a primary active transporter and transports protons from the cytosol into the lumen of the vacuole via the hydrolysis of ATP (25). Since natamycin had no effect on vacuolar acidification indicates that the V-ATPase function was not disturbed by natamycin. Even if natamycin is able to inhibit more transport proteins in the plasma membrane, it is probably not enough to completely explain its antifungal activity. The fact that starved yeast cells can survive for more than three weeks in water (26), while a constant natamycin pressure gives the onset of cell death after 1 – 2 hours (unpublished observations), indicates that there has to be an additional factor accompanying natamycin toxicity than the mere starvation of yeast. Therefore, the results suggest that the mode of action of natamycin may not be exclusively based on the inhibition of transport proteins, but may entail other factors like the inhibition of other important membrane proteins or membrane processes.

How would natamycin be able to inhibit the function of the transport proteins described? There are 24 family members of amino acid transporters in yeast and their specificity ranges from very narrow, with only a few or even one amino acid such as known for Can1p or Put4p, to very broad, like the general amino acid permease Gap1p (6,21). Amino acid permeases are considered to be secondary active transporters that use the electrochemical gradient as a source of energy for the symport of protons and amino acids (27). Hxt proteins and all family members of the MFS family, transport their substrates by passive, energy-independent facilitated diffusion, with glucose moving down a concentration gradient (11). The differences in substrate specificities and transport mechanisms of the amino acid and glucose transporters, makes it likely that natamycin inhibits these proteins via a general mechanism. Since the amino acid permeases do not have any domains/motifs in common with the glucose transporters, the existence of specific binding pockets on these different transport proteins for natamycin is unlikely (11,21,24). What these transporters do have in common is their similarity in length and hydrophobicity profiles. The amino acid permeases are between 60-70 kDa and contain between 10-12 putative transmembrane (TM) domains (21,28). All MFS members have calculated masses in the range of 60-65 kDa with 12 putative TM domains (29). Therefore, it is most

likely that natamycin will affect these proteins through alterations in membrane properties instead of inhibiting in a specific way for each protein involved.

Several possibilities have to be considered what these altered membrane properties are and how they affect the transport proteins, but all start with the premise that the specific binding of ergosterol by natamycin is part of the mechanism because it is the basis for the antifungal effect (1). One possibility is that natamycin is able to affect ergosterol-enriched lipid domains in the membrane, which have been speculated to be important for the correct functioning of membrane proteins (30). We showed in a model system that natamycin is able to bind efficiently to tightly packed sphingolipid-ergosterol domains, without disrupting them. This suggests that the membrane transport inhibitory effect of natamycin is not due to a disruption of putative sphingolipid-ergosterol domains in the plasma membrane. Inhibition might be due to a change in properties of the putative domains, because it can be expected that the insertion of the hydrophobic moiety of natamycin into the sphingolipid-ergosterol domains might alter the packing and interfacial properties of these domains. Changing the sterol composition of yeast plasma membranes in ergosterol biosynthesis mutants did cause a decrease in arginine uptake in these strains, but this was concluded to be due to the mistargeting of Can1p from the plasma membrane to the vacuole and not to altered protein function because of changed domain properties (9). There is no direct evidence that the function of the transporters described are dependent on ergosterol-sphingolipid domain properties, but Can1p, Gap1p, Hxt1p and Hxt2p have been found in detergent extracted sphingolipid-ergosterol fractions from yeast membranes and localization studies indicated confinement of Can1p to subregions in the plasma membrane of yeast (9,31,32). However, using yeast plasma membrane vesicles fused with ergosterol-free phospholipid vesicles, Can1p was still functional in an estimated presence of 1% ergosterol (33). Given the uncertainty of the existence of these domains in the plasma membrane of yeast and their importance in function of the various transporters tested, the explanation that natamycin disrupts membrane transporters through influencing ergosterol-sphingolipid domains remains speculative.

Perhaps a direct antibiotic-protein interaction could explain the transport protein inhibition by natamycin. In this mechanism, natamycin would first bind to ergosterol, thus gaining entrance to the plasma membrane and then through its different oxygen functions bind to proteins and thereby inhibit their function. This hypothesis has been proposed before for other polyenes (34,35).

Disturbing the equilibrium of free sterols to sphingolipids by the binding of natamycin to ergosterol may also be responsible for the inhibition of ergosterol dependent protein functions. A recent study showed that sterols and sphingolipids function together to carry out a wide variety of functions and it was stated that cells may have a mechanism to sense the quality of their membrane sterol composition (36). Any integral or peripheral membrane protein may recognize and interact differently with either free sterols and sphingolipids over sphingolipid-sterol complexes. When natamycin binds to ergosterol, it will disturb this equilibrium possibly resulting in inhibition of protein function. In the general discussion (Chapter 5) a more comprehensive discussion of the various potential mechanisms for inhibition of protein function by natamycin will be given.

Given the similarities in properties of all polyene antibiotics, the possibility has to be considered that next to their potential membrane permeabilisation activity they all act via inhibition of membrane protein functions. An argument that supports this hypothesis is the report that nystatin affected properties of the arginine and glucose transporters in yeast plasma membrane vesicles (33). An analogue of amphotericin B, amphotericin B methyl ester was shown to inhibit the replication, assembly and release of HIV-1 by interfering with the ion channel VPU (35,37). However, membrane permeabilisation by these polyene antibiotics does complicate the interpretation of these types of results because these polyenes unlike natamycin will collapse vital ion or substrate gradients. This theory could be tested by studying the effects of polyene antibiotics on a plasma membrane protein that does not depend on these factors, like integral membrane proteins that act as enzymes without being dependent on membrane barrier function.

Acknowledgments

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Addendum to Chapter 4

Synthesis and properties of NBD labelled natamycin

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Abstract

The antifungal polyene antibiotic natamycin has been used for many years in medical treatments, as well as food preservation. The activity of natamycin is based on the specific binding of ergosterol in the membrane of fungi. In contrast to other family members of the polyene antibiotics, natamycin does not disrupt the membrane barrier. The mode of action of natamycin is most likely based on the disturbance of different ergosterol dependent membrane protein functions. This was indicated from the observation that natamycin blocked substrate uptake over the plasma membrane and the early priming phase of vacuole fusion in yeast (Chapter 3 and 4). This raises the question which compartments of fungal cells are accessible to natamycin? To address this question, natamycin was labeled at the amine function of the mycosamine group with the fluorescent molecule NBD. The plasma membranes of yeast were stained after incubation with natamycin-NBD and faint internal membrane structures were observed as well. Germinating spores, in which natamycin was shown to block endocytosis (Van Leeuwen, M. R. et al. (2009) J. Appl. Microbiol. 106, 1908), showed similar results. This indicates that even though natamycin is also able to inhibit endocytosis, a small portion of natamycin-NBD is able to enter the spores via this pathway. The intensity of staining of the internal membrane structures was increased in time after washing the spores, which indicates that the block in endocytosis can be reversed. The antifungal activity of natamycin-NBD was found to be significantly lower in comparison to natamycin. Even though the membrane affinity of natamycin-NBD was increased, the specificity in binding ergosterol was lost, which explains the loss in antifungal activity. To test if the loss in ergosterol binding was due to the NBD group itself or the block of the amine function, the amine group of natamycin was derivatized with small methyl groups. The decrease in antifungal activity of natamycin substituted with methyl groups was substantially reduced as compared to NBD, indicating that the loss in interaction of natamycin-NBD with ergosterol is largely due to the membrane active property of the NBD group. These results emphasize the importance of the specific interaction between natamycin and ergosterol for the efficiency of the antifungal activity of natamycin.

Introduction

Polyene antibiotics exert their anti-fungal activity via an interaction with the main sterol, ergosterol, in the plasma membrane of fungi. Natamycin, a family member of the polyenes produced by *Streptomyces natalensis,* is widely utilized in food preservation and pharmacotherapy. Although it has been the understanding that this antibiotic would act by permeabilizing the plasma membrane, similar to other polyenes (like amphotericin B, nystatin and filipin), natamycin is unable to cause leakage in model membranes nor in yeast cells (1). The growth of yeast is effectively inhibited by natamycin via a specific interaction with ergosterol and the antifungal activity was shown to be dependent on this interaction (1). The antifungal activity of natamycin involves the inhibition of plasma membrane uptake processes and the fusion of vacuoles in yeast (Chapter 3 and 4). In addition, natamycin was shown to inhibit endocytosis in germinating spores of *Penicillium discolor* (*P. discolor*) (2). This raises the question which compartments of fungal cells and spores are accessible to natamycin. Fluorescence microscopy is a commonly used technique to study the cellular location of a given compound. Natamycin has an intrinsic fluorescence, but the excitation wavelength is too low to be observed by conventional fluorescence microscopy. Therefore, natamycin was fluorescently labeled by attaching a NBD moiety to the amine function on the mycosamine group. The cellular location of natamycin-NBD in cells of *Saccharomyces cerevisiae* (*S. cerevisiae*) and germinating spores of *P. discolor* was studied and the consequences of labeling of natamycin for its antifungal activity and specific binding to ergosterol in model membrane systems were analyzed. The results demonstrate that natamycin-NBD localizes preferentially to the plasma membrane with some staining of internal membrane structures. Labeling natamycin with NBD causes a large reduction in antifungal activity, which was paralleled by a loss in the specific interaction with ergosterol, underscoring the importance of the natamycin-ergosterol interaction for the antifungal activity of natamycin.

Experimental procedures

Chemicals and general experimental procedures – 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Ergosterol was obtained from Larodam AB (Sweden). Lipids were dissolved in chloroform. Phospholipid concentrations were determined by phosphate analysis according to Rouser (3).

The polyene antibiotic natamycin was obtained from Sigma Chemical (St. Louis, MO) and dissolved in 85 : 15 DMSO to H_2O (v/v). The concentration of natamycin was determined by UV absorption on a Perkin Elmer UV / Vis Spectrometer (Lambda 18). The molar extinction coefficient and corresponding wavelengths in methanol for natamycin was 7.6 x 10^4 M^{-1} cm^{-1} (318 nm). MS measurements were carries out on an Applied Biosystems Voyager DE-STR MALDI-TOF MS using 9-nitroanthracene (Sigma Chemical) as matrix.

4-Chloro-7-nitrobenzofurazan (NBD-Cl) was obtained from BioChemika Fluka (Switzerland). Formaldehyde solution (37 wt. % in H_2O), *N,N*-diisopropylethylamine (DIPEA), sodium cyanoborohydride ($NaCNBH_3$), N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) and poly-L-lysine were obtained from Sigma Chemical (St. Louis, MO). Thin layer chromatography (TLC) was performed using high performance TLC plates consisting of Silica gel 60 (Merck, Germany).

Strains and growth conditions – The *Saccharomyces cerevisiae* (*S. cerevisiae*) strain used in this study was RH448. The cells were grown at 30ºC on rich [10 g/l yeast extract, 20 g/l Bacto-peptone and 20 g/l dextrose (YPD)] medium. Minimum inhibitory concentrations (MICs) were performed in YPD as described in reference (1). The amount of methanol used to dissolve natamycin-NBD, gave no inhibition of growth of the yeast strain. *Penicillium discolor* (*P. discolor*) CBS 112557 was grown on Malt Extract Agar (MEA, (4)) at 25ºC. Conidia of 10 - 12 days old colonies were harvested in cold ACES buffer (10 mM ACES, 0.02% Tween-80, pH 6.8), as described in reference (5).

Preparation and purification of natamycin derivatives – NBD-labeled natamycin was prepared essentially based on (6,7), with the adaptations described below. 200 mg natamycin (1 equivalent) was dissolved in methanol to a final concentration of 1 mg/ml. 300 mg of NBD-Cl (5 equivalent) and 0.5 ml DIPEA (10 equivalent) were added to the solution, which was stirred at room temperature and the reaction was followed in time by HPLC. After completion of the reaction, methanol of the reaction mixture was evaporated using a rotary evaporator and the dry mixture was stored at -20ºC. The mixture was purified by dissolving it in 50% methanol in water and applying it onto a C18 HPLC column (Supelco) eluting with a linear gradient from 50% methanol in water to 100% methanol. Elution of the products and reagents were monitored at 304 nm. The identity of natamycin-NBD was confirmed using mass-spectroscopy; MALDI-TOF (m/z): $[M + Na]^+$ calculated for $C_{39}H_{47}N_4O_{16}$: 850.8 found: 849.6. The yield of the purified compounds was estimated at 30%.

Natamycin was labeled with a methyl group by reductive methylation based on references (8,9), except that the reaction was performed in methanol instead of buffer. In brief, natamycin was dissolved in methanol up to 1 mg/ml, followed by addition of formaldehyde and $NaCNBH_3$ (dissolved in methanol). The final proportions in the reaction mixture were natamycin/formaldehyde/$NaCNBH_3$ of 1:10:1. The reaction was stirred at room temperature and dried by using a rotary evaporator. The solid reaction mixture was stored at -20°C. The reaction products were purified by column chromatography using silica gel (J.T. Baker) and a solvent system of acetonitrile : H_2O of 10:1 to remove breakdown products. Elution of the products and reagents were monitored by TLC using methanol/water (10:1) and the spots were visualized by iodine vapor or spraying with sulfuric acid and heating the TLC plate. After elution of impurities, the solvent system was changed to methanol : H_2O (5:1) to separate natamycin and labeled natamycin. Products were pooled together and dried using sequentially a rotary evaporator and lyophiliser. The identity of labeled natamycin was confirmed using mass-spectroscopy. Natamycin-CH_3: MALDI-TOF (m/z): $[M + Na]^+$ calculated for $C_{34}H_{49}NO_{13}$: 702.8 found: 701.1. Natamycin-$(CH_3)_2$: MALDI-TOF (m/z): $[M + Na]^+$ calculated for $C_{35}H_{51}NO_{13}$: 716.8 found: 715.6. The concentration of the natamycin derivative was determined by UV absorption using the molar extinction coefficients of natamycin.

Incubating fungi with natamycin-NBD – A full grown culture of *S. cerevisiae* was incubated with 100 µM of natamycin-NBD in YPD for approximately one hour at 30°C, after which the cells were washed and resuspended in YPD. 200 µl of the culture was transferred into a well of an 8-well coverglass chamber (Lab-Tek chambered coverglass, Nunc) and examined using a confocal fluorescence microscope.

Spores of *P. discolor* were diluted in Malt Extract Broth (MEB) and 100 µl was placed on poly-L-lysine coated glass coverslips as described in (5). After 6 hours of germination at 25°C, the MEB was removed and the spores were incubated with 100 µM of natamycin-NBD in MEB for approximately one hour at room temperature. The spores were washed and supplemented with MEB and either examined immediately or after 15 min. using a fluorescence microscope.

(Confocal) fluorescence microscopy – Images of *S. cerevisiae* cells incubated with natamycin-NBD were acquired with a Nikon Eclipse TE2000U inverted microscope, equipped with C1 confocal laser scanning unit and CFI Plan Fluor 60x objective (Nikon N.A 1,4). The green fluorescence signal was acquired using

excitation (488 nm line from a He-Ne laser) and detection (emission bandpass filters 515/30). Images were generated and analyzed using software of EZ-C1 (Nikon) and Image J (NIH, USA). The images of spores of *P. discolor* treated with natamycin-NBD were acquired by automatic exposure at a magnification of 100 x 2.0 with a Zeiss Axioplan II microscope, equipped with a Plan-ApoChromat 100×/1.4 oil objective and a Zeiss filterset #09 (450-490, FT510, LP520). Images were captured with a Zeiss AxioCam MRc digital camera and a shutter system (Ludl Electronic Products Ltd., USA) run by Zeiss AxioVision 4 software.

Binding assay using model membranes – Large unilamellar vesicles (LUVs) with a mean diameter of 200 nm of DOPC with or without 10 mol % of ergosterol or cholesterol were prepared as described before (1). The centrifugation based binding assay was carried out as described in reference (1). In short, LUVs (2 mM final phospholipid concentration) were incubated with natamycin-NBD (varying from 0 to 0.1 mM concentrations) for 1 hour in an Eppendorf incubator (22°C, 650 rpm), with a maximum of 15% methanol present. 1 ml of the mixture was centrifuged in a TLA 120.2 rotor in a Beckman Ultracentrifuge (TL-100) for 1,5 hrs at 100 krpm and 20 °C. The amount of antibiotic before centrifugation and in the supernatant and pellet was determined by UV absorption after 7 times dilution in methanol followed by centrifugation to remove any precipitated salts. Under these conditions less than 3% of the phospholipids remained in the supernatant. Natamycin-NBD was not pelleted in the absence of lipid below a concentration of 100 μM.

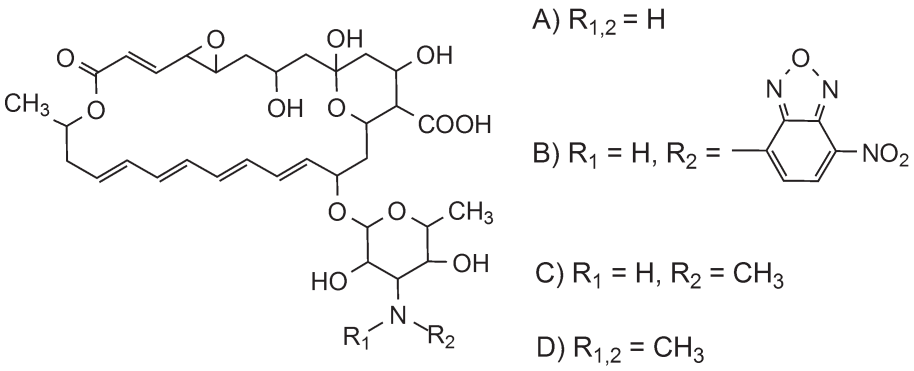

Figure 1. Structures of natamycin and several derivatives prepared in this study. (A) natamycin, (B) natamycin-NBD, (C) natamycin-CH_3 and (D) natamycin-$(CH_3)_2$.

Results

Derivatization of natamycin with NBD – To be able to determine if natamycin will enter fungal cells, natamycin was labeled with the fluorescent dye NBD (for structures see Figure 1). Natamycin-NBD was prepared by covalently attaching NBD to the amine of natamycin, using a reaction mixture of natamycin, NBD-Cl and DIPEA in methanol. After testing different ratios of the reagents in small scale reactions to gain the optimal reaction conditions, the ratio of natamycin : NBD-Cl : DIPEA of 1:5:10 was found to be optimal and used for a large scale reaction (200 mg natamycin). The reaction mixture was purified using HPLC and a typical elution pattern is given in Figure 2A. The injection peak most likely contained breakdown products and was followed by unreacted NBD-Cl, natamycin-NBD and unreacted natamycin as confirmed by TLC and/or MS. The unreacted natamycin, as well as free NBD-Cl were completely eliminated during the purification by HPLC. However, drying the fractions by rotary evaporator caused a decomposition of the compound, observed by a decrease in product and increase in unidentified peaks in the HPLC chromatogram (Figure 2B). This decomposition could be due to heating

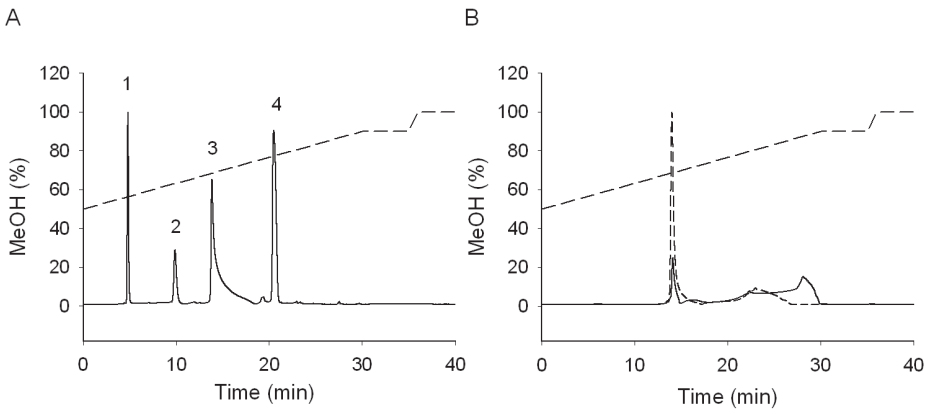

Figure 2. HPLC chromatograms of the natamycin-NBD reaction mixtures. (A) HPLC elution pattern of the crude reaction mixture (–). The injection peak (1) is followed by NBD-chloride (2), NBD-natamycin (3) and natamycin (4). (B) HPLC chromatograms of the pooled natamycin-NBD fractions (- - -) and the same fraction after drying by rotary evaporator (–). The mixtures were purified on a C18 HPLC column (Supelco), using a linear MeOH / H_2O gradient (– – –, with MeOH in %). Elution of the products and reagents were monitored at 304 nm and the identities of the products were confirmed using TLC or MS.

or light exposure and further removal of solvents were performed by paying careful attention to these factors. However, natamycin is known to be unstable in methanol and this was also observed for natamycin-NBD after storage overnight at 4ºC in methanol (estimated breakdown between 23 - 53 % by HPLC) (10). To overcome this problem in all experiments, natamycin-NBD was freshly purified by HPLC and immediately used in the experiments. The concentration of natamycin-NBD was determined by UV absorption.

The absorption spectra of pure natamycin, NBD-chloride and natamycin-NBD are given in Figure 3. The absorption spectrum of purified natamycin-NBD presented the characteristic absorption bands of the tetraene from natamycin and the typical absorption band of the NBD chromophore in the 250–350 and 400–550 nm regions, respectively (Figure 3) (1,10-12). The absorption ratios of the characteristic peaks of natamycin at 318/303 and 290/303 nm respectively were determined to be the same for natamycin and purified natamycin-NBD and indicated that the labeling did not affect the tetraene part of natamycin. By using the molar extinction coefficients of natamycin and the NBD chromophore in methanol (also used for nystatin-NBD (12)), the concentration of natamycin-NBD was determined spectrophotometrically ($\varepsilon_{290nm} = 5.3 \times 10^4 M^{-1} cm^{-1}$, $\varepsilon_{304\ nm} = 8.3 \times 10^4\ M^{-1}\ cm^{-1}$, $\varepsilon_{318\ nm} = 7.6 \times 10^4\ M^{-1}\ cm^{-1}$ for natamycin and $\varepsilon_{467\ nm} = 2.3 \times 10^4\ M^{-1}\ cm^{-1}$ for the NBD chromophore) (1,12,13). In addition, the 1:1 labeling stoichiometry of the antibiotic was confirmed in this way, which indicated that this method could be used to determine the concentration of natamycin-NBD.

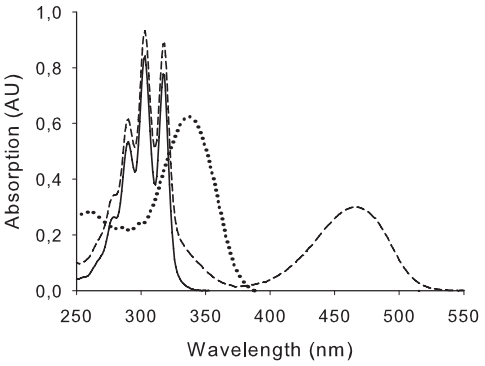

Figure 3. Absorption spectra comparing derivatized with underivatized natamycin. Normalized spectra of natamycin (—), NBD-chloride (···) and natamycin-NBD (- - -) were measured in methanol.

Incubating fungi with natamycin-NBD – Incubation of natamycin-NBD with a culture of *S. cerevisiae* resulted in clear labeling of the plasma membrane (Figure 4A). Internal membrane structures were faintly distinguished as well. A small proportion of the cells showed a complete fluorescent staining, most likely because they are dead cells in which natamycin-NBD can enter freely. These results show that natamycin-NBD interacts strongly with the plasma membrane of yeast cells and to some extent can enter the cells to stain internal membrane structures.

Because endocytosis was shown to be inhibited in germinating spores of the fungus *P. discolor*, the localization of natamycin-NBD in these spores was examined as well (2). Spores incubated with natamycin-NBD examined immediately after washing the spores, showed intensively stained plasma membranes (Figure 4B I and II). Very faint internal membrane structures were observed as well (Figure 4B II). This indicates that natamycin-NBD also in spores is able to bind to the plasma membrane and that a low amount of natamycin-NBD is able to enter the spores.

To gain more information on natamycin-NBD entrance into fungal spores, the spores were incubated in medium for 15 min. after washing (Figure 4B III and IV). The plasma membranes of the spores were still stained intensively with natamycin-NBD, compared to spores examined immediately after washing, but a more intense staining of internal structures was observed. These results indicate that after washing, some natamycin-NBD stays in the plasma membrane and the entrance of natamycin-NBD into fungal spores can be increased in time.

Sensitivity of yeast towards natamycin-NBD – An MIC assay was performed to determine the sensitivity of yeast towards natamycin-NBD (Table 1). The MIC value of natamycin is given as a reference (1). *S. cerevisiae* was almost 26 times less sensitive towards natamycin-NBD compared to unlabeled natamycin. These results show that natamycin-NBD has a largely reduced antifungal activity in comparison to natamycin.

Table 1. The minimum concentration of natamycin and its derivatives needed to inhibit the growth of *S. cerevisiae*.

	MIC (µM)
Natamycin	1.7
Natamycin-NBD	44
Natamycin-$(CH_3)_{n=1/2}$	6.3

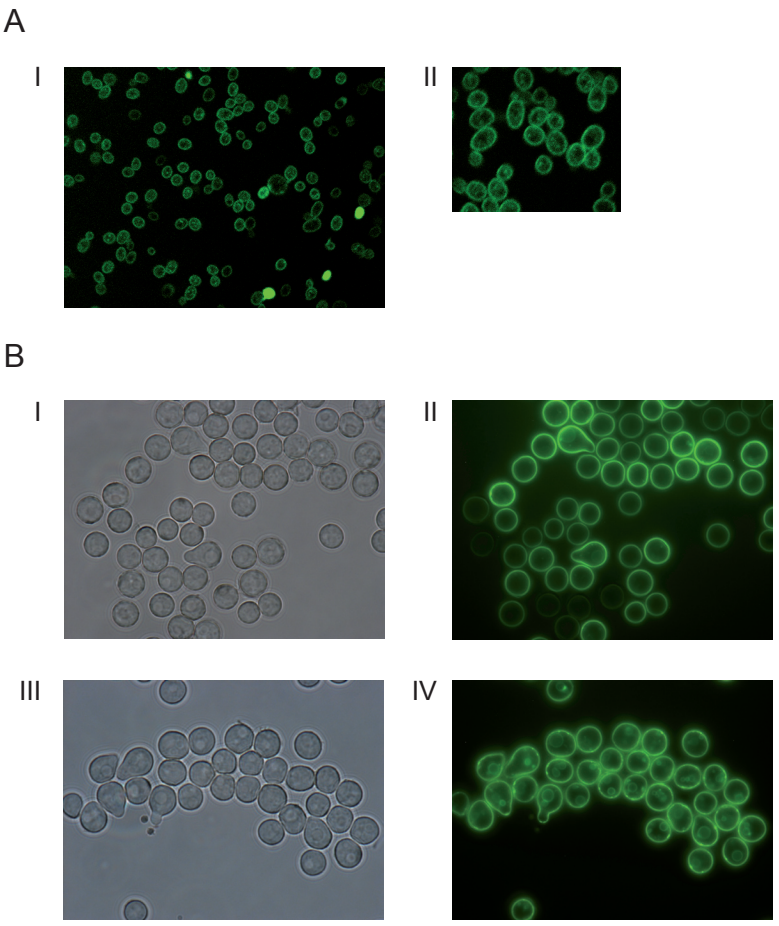

Figure 4. Fluorescent natamycin-NBD staining of fungi. (A) Confocal fluorescence microscope images of *S. cerevisiae* cells treated with natamycin-NBD. Cells were incubated with 100 µM natamycin-NBD for one hour, after which cells were spun down and resuspended in YPD. An overview (Panel I) and enlargement (Panel II) of the stained yeast with natamycin-NBD are given. (B) Images of labeled *P. discolor* spores with natamycin-NBD. Germinating spores incubated with 100 µM natamycin-NBD for one hour, were washed and examined by fluorescence microscopy, either immediately (I / II) or after 15 min (III / IV). The left panels (I and III) display the bright field and the right panels (II and IV) the fluorescent images.

Binding of natamycin-NBD to ergosterol – Because the antifungal activity of natamycin is related to its specific interaction with ergosterol, the loss in antifungal activity of natamycin-NBD could indicate a loss in the interaction between natamycin-NBD and ergosterol. Binding assays to determine the specificity of natamycin-NBD towards ergosterol were performed with model membranes composed of 10% ergosterol, cholesterol or no sterols in DOPC (Figure 5A).

Natamycin-NBD binds with similar affinity to model membranes whether they contain ergosterol, cholesterol or no sterols at all (Figure 5A). In contrast, natamycin binds strongly to ergosterol containing model membranes, but hardly to model membranes without sterols (Figure 5B, adapted from (1)). These results show that derivatizing the amine group with NBD increases the membrane affinity of natamycin, but causes a loss in ergosterol dependency of binding.

Importance of an underivatized mycosamine group for the toxicity of natamycin – To determine whether the NBD group itself or the derivatization of the amine of natamycin was responsible for the loss in natamycin-NBD activity, natamycin was derivatized with relatively small methyl groups (Figure 1D). During the reaction, products with either one or two times methylated amine groups were formed.

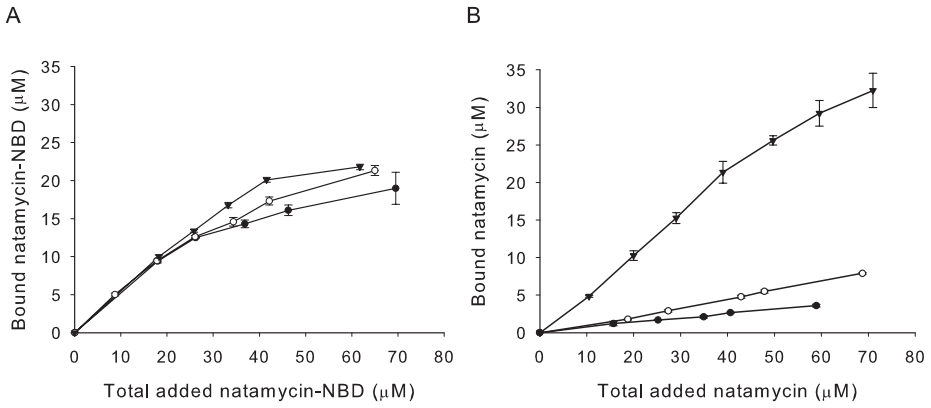

Figure 5. Effect of labeling natamycin with NBD on the sterol dependency of the natamycin model membrane interaction. Binding of natamycin-NBD (A) or natamycin (B) to vesicles containing 10% ergosterol (▼), 10% cholesterol (○) or no sterols (●). The binding data for natamycin were taken from (1). The assay was performed in 10 mM MES / Tris, 15 mM K_2SO_4, pH 7.0 and the vesicles had a 2 mM final phospholipid concentration.

We were unable to purify the individual reaction products; however, we were able to separate them from un-reacted natamycin as determined by TLC (Figure 6). One reaction product was isolated containing natamycin with one methylated amine group (for structure see Figure 1C) together with unidentified (breakdown) products. Another product contained natamycin with both one and two times methylated amine groups (a mixture of the structures of Figure 1C and D).

The rest of the study was performed with this product, because it contained natamycin-$(CH_3)_2$ as the major component (Figure 6) and had no significant amount of breakdown products. The absorption spectrum of the product was almost identical to the spectrum of natamycin (data not shown) The absorption ratios of the peaks, respectively at 318/303 and 290/303 nm were determined to be the same for natamycin and the natamycin-$(CH_3)_2$ mixture, demonstrating that the derivatization reaction did not affect the polyene part of the molecule. The sensitivity of the yeast *S. cerevisiae* towards the mixture containing natamycin with one and two methylated amine groups was approximately 3.7 times lower compared to natamycin (Table 1). This indicates that the amine is functionality important for the antifungal activity of natamycin. However, the results also indicate that the high loss in activity of natamycin-NBD is most likely because of the size or nature of the NBD group and not simply because of the derivatization of the amine on natamycin.

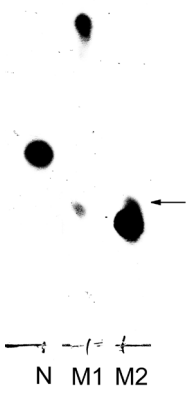

Figure 6. TLC of methyl labeled natamycin reaction products. Natamycin (N) was spotted together with a mixture (M1) containing natamycin-CH_3 and breakdown products and a mixture (M2) of natamycin-CH_3 and natamycin-$(CH_3)_2$. The arrow indicates natamycin-CH_3. The TLC was performed with a solvent system of methanol/water (10:1) and the spots were visualized by spraying with sulfuric acid and heating the TLC plate. The identity of the products in the mixtures was confirmed by MS.

Discussion

The main goal of this study was to synthesize a natamycin derivative that could be used to study the cellular localization of natamycin when it is added to fungi. To this end, the fluorescent dye NBD was covalently attached to the amine group of natamycin, resulting in natamycin-NBD (Figure 1). NBD was chosen as the fluorescent group, because it has a relatively small size and therefore the influence on the activity of natamycin was expected to be minimal.

Natamycin-NBD was observed to stain the plasma membrane of yeast and faint internal membrane structures were observed as well. This indicates that natamycin-NBD binds to the plasma membrane of yeast and that a small portion of natamycin-NBD can enter the cell and stain internal membrane structures. These results are similar to a study performed with a fluorescein labeled amphotericin B derivative, where no significant internalization of the derivative was observed in yeast cells (14). Because of the amphiphatic nature of natamycin and the inability to permeabilise membranes (1,2), it seems unlikely that natamycin will be able to traverse the membrane. In addition, amphotericin B and nystatin were indicated to be unable to cross the bilayer (15,16).Thus, it is unlikely that NBD labeled natamycin will be able to cross the membrane. If natamycin-NBD only binds to the outer leaflet of a bilayer, it is most likely that if it does enter cells, it will do so via endocytic vesicles.

Similar results were obtained with the germinating spores of *P. discolor,* that also showed intense staining of the plasma membrane and a faint staining of internal membrane structures. The intensity of the labeling of the internal structures of the germinating spores with natamycin-NBD could be increased in time after washing the spores. In this respect, the behavior of natamycin-NBD is similar to the behavior in yeast cells.

We expect a similar behavior of the membrane marker FM4-64, which is believed to be unable to cross membranes because of its amphiphilic nature and to be anchored in the outer leaflet of the bilayer (17,18). FM4-64 is reported to enter cells primarily by endocytic vesicles from the plasma membrane and to reach vacuoles as well (19). This probe was used to stain the plasma membrane and internal membrane structures of germinating *P. discolor* spores (2). The membrane structures stained by natamycin-NBD in the spores are very similar to the structures stained by FM4-64 (2). This indicates that the internal membrane structures stained by natamycin-NBD are most likely endocytic

vesicles and vacuoles. FM4-64 was used in spores to show the inhibition of natamycin on endocytosis as well (2). In the absence of natamycin, FM4-64 stained the plasma membrane and internal membrane structures with approximately the same intensity after a one hour incubation (2). The low intensity of stained internal membrane structures by natamycin-NBD, as compared to staining with FM4-64, could relate to this finding and suggests that natamycin-NBD inhibits endocytosis, an inhibition that can be relieved after washing the spores. If a portion of natamycin-NBD is able to reach vacuoles in spores, it might also reach vacuoles in yeast cells, strengthened by the faint staining of internal membrane structures by natamycin-NBD in yeast. These observations could explain the vacuole fusion inhibition of natamycin in yeast cells (Chapter 3). However, caution has to be taken in the interpretation of the results obtained with natamycin-NBD, because of the large decrease in activity compared to natamycin.

The decrease in antifungal activity of natamycin-NBD was accompanied by a higher affinity of natamycin-NBD towards membranes with a loss in specificity to ergosterol, as demonstrated by model membrane binding studies. The high affinity of natamycin-NBD towards membranes was also inferred from the localization studies, where even after washing the fungal species retained the fluorescent staining of the plasma membrane by natamycin-NBD. The loss in interaction between natamycin-NBD and ergosterol could be due to the nature of the NBD group or to the derivatization of the amine of natamycin. Derivitizing the amine of natamycin with smaller methyl groups does lead to a reduced sensitivity of yeast. However, this decrease in sensitivity is much smaller than that observed for coupling NBD to natamycin. The amine group, or more specifically a positively charged nitrogen atom, has been implicated to be necessary for the activity of other polyene antibiotics (20-22) (and reviewed in (23,24)). It has been speculated that the amine group on polyenes may be important for forming hydrogen bonds with the hydroxyl group on sterols (21). Derivatizing natamycin with methyl groups could cause a steric hindrance and reduction in hydrogen bond formation, which could explain the decrease in activity of this derivative. Given the larger decrease in activity observed for natamcyin-NBD, it is more likely that the NBD group itself disturbed the interaction between ergosterol and natamycin or overruled the membrane affinity. When a NBD group attached to the end of a short acyl chain of a phospholipid is incorporated into a bilayer, the membrane active property of NBD causes a looping back of the acyl chain towards the membrane surface (25-27). This

membrane active property of NBD could explain the loss in ergosterol dependency of binding and enhanced membrane affinity observed for natamycin-NBD.

In conclusion, although interesting results were obtained with natamycin-NBD in terms of the localization of this molecule in fungal species, caution has to be taken into the extrapolation of these results to the localization of natamycin, due to the reduced antifungal activity and ergosterol affinity of natamycin-NBD in comparison to natamycin. Yet this study has underlined the importance of the interaction between natamycin and ergosterol for the efficiency in the antifungal activity of natamycin.

Acknowledgments

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Chapter 5

Summarizing discussion

The work presented in this thesis focuses on the mechanism by which the polyene antibiotic natamycin is able to exert its antifungal effect. Our studies have shown that the mode of action of natamycin is related to its ability to block fungal growth via binding specifically to ergosterol, without causing membrane permeabilisation (Chapter 2). Through its specific interaction with ergosterol, natamycin was shown to inhibit vacuole fusion. The cellular morphology of intact yeast cells showed fragmented vacuoles after incubation with natamycin, underlining the ability of natamycin to act on ergosterol dependent fusion processes in yeast cells. The inhibition of natamycin on vacuole fusion took place at the priming phase, which indicated an inhibition of ergosterol dependent protein function (Chapter 3). This was further examined using substrate uptake assays of plasma membrane transport proteins in yeast. Natamycin was shown to inhibit the transport of different substrates, suggesting that it is able to inhibit different plasma membrane transport proteins in fungi. Inhibition of transport by natamycin was most likely not related to a reorganization of sphingolipid-sterol domains, but most probably is a direct result of the disturbance of ergosterol dependent protein functions (Chapter 4).

In the mechanism of action of natamycin three aspects can be distinguished; (**1**) its highly specific interaction with ergosterol, (**2**) the nature of the putative complex formed between ergosterol and natamycin in the membrane, (**3**) the effect of natamycin or the natamycin-ergosterol membrane complex on fungal cells in relation to the ability of natamycin to inhibit membrane transport protein function. As discussed in Chapter 4, the mode of action of natamycin may not be exclusively based on the inhibition of transport proteins, but may entail other factors like the inhibition of other important membrane proteins or membrane processes. These aspects will be reviewed and discussed. Other topics that will be reviewed in this discussion are the effect of natamycin on protein levels in general, the mechanism of action of natamycin in relation to other polyene antibiotics and possible improvements in antifungal formulations.

Natamycin-ergosterol interaction

Natamycin was shown to interact specifically with ergosterol and the most important chemical features of the sterol for this interaction were proven to be the double bonds in the B-ring. The loss of natamycin binding to sterols was higher when the double bond at the C-7 position was lost compared to the C-5 position of the sterol B-ring, demonstrating that the double bond at the C-7 position is most

important for the interaction with natamycin. Changes to the side chain of the sterol molecule did not seem to affect the affinity of natamycin significantly (Chapter 2). These findings explained the higher affinity of natamycin for the fungal sterol ergosterol compared to the animal sterol cholesterol (structures are given in Figure 1), which is a basis for the safe use of natamycin in various applications.

As discussed in Chapter 2, the tightly constrained planar molecular topology of natamycin and the conformation of the B-ring in ergosterol are likely responsible for the efficient interaction between natamycin and ergosterol (1,2). A docking simulation between natamycin and ergosterol was performed to get insight into the natamycin-ergosterol interaction (Figure 2A)[#].

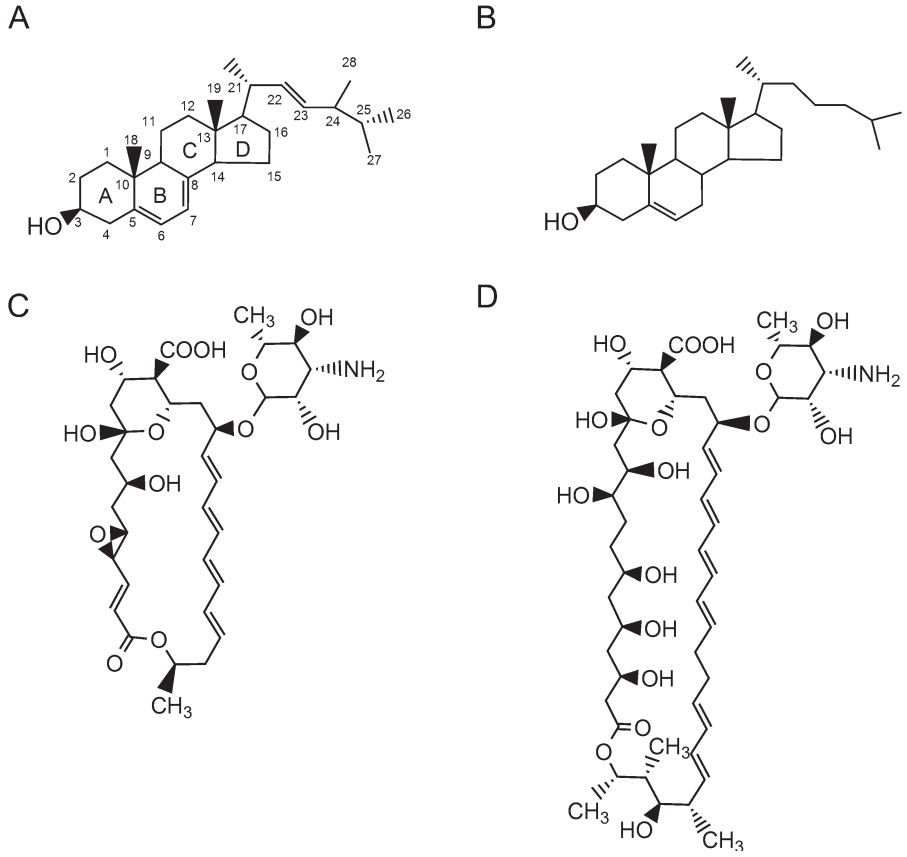

Figure 1. Molecular structures of sterols and polyene antibiotics. The structure of ergosterol (A) and cholesterol (B) is given together with natamycin (C) and nystatin (D). The assignment of the structure of ergosterol is displayed (A).

[#]A.M.J.J. Bonvin, unpublished results.

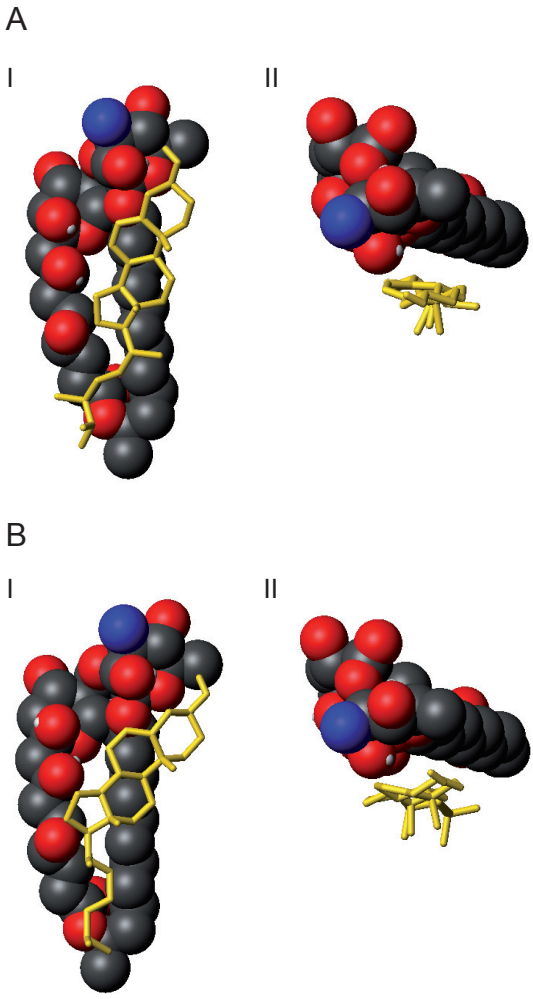

Figure 2. Hypothetical molecular model of the natamycin-sterol interactions. Interaction between natamycin and ergosterol (A) or cholesterol (B) viewed from the front (panel I) or top (panel II). A space filling representation of natamycin is given, together with a licorice representation of the sterols (gold). The models were created using HADDOCK based on the crystal structures given in refs (1,3,9) and manipulated using the program MolMol (ETH Zurich).

The docking was performed initially in water and refined in DMSO using High Ambiguity Driven biomolecular DOCKing (HADDOCK), based on the NMR solution structure of natamycin in methanol and the crystal structures of ergosterol in a protein complex (1,3). The space filling representation of natamycin is shown together with ergosterol in gold (Figure 2A). The representation of natamycin shows its hydrophilic side with three hydroxyl groups, epoxide functionality, keto group and the ester linkage aligned along the left side (panel I of Figure 2A, for the molecular structure see Figure 1). The hydrophobic side of the ring structure, the more rigid side due to the conjugated double bond system, is on the right side of the ring structure. At the top of the ring structure, the mycosamine and carboxyl groups are located, with the carboxyl

group oriented away from the viewer. Ergosterol (in gold, Figure 2A) is positioned in front of the ring structure of natamycin and occupies the space between the polyene side chain and the hydrophilic side, with its hydroxyl group placed near the mycosamine. The long axes and mean plane of ergosterol is approximately parallel to the polyene plane of natamycin. The methyl groups on ergosterol are pointed away from natamycin, which enhances the interaction between the planar molecules. The ring structure, particularly the B and C-ring on ergosterol seem to be positioned and overlapping the polyene side of natamycin. This could explain why a more planar B-ring via the double bond at the C-7 position is important for the interaction of natamycin with ergosterol. In this manner, the molecules have the highest overlap in structures increasing the van der Waals interactions, because of their planar and rigid structures.

The hydroxyl group on ergosterol seems to be oriented towards the mycosamine of natamycin, which could be an indication for hydrogen bond formation. Derivitizing natamycin at the amine with methyl groups did reduce the antifungal activity, indicating the importance of the mycosamine of natamycin for its antifungal activity (Addendum to Chapter 4). The amine group and carboxyl group have been implicated to be important for the interaction of other polyene antibiotics with ergosterol (4-6) (and reviewed in (7,8)). It was suggested that these groups are important for the correct functioning and orientation of these polyenes into the bilayer (4,7,8). For example, filipin, which lacks the sugar moiety and carboxyl group, is able to insert into the hydrophobic core of the membrane and form large aggregates, perpendicular to the phospholipid acyl chains. This polyene antibiotic displays the least sterol specificity to elicit its mode of action (Chapter 2).

The side chain of ergosterol is the least conformational restricted part of the sterol molecule and since it does not seem to specifically interact with the more hydrophobic polyene side of natamycin, this suggests that it is probably less involved in the interaction with natamycin in comparison to the ring structure of ergosterol. This relates to the observation that changes in this side chain were of less importance for the interaction with natamycin (Chapter 2).

A similar docking simulation was formed with cholesterol, which was based on the crystal structure of cholesterol in a sterol carrier protein (Figure 2B)[#] (9). This simulation resulted in less clear results, because different energetically favorable complexes were obtained, including ones in which the hydroxyl group was

oriented towards the lactone side of natamycin. This already suggests that the structure of the cholesterol-natamycin complex is less favorable than the structure of the natamycin-ergosterol complex, in agreement with the experimental results (Chapter 2). The only simulation showing cholesterol oriented towards the mycosamine side of natamycin is shown in Figure 2B. The A-ring of cholesterol is folded slightly towards the polyene side (Figure 2B, panel I and II). However, no insights into the structural basis of the specificity of natamycin for ergosterol in comparison to cholesterol can be obtained via this docking simulation.

As described in Chapter 2 and illustrated in Figure 2A, natamycin is able to bind to ergosterol and will presumably adapt an orientation, with its polyene plane approximately parallel to the long axes and mean plane of ergosterol in a bilayer. Similar to what has been suggested for polyene antibiotics, like amphotericin B or nystatin (10,11). The orientation of ergosterol in a bilayer has been assumed to be similar to cholesterol, which is oriented with its hydrophobic long molecular axis parallel to the lipid acyl chains and with the hydroxyl group close to the phospholipid ester carbonyl oxygen within the water/lipid interface (12-14). Recent molecular dynamics simulations and solid state NMR studies using magic-angle spinning have indicated that ergosterol might tilt slightly with respect to the bilayer normal (15,16). If natamycin will insert into a bilayer parallel to the ring system of ergosterol, this insertion will lead to the hydrophilic oxygen functions on natamycin being exposed to the hydrophobic environment of the bilayer, which is energetically unfavorable and will most likely lead to the formation of a complex consisting of several natamycin and ergosterol molecules.

Natamycin-ergosterol complex

The formation of polyene-ergosterol complexes has been described before and although the exact nature of these complexes are still under debate, the well known models are for the pore forming model (amphotericin B and nystatin) and cluster forming model (filipin) that have been described in the general introduction (Chapter 1) (10,11,17). In Chapter 2 it was shown that the sterol specificity of natamycin in model and biomembranes was more comparable to nystatin than to filipin. It was speculated that natamycin would form complexes with ergosterol similar to the pore complex formed by nystatin given the similarity in overall chemical structure in which the charged parts are believed to anchor the molecule to the *cis*-side of the membrane.

In contrast to nystatin, natamycin was shown not to permeabilise model membranes nor yeast plasma or vacuolar membranes (Chapter 2 and 3). Additionally, natamycin did not permeabilise the plasma membrane of germinating fungal conidia (18). Two possible explanations could account for the differences in membrane permeabilisation activity between natamycin and nystatin. One is that the pore in the complex of natamycin and ergosterol might be too tight to pass anything, even something as small as a proton. Secondly, the complex could be too short to span the complete bilayer. The complex between nystatin and ergosterol is not long enough to span a complete membrane either, but still this antibiotic is proposed to form single length pores through the membrane (19-21). It is presumed that when a membrane is thicker than the length of a channel, a deformation or dimpling must occur in order to accommodate a complex, which has been extensively studied for the gramicidin protein channel (22,23). It was shown that at some point, the mismatch between the membrane thickness and pore length will become too large to overcome via dimple formation, in which case gramicidin channels were no longer sustained (22,23). Also for amphotericin B dimple formation plays a role in the pore forming mechanism (24) and has been suggested for nystatin (19). Natamycin is even smaller in comparison to nystatin (head to tail, hydrophilic side; 15 versus 21 atoms, Figure 1) and perhaps dimple formation will be energetically too costly to sustain a natamycin-ergosterol complex over an entire bilayer in fungi. Additionally, sterols and sphingolipids are known to increase the bilayer thickness in for example the plasma membrane of fungi (14) and this will increase the mismatch of the bilayer with the natamycin-ergosterol complex even further. Therefore, I propose that natamycin will form a pore complex with ergosterol that spans only half the membrane and is thus non-conducting.

The insertion of nystatin into a model membrane bilayer was described by a cooperative partition model, where nystatin binds to the membrane surface, after which binding of one nystatin molecule to ergosterol would favor binding of the next one and so on, leading to the ergosterol complex, described in the general introduction (Chapter 1) (21,25). If the natamycin-ergosterol complex is similar to nystatin, the complex formation will likely occur in the same manner and is illustrated in Figure 3A. The insertion process of natamycin, where it has to change from a perpendicular to a parallel orientation with respect to ergosterol, may prove to be important in its mechanism to inhibit membrane protein function.

Nystatin is proposed to form complexes with ergosterol in a 1:1 molar ratio. The total number of molecules in the complex reported varies between 4 – 10 polyene to sterol molecules, but the combination of eight antibiotic and eight sterol molecules has been reported most often (8,10,21). In Chapter 2, natamycin was shown to have an interaction with ergosterol in a stoichiometry of 1 to 1 or 1 to 2, depending whether the sterol is available for interaction only in the outer leaflet or in both leaflets of the membrane. It was shown that amphotericin B and nystatin are unable to cross the bilayer (19,24). If the same is assumed for natamycin, it can only interact with sterols on the outer leaflet of the bilayer and although the exact nature of the complex is not known, I propose a half pore model

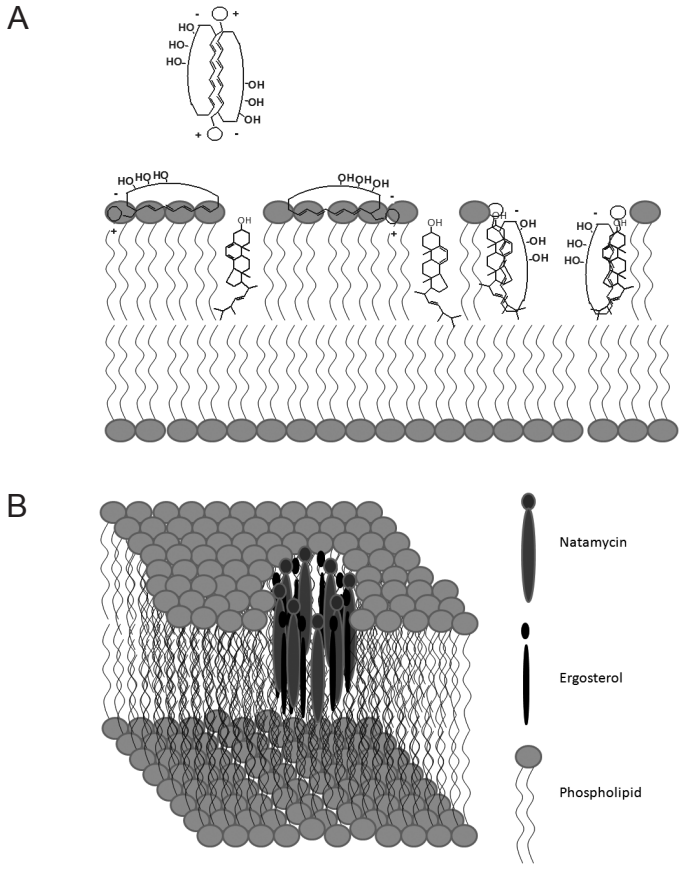

Figure 3. Hypothetical model of the natamycin-ergosterol complex formed in a bilayer. (A) Insertion process of natamycin into the bilayer. (B) Natamycin-ergosterol complex in a bilayer consisting of 8 molecules natamycin with 8 molecules ergosterol.

of natamycin containing 8 natamycin with 8 ergosterol molecules (illustrated in Figure 3B). Either the complex or the insertion of natamycin into the bilayer may be responsible for the inhibition of protein function of membrane processes by natamycin, which most likely only involves the *cis* side of the membrane.

The ability of natamycin to inhibit membrane protein function or membrane processes

Natamycin was shown to inhibit membrane vacuole fusion via perturbation of ergosterol dependent priming reactions, which preceded membrane fusion in yeast (Chapter 3). This pointed to an effect of natamycin on ergosterol dependent protein function and led to the discovery of the impaired functions of plasma membrane transport proteins by natamycin (Chapter 4). It is likely that natamycin will inhibit the functions of these transport proteins via a general mechanism, because of the differences in substrate specificities, transport mechanisms and lack in common domains/motifs of these proteins (26-28). The similarity in size and transmembrane domains may be an indication that the function of these proteins could be influenced by a change in membrane properties (28-30). If natamycin will form complexes in the outer leaflet of a bilayer as suggested in Figure 3, most likely the part of a protein that is present in this leaflet of the bilayer will be affected. Interestingly, this is the exact region of the bilayer where the interaction of transport proteins with the substrates occurs, that were discussed in Chapter 4.

It was suggested that the mode of action of natamycin may not be exclusively based on the inhibition of transport proteins, but may involve for example the inhibition of other important membrane proteins or membrane processes through the interaction with ergosterol. There are several possibilities to consider altered membrane properties and how they could affect membrane proteins. Since the antifungal activity of natamycin is based on the binding to ergosterol, the mechanism of membrane protein inhibition most likely involves this interaction. Sterols have the unique ability to increase lipid order in fluid membranes and maintain a relative fluid membrane at the same time. In addition, they are believed to play a crucial role in the organization of membranes in the form of specialized lipid domains (31-33). The higher order in membrane lipids leads to an increased bilayer thickness, which is believed to be important for protein function (14).

Theoretically natamycin may be able to influence the fluidity of the membrane by its insertion or complexation of ergosterol and thereby influence membrane protein function or inhibit membrane processes. In a model membrane study, natamycin was shown to withdraw cholesterol from its interaction with phosphatidylcholine (18:1), thereby reversing the effects of cholesterol on membrane fluidity (34). In Chapter 4, however, no changes to the membrane domains in GUVs were observed by the addition of natamycin. Even if natamycin did not dissipate ergosterol-enriched domains in a model membrane system, the binding of the hydrophobic moiety of natamycin into the tight ergosterol-sphingolipid domains may influence the function of residing proteins in these putative domains *in vivo*. The membrane transporters indicated to be inhibited by natamycin in Chapter 4 have not been described to depend on ergosterol for their function, however, other membrane proteins have been indicated to interact and depend on sterols, or sterol induced changes in bilayer thickness for their function (35-39) (reviewed in (40)). Therefore, natamycin may be able to influence proteins by complexing ergosterol and influencing membrane properties like bilayer thickness.

A recent study has shown that together, sterols and sphingolipids carry out a wide variety of functions in yeast (41). In response to changes in the structure of sterols, cells will adjust their sphingolipid composition. Changing their membrane composition nevertheless resulted in a large number of synthetic and suppression phenotypes, with for example altered functionalities of an ATP-dependent proton pump, Pdr12p and reduced TORC2 activity (41). In addition, proteins that were shown to be localized in a specific plasma membrane microdomain in yeast, showed a different dependency on the presence of sterols and sphingolipids for their localization (41). The localization study did not correlate with plasma membrane fluidity measurements, which indicated that the ordered state of the membrane was not the major determinant in the measured phenotypes (41). The complex pattern of phenotypes was suggested to result from a disturbance in lipid equilibrium, between free sterols or sphingolipids over sphingolipid-sterol complexes. It was suggested that any integral or peripheral membrane protein may recognize and interact differently with either of these lipids or sphingolipid-sterol complexes (41). A change in lipid equilibrium via the binding of natamycin to ergosterol may theoretically result in impaired protein functions.

An indication that natamycin may cause a segregation of membrane proteins was obtained from a freeze-fracture study, where natamycin was shown to produce a network of membrane structures containing particle enriched and particle free areas in fungal membranes (42). Assuming the particles are membrane proteins, this might indicate that natamycin is able to segregate proteins into specific areas in the membrane (42). This may correlate to the observation that the initial stage of vacuole fusion was inhibited by natamycin (Chapter 3), which requires the reorganization of different membrane protein complexes (43,44). The reorganization of protein complexes is also required in endocytosis, which natamycin was shown to inhibit in spores of *Penicillium discolor* (*P. discolor*) (18).

Natamycin may also have a more direct effect on protein function. As discussed in Chapter 4, this may involve a direct binding of natamycin to proteins after entering the membrane via the interaction with ergosterol. The insertion of natamycin into the bilayer will most likely occur via the interaction with ergosterol, as illustrated in Figure 3A. During its insertion, natamycin may encounter proteins with which it theoretically may bind, thereby disturbing their function. Similar mechanisms have been speculated for other polyene antibiotics as well (11,45). In addition, using the fluorescence energy transfer of nystatin to tryptophan, nystatin was shown to locate closely to proteins in the presence of ergosterol (46). In this respect, the much lower antifungal activity of natamycin-NBD was interesting to note (appendix to Chapter 4). Perhaps the membrane active property of natamycin-NBD will inhibit natamycin to insert into the membrane parallel to ergosterol. Only at very high natamycin-NBD concentrations, perhaps some molecules will be able to insert and interact with ergosterol, which could result in the observed inhibition on endocytosis.

Another more direct way in which natamycin could theoretically inhibit protein function is through binding ergosterol in ergosterol binding pockets on proteins. Although it is unlikely the mechanism in which the transporters studied in Chapter 4 were inhibited, because of the lack in common domains or motifs, ergosterol binding domains in specific fungal proteins exist that regulate sterol homeostasis and contain sterol-sensing domains (SSD) (47,48). Other proteins that can interact directly with ergosterol include sterol transport proteins, like the oxysterol-binding protein homologues, or OSH proteins, of which seven have been found in yeast (49,50). Additionally, the homologues to the human sterol transport Niemann–Pick C genes (Ncr1p, Npc2p) are believed to bind ergosterol and facilitate sterol movement from the vacuole to other organelles in

yeast (51,52). Ergosterol is taken up from the extracellular environment under anaerobic conditions by ABC transporters, like Aus1p and Pdr11p, and facilitate their movement through the cell (53,54). Theoretically, natamycin may inhibit the function of these proteins by binding to ergosterol in these binding pockets.

A summarizing representation of the mechanisms in which natamycin could hypothetically influence membrane proteins or membrane properties is given in Figure 4. As discussed, there are many possibilities in which natamycin may inhibit protein function. Some possibilities have more potential in describing the mechanism of action of natamycin compared to others. For example, if natamycin would be able to interact directly with proteins, it may be a very a-specific effect, which may involve all proteins in the plasma membrane of fungi and as discussed in Chapter 4, the V-ATPase was most likely not inhibited by natamycin. In addition, natamycin may bind to ergosterol in binding pockets on proteins, but the chances of natamycin coming across these binding sites seems relatively small in comparison with the high presence of free ergosterol molecules in the

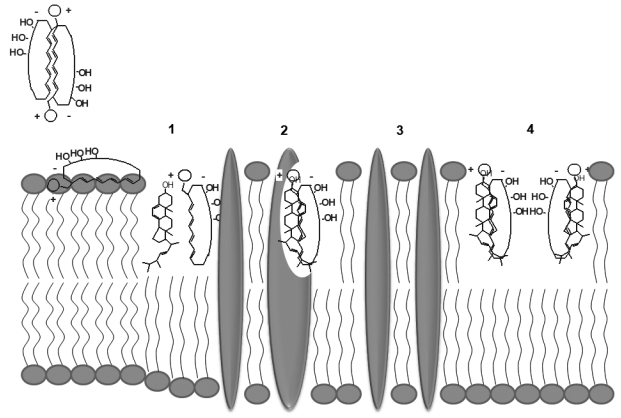

Figure 4. Representation of the hypothetical mechanisms in which natamycin can influence membrane proteins or membrane properties. Membrane protein functions can be directly affected via the insertion of natamycin and forming interactions with proteins (**1**), or binding to ergosterol in binding pockets on a protein (**2**). Alternatively the complexes formed between natamycin and ergosterol may cause a segregation of membrane proteins and interfere with membrane processes (**3**). Natamycin may be able to influence the membrane properties via influencing sphingolipid-ergosterol domains, a sphingolipid-ergosterol equilibrium or bilayer thickness (**4**).

plasma membrane. Thus, the most likely method by which natamycin is able to inhibit protein function is through its interaction with ergosterol by disturbing membrane properties, like bilayer thickness or the lipid equilibrium between ergosterol and sphingolipids, resulting perhaps in the segregation of proteins.

The effect of natamycin on protein levels

The mechanism of action of natamycin that involves the interaction with ergosterol, inhibition of membrane fusion and fission, and membrane transport proteins may have an effect on protein expression in fungi. In an unpublished study the transcriptome of *Aspergillus niger* (*A. niger*) was analyzed during germination of conidia in the absence or presence of natamycin (55). The changes in expression patterns caused by natamycin showed similarities in the effects observed for amphotericin B or nystatin on fungi in different genomic and proteomic studies (56-59). In general, the polyene antibiotics elicited changes in gene expression reflecting cell stress, membrane reconstruction, transport and cell wall integrity. Cell stress and membrane reconstruction are indications of the cells response to these antifungal agents. Membrane reconstruction was in general observed by an induction of genes involved in the ergosterol biosynthesis or lipid and fatty acid metabolism (55-57). In this respect it is interesting to note that ergosterol has been shown to play an important role in activities of phospholipid biosynthetic enzymes, which could be inhibited by amphotericin B in an *in vitro* assay (60,61). The most interesting observation is the effect of polyene antibiotics on transport proteins. For amphotericin B and nystatin it can be related to their pore forming abilities for which the cell may try to compensate. However, it cannot explain the similar effect observed in spores incubated with natamycin, which may be an indication of the response of the spore to the inhibition of membrane transporter proteins by natamycin. This hypothesis is strengthened by the observation that the spores incubated with natamycin showed reduced levels of intracellular glucose compared to spores that were not incubated with natamycin (55).

The mechanism of action of natamycin in relation to other polyene antibiotics

As discussed in Chapter 3 and 4, the ergosterol dependent mechanism of action of natamycin could perhaps be viewed as a basic mechanism of action

for all polyene antibiotics. In this hypothesis, all members of the polyene antibiotic family have the basic ability to act through the inhibition of ergosterol dependent protein functions, while some members have the additional ability to permeabilise the membrane. Arguments supporting this hypothesis are the similarities in inhibition of vacuole fusion at the priming stage in Chapter 3 and the report that nystatin affected properties of the arginine and glucose transporters in yeast plasma membrane vesicles (62). It has been speculated for many years whether the permeabilising properties of polyene antibiotics would be the sole mechanism of action. Mainly because an observed permeabilisation of the membrane did not always lead to cell death (63-65). Other modes of actions have been proposed such as oxidative damage of membrane structures (66-68). In addition, polyene antibiotics have been studied as human therapeutics in diseases unrelated to fungal infections, via the interaction with cholesterol, perhaps unrelated to a membrane permeabilisation action (69-72). Because natamycin is the only family member known not to permeabilise the membrane, it is so far the only member which can be used unambiguously to study this putative basic mode of action of the polyene antibiotics.

As indicated in a previous section, a few key questions in the mechanism of action of natamycin and possibly other polyene antibiotic family members remain. One of these key questions involves the ergosterol dependency of the inhibition on transport protein function by natamycin. This question can be addressed by purifying a specific protein and testing its activity after reconstitution in model membranes with different lipid compositions. Another key question involves the conformation of the putative half pore complex of natamycin with ergosterol. A molecular dynamics simulation of natamycin with an ergosterol containing bilayer may provide more information on this complex. The final key question is whether all polyene antibiotics have the same basic mechanism of action. This can be determined by studying the effect of polyene antibiotics on integral membrane proteins that act as enzymes without being dependent on the membrane barrier function as described in Chapter 4.

Speculations on improved antifungal formulations

As described in the general introduction, we wanted to gain more insight into the mode of action of natamycin, which could perhaps aid the development of new or improved antifungal formulations or result in novel strategies to prevent fungal spoilage. One way to develop improved antifungal formulations is to enhance the

antifungal activity of natamycin. An improved antifungal activity of natamycin has already been shown for the bis(aminopropylene) derivative of natamycin (73).

As described in this thesis, natamycin was shown to inhibit fungal growth via a specific interaction with ergosterol that leads to the impairment of membrane protein functions in an ergosterol dependent manner. Thus, the presence of ergosterol in fungi is required for natamycin to elicit its mode of action. Dormant fungal conidia contain low levels of ergosterol and the lowest levels of ergosterol are observed during the first initial 3 hrs of germination, as shown in conidia of *P. discolor* (74,75). Because of these low levels of ergosterol, natamycin is unlikely to have an effect on dormant conidia. Spores do not respond to natamycin in the first 2 hours, but do start to change their protein expression profile after this period (55). The germination of spores of *A. niger* could be inhibited by natamycin for at least 8 hours (55). In addition, natamycin was shown to inhibit endocytosis of spores of *P. discolor* that had been germinating for 6 hours, without causing membrane pemeabilisation (18). But it was also shown that the effect of natamycin on transport protein inhibition and possibly the effect of natamycin-NBD on the block of endocytosis could be reversed (Chapter 4 and its appendix). Spores could remain in a relatively dormant state until the decomposition of natamycin in time, after which they can start germination (76). A similar dormancy and reawakening process has been reported for *Candida albicans* in response to amphotericin B (77).

If a more effective antifungal formulation was to be created, it should focus on the germination of spores. Germination often requires environmental nutrients that trigger the spores (78,79). However, water, oxygen, and carbon dioxide are known to activate spore germination as well. Other low molecular weight nutrients (e.g. specific amino acids and vitamins) and for example inorganic salts can activate germination (78,79). Alternatively, ergosterol levels in spores may be increased. Although this seems like a difficult task, low levels of the antifungal agents of azoles have been shown to increase ergosterol levels in fungal cells (80,81). Additionally, by increasing the activity of proteins that regulate sterol homeostasis and contain sterol-sensing domains (SSD) could cause an increase in ergosterol levels, which has been observed after azole treatment with *Candida albicans* (82). An example of a compound that is able to increase SSD in mammalian cells is the HIV protease inhibitor, ritonavir (83). If this compound is able to have similar effects in dormant spores, a combined formulation could prove interesting for fungal infected immune-compromised HIV patients.

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Nederlandse samenvatting

De laatste jaren is de vooruitgang in de behandeling van patiënten met kanker, AIDS of orgaan transplantaties vergezelt door een toename in het aantal gevallen van schimmelinfecties die bij deze groep van patiënten met een laag immuunsysteem als levensbedreigend worden beschouwd. Op medisch gebied zijn deze schimmelinfecties een grote uitdaging, omdat er maar een klein arsenaal aan antibiotica bestaan om deze infecties te bestrijden en er een wezenlijke toename in het aantal resistente schimmelsoorten is waargenomen. Tegen de polyeen antibiotica, wat een familie van antischimmel middelen is die het membraan van schimmels aanpakt, wordt nog weinig resistentie waargenomen. Het membraan is het omhulsel van een cel die de buitenkant van de binnenkant van een cel afschermt. Het bestaat uit lipiden die opgebouwd zijn uit een waterlievende kop en een waterafstotende staart. Het membraan zelf bestaat uit twee lagen van lipiden, waarbij de waterlievende koppen aan de buitenkant zitten en daarbij de waterafstotende staarten aan de binnenkant afschermen. De membranen van schimmels bevatten ergosterol, een variant van het menselijk cholesterol, waarmee sommige polyeen antibiotica een interactie kunnen aangaan. Van de familieleden van de polyeen antibiotica waarvan het werkingsmechanisme bekend is, is gevonden dat ze via de interactie met ergosterol de membranen lek kunnen maken. Dit gebeurt of door de vorming van poriën (nystatine) of door grote clusters die het membraan openscheuren (filipine). Hierdoor lekken belangrijke bestanddelen uit de cel wat celdood tot gevolg heeft. Natamycine is een familielid van de polyeen antibiotica en wordt al vele jaren gebruikt in de voedselindustrie voor het conserveren van kaas, worst of wijn. Verder wordt natamycine toegepast als geneesmiddel voor uitwendig gebruik, bijvoorbeeld op de huid of ogen. Hoewel dit antibioticum al jaren toegepast wordt, is het werkingsmechanisme niet bekend. Het doel van deze studie is om inzicht te verkrijgen in het werkingsmechanisme van natamycine. Mogelijk kunnen deze nieuwe inzichten leiden tot vernieuwde of verbeterde antischimmel formulaties.

Omdat van andere polyeen antibiotica bekend is dat ze een interactie aan kunnen gaan met sterolen in membranen, is dit als uitgangspunt genomen in de studie naar het werkingsmechanisme van natamycine. Om de interactie van natamycine met sterolen in een membraan te bestuderen, hebben we de membraan van een cel nagebootst door model membranen te maken die ergosterol (schimmel sterol), cholesterol (menselijk sterol) of geen sterolen

bevatten (Hoofdstuk 2). De binding van natamycine aan deze model membranen is bestudeerd met verschillende technieken, waaronder een direct bindingsexperiment en een thermodynamische studie genaamd *Isotherme Titratie Calorimetrie* (ITC). Uit deze studies bleek dat natamycine het meeste bindt aan model membranen als ze ergosterol bevatten. Verder hebben we het effect van natamycine op een schimmel soort getest; gist. Deze gisten waren genetisch gemodificeerd waardoor ze verschillende soorten sterolen in hun membranen bezitten. Uit deze studie bleek dat de gisten die ergosterol bevatten het meest gevoelig waren voor natamycine en veranderingen in de ring structuur van ergosterol zorgde ervoor dat de gisten minder gevoelig waren voor natamycine. Daarbij kwam de mate van binding van natamycine aan deze gisten goed overeen met de gevoeligheid van deze stammen voor natamycine. Uit deze studie kon opgemaakt worden dat de binding van natamycine aan ergosterol in het membraan belangrijk is voor het werkingsmechanisme van natamycine.

Vervolgens hebben we in Hoofdstuk 2 onderzocht of natamycine, net zoals andere polyeen antibiotica, membranen lek kan maken. Dit hebben we bestudeerd door model membranen te gebruiken waarin een verbinding als sensor was ingesloten waarmee de mate van membraan lek bepaald kon worden. Een soortgelijk experiment hebben we ook uitgevoerd met levende gistcellen. Uit deze experimenten bleek dat natamycine, in tegenstelling tot andere polyeen antibiotica zoals nystatine of filipine, de model membranen niet lek kon maken. Ook de membranen van de gistcellen kon natamycine niet lek maken, terwijl het wel de groei van de gist efficiënt kon remmen. Hieruit konden we concluderen dat het werkingsmechanisme van natamycine niet gebaseerd is op het lek maken van membranen.

Om te bepalen hoe natamycine de schimmel kan doden is het belangrijk om te bepalen wat de interactie van natamycine met ergosterol in een membraan voor effect zou kunnen hebben op het functioneren van een schimmel cel. Van ergosterol is bekend dat dit membraan component een belangrijke rol speelt bij het samensmelten (fuseren) of afscheiden (splitsen) van membranen. Het fuseren en splitsen van membranen zijn belangrijke processen die nodig zijn voor het transportsysteem binnen een cel. Mogelijk zou natamycine via de interactie met ergosterol de fusie of splitsing van membranen kunnen beïnvloeden. Een bekend model systeem om de fusie van membranen te testen is via de isolatie van een organel van een gistcel, genaamd de vacuole. De vacuole van een gist is soortgelijk aan het lysosoom van menselijke of dierlijke cellen en kan opge-

vat worden als de vuilnisbak van een cel. Alle afvalstoffen van een cel worden verwerkt in de vacuole. De vacuole is in feite een met vocht gevuld blaasje die omringd is door een membraan, waarin ook ergosterol aanwezig is. De meeste membraansystemen, waaronder die van de vacuole, hebben dezelfde soort eigenschappen die nodig zijn voor membraan fusie en daardoor kan de vacuole goed als model systeem dienen. Als twee vacuolen met elkaar fuseren, dan treedt er een vermenging van de inhoud van beide vacuolen op. Door twee vacuolen te gebruiken die verschillen van inhoud, kun je achteraf bepalen of de inhoud van deze vacuolen met elkaar vermengd zijn. Op deze wijze kan de hoeveelheid fusie gemeten worden. Aan de hand van deze methode hebben we bepaald dat natamycine de fusie van vacuolen kan remmen, zonder deze vacuolen lek te maken (Hoofdstuk 3). Daarbij hebben we, net als in Hoofdstuk 2, gebruik gemaakt van genetisch gemodificeerde giststammen die verschillende soorten sterolen in hun membranen bezitten. De fusie van de vacuolen kon alleen door natamycine geremd worden als de vacuolen van deze giststammen sterolen bevatten waarmee natamycine kon binden. Toen natamycine aan intacte gistcellen werd toegevoegd, waren er vele kleine blaasjes zichtbaar in plaats van een of twee grote vacuolen als er geen natamycine was toegevoegd. Dit resultaat duidde erop dat natamycine de fusie van vacuolen ook in een intacte cel kan remmen.

De fusie van de geisoleerde vacuolen wordt in een heel vroeg stadium geremd door natamycine, zelfs voordat de membranen van de vacuolen elkaar raken (Hoofdstuk 3). Doordat de lipiden in de membranen elkaar nog niet raken in dit vroege stadium van membraanfusie, is het onwaarschijnlijk dat natamycine de vermenging van de lipiden van de vacuolen beïnvloed. In dit vroege stadium is alleen sprake van de reorganisatie van eiwitcomplexen die de fusie van de membranen en menging van lipiden voortdrijven. Deze bevindingen lijken erop te wijzen dat natamycine via de interactie met ergosterol, mogelijk een effect heeft op eiwitten die afhankelijk zijn van ergosterol voor hun functie.

Om meer inzicht te verkrijgen of natamycine inderdaad in staat is eiwit functies te beïnvloeden via de interactie met ergosterol, hebben we naar het meest ergosterol rijke membraan gekeken: het plasma membraan. Het plasma membraan is het membraan wat direct om de cel aanwezig is en de binnenkant van een cel van de buitenkant afschermt. Om ervoor te zorgen dat belangrijke voedingsstoffen, zoals suikers en aminozuren, toch de cel binnen kunnen komen, bevinden er zich speciale transporteiwitten in het plasma membraan. Om het effect van natamycine op dergelijke membraan

transporteiwitten te bestuderen, hebben we gekeken naar de opname van suiker en de aminozuren arginine en proline door gistcellen na het toevoegen van natamycine. Uit deze studie bleek dat natamycine de opname van al deze voedingsstoffen in gist remde, zonder dat ze uit de gistcel lekten (Hoofdstuk 4). De door natamycine veroorzaakte blokkering van de opname van arginine in gist bleek een omkeerbaar proces te zijn na het wassen van de gistcellen.

In het plasma membraan van een gistcel wordt verondersteld dat er zich bepaalde regio's of domeinen bevinden die verrijkt zijn met ergosterol. Van deze verrijkte ergosterol domeinen wordt gedacht dat ze belangrijk zijn voor de ophoping van eiwitten die daardoor hun functie beter uit kunnen voeren. We hebben grote model membranen gemaakt om te testen of natamycine deze ergosterol verrijkte domeinen kon verstoren, wat mogelijke tot de verklaring voor de functieverstoring van de transporteiwitten door natamycine zou kunnen leiden. In deze grote model membranen zaten lipiden met een fluorescerende groep, die niet in het ergosterol verrijkte domein konden komen. Met behulp van een fluorescentie microscoop konden we de ergosterol rijke en arme domeinen in de grote model membranen zichtbaar maken (Hoofdstuk 4). Natamycine bleek geen effect te hebben op de ergosterol verrijkte domeinen in de model membranen. Door deze resultaten leek het niet waarschijnlijk dat de remming van de membraan transporteiwitten door natamycine afhankelijk zou zijn van een verstoring van ergosterol verrijkte membraan domeinen in een gist of schimmel. Het is echter nog niet duidelijk hoe deze transporteiwitten precies geremd worden en of er ook andere membraan onderdelen beïnvloed kunnen worden door natamycine. Maar het meest waarschijnlijke is dat het werkingsmechanisme van natamycine gebaseerd is op de interactie van natamycine met ergosterol. Omdat andere polyeen antibiotica ook een interactie met ergosterol hebben, is het mogelijk dat deze antibiotica ook membraan transport functies kunnen verstoren. Dit is nog niet eerder aangetoond, omdat andere polyeen antibiotica het membraan lek kunnen maken, wat soortgelijke studies altijd bemoeilijkte. Immers, als de opname van de voedingsstoffen in de cel geremd wordt, maar de voedingsstoffen er tegelijkertijd ook uit kunnen lekken, dan kan er geen onderscheid worden gemaakt tussen de remming van opname en lek van de voedingsstoffen. Doordat natamycine het membraan niet lek kan maken is natamycine het meest interessante familie lid van de polyeen antibiotica om een mogelijke "dubbele" werking van de polyeen antibiotica te bestuderen.

De indicatie dat natamycine via de interactie met ergosterol effect heeft op de functie van eiwitten in het plasma membraan, maar ook in het vacuole membraan, leidde tot de vraag of natamycine toegang heeft tot meerdere membraan onderdelen van een schimmelcel. Deze vraag hebben we proberen te beantwoorden door natamycine aan te passen met een fluorescerende groep. Met behulp van een fluorescentie microscoop konden we de locatie van natamycine in een cel bestuderen. Na toevoeging van het aangepaste natamycine aan gistcellen werd het plasma membraan van gistcellen fluorescent gekleurd en ook membraan systemen binnenin de gistcel werden deels gekleurd. Aangetoond is dat natamycine de afsplitsing van kleinere membraan blaasjes van het plasma membraan kon remmen in bepaalde schimmelsporen. Na toevoeging van het aangepaste natamycine aan deze schimmelsporen werd zowel het plasma membraan als gedeeltelijk de membraan systemen in de schimmelsporen gekleurd. Hoewel natamycine de afsplitsing van membranen kan remmen, is het mogelijk dat toch een klein gedeelte van het aangepaste natamycine de spore binnen kan komen. De hoeveelheid kleuring van de membraan systemen in de sporen nam na het wassen van de sporen in de tijd toe, wat misschien aangeeft dat de remming van de afsplitsing van membranen omkeerbaar kan zijn.

Helaas bleek door de aanpassing dat natamycine veel minder de groei van gist kon remmen en dus veel minder actief was als antibioticum. Door middel van een directe bindingsstudie bleek dat door de aanpassing, natamycine wel aan membranen kon binden, maar niet meer specifiek aan ergosterol. Als natamycine met een kleinere groep werd aangepast was de afname in gevoeligheid van de gisten veel minder vergeleken met de toevoeging van de fluorescente groep aan natamcyine. Dus de verminderde werking van het fluorescente natamycine komt waarschijnlijk door de fluorescente groep. Deze resultaten geven wel duidelijk aan dat natamycine de interactie met ergosterol nodig heeft om zijn toxische werking op de schimmel uit te voeren, omdat alleen binden aan membranen niet voldoende is voor natamycine om zijn werking uit te voeren.

Deze studie heeft geleid tot nieuwe inzichten in het werkingsmechanisme van natamycine, dat via de interactie met ergosterol in het membraan in staat is eiwitfuncties te beïnvloeden. Toekomstig onderzoek zal moeten uitsluiten of dit werkingsmechanisme specifiek voor natamycine is of dat ook andere familieleden van de polyeen antibiotica via een “dubbel werkingsmechanisme” werken.

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Curriculum Vitae

Yvonne te Welscher was born on July 26th 1980 in Nijkerk, the Netherlands. After graduating in 1998 at the Oostvaarders College in Almere where a VWO degree was obtained, she studied Chemistry at the Free University in Amsterdam. A research project was attended in Organic Chemistry under supervision of Prof. dr. ir. Romano Orru. In this project, a synthesis method for the substitution of isoflavones was developed. Next, she attended a research project in Molecular Toxicology under supervision of Prof. dr. Nico Vermeulen and dr. Sebastiaan Liemp into the development of a fluorescence energy transfer-based DNA alkylation assay. In January 2005 she obtained her masters degree and in February she started a research position in the Biochemistry of Membranes group at the Utrecht University under supervision of Prof. dr. Ben de Kruijff and dr. Eefjan Breukink. During this time she was funded an EMBO short-term fellowship to work for three months in the group of dr. Gary Eitzen in Edmonton, Canada. The total work on the mode of action of the polyene antibiotic natamycin has resulted in this thesis. In July 2009, she started a PostDoc position in the same group where she works on the expression and purification of Shape, Elongation, Division and Sporulation (SEDS) proteins of different bacteria, which are a family of membrane proteins.

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