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minireview

Membrane Transport Inhibition as Mode of Action of Polyene Antimycotics: Recent Data Supported by Old Ones

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Summary

Recent studies demonstrate that the actual interactions of polyenes with membrane sterols are the basis for their antimycotic toxicity and not, as previously assumed, their potential to form pores. Therefore, sterols must play a vital role in membranes and this function is seriously disturbed once polyenes bind to and/or sequester them. Essential membrane proteins obviously require sterols for their activity. Among the various membrane protein/ergosterol interactions discussed herein, transport proteins of the plasma membrane are most likely the primary target for polyenes. All data available which support this notion are summarized in this review. Even data obtained almost 40 years ago could be useful in guiding future research.

Key words: amphotericin B, nystatin, natamycin, sugar and amino acid transport

Introduction

The number of fungal infections, mainly of immunocompromised patients, has greatly increased over the past 30 years and with its high degree of mortality has become a major health threat (*1*). In addition, fungi represent a serious problem for animals and plants as well as for food security (*2*). Among the available antifungal agents, the polyenes stand out for low rates of drug resistance (*3*). In spite of their general toxicity for all eukaryotic cells and, consequently, the low therapeutic index, polyene antimycotics are widely used for treating fungal infections. Their mode of action has not been fully understood, although it has been intensely studied and discussed for decades (*4*).

Numerous studies have established that polyenes specifically interact with membrane sterols, preferentially with the fungus-specific ergosterol, and they do require sterols for their antimycotic activity (*4–7*). Although apoptosis-like responses triggered by amphotericin B have recently been suggested as possible mechanisms of toxicity (reviewed in ref. *8*), it has been widely accepted that polyenes kill fungal cells by permeabilizing plasma membranes (PM) (*4,6,8*), either by forming pores (amphotericin B and nystatin) or by destabilizing the membrane (filipin). Nevertheless, doubts had been raised concerning this mode of action as early as in 1974 (*9–11*). Therefore, until recently a typical statement in reviews read: 'the precise way in which this fungicidal effect occurs still remains unclear' (*12*). The situation has changed due to a number of recent publications (*7,13–16*), which will be summarized in the first part of this review. These studies demonstrate that the interaction of polyenes with membrane sterols as such is the basis for their toxicity and the documented permeabilizing actions may only be an additional complementary effect. In other words, sterols must play a vital role in membranes, and this function is seriously disturbed once polyenes bind to and/or sequester them. In principle, these studies suggest that a number of essential membrane proteins, like for example transporters, require sterols for their activity, stability and/or regulation. In the second part of the review, we

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Dedicated to the late Prof. Pavao Mildner, who was a whole-hearted biochemist and a friend through many years.

summarize sterol-protein interactions so far postulated for fungal cells (*17–26*) and include a number of observations from the transport field (*27–40*); the latter may offer guidance for future research.

Polyenes 'Primarily Kill Yeast by Simply Binding Ergosterol'

This is the title of a last year's paper by Gray *et al*. (*14*), except that they used 'amphotericin' instead of 'polyenes'. Testing natamycin, the laboratory of de Kruijff and Breukink in Utrecht in 2008 (*7*) was the first that clearly demonstrated polyene antimycotic toxicity caused by its specific binding to ergosterol. Natamycin is a natural product of *Streptomyces natalensis* and a typical polyene of intermediate size between nystatin and amphotericin B. Precise *in vitro* and *in vivo* studies showed that natamycin binds to ergosterol, but does not form pores, not even small ones suited for protons. Nevertheless, the toxicity of natamycin hardly differs from that of pore-forming nystatin; its MIC value (minimal concentration of inhibition) for *S. cerevisiae* was determined at (1.7±0.5) μM as compared to (1.1±0.2) μM for nystatin (*7*).

The studies of Burke's group (*13,14*) were based on the syntheses of various amphotericin B derivatives, and their testing for ergosterol-binding and/or pore-forming activities. The authors proved that the amino dideoxy sugar mycosamine, typical for all mycotoxic polyenes (Fig. 1), is essential for binding to ergosterol. They also showed that the aglycone of natamycin does not bind ergosterol anymore and is consequently non-toxic. Deletion of the C_{35} hydroxy group of amphotericin B gave rise to the compound C35deOAmB (Fig. 1), which did not cause pore formation, was able to bind to ergosterol, and its toxicity was only slightly reduced (*14*). The MIC value was 3 as compared to 2 and 0.5 μM for natamycin and amphotericin B, respectively. In principle, similar results were obtained with the methyl ester of 35-deoxy amphotericin B synthesized and tested by Szpilman *et al*. (*16*). However, the difference in the toxicity of the deoxy compound was more than an order of magnitude lower than that of amphotericin B; the deviation from that of C35deOAmB was explained by the presence of the methyl ester group (*14,15*).

Which and in What Way are Membrane Proteins Affected by Interacting with Sterols?

As proven in the above studies, the main mode of action of polyenes is their binding to ergosterol and in this way interfering with fundamental sterol functions. A most pertinent question therefore is which these functions are. It is known that membrane sterols play a role in a vast number of cellular reactions. Especially reactions participating in endocytosis and secretion only function efficiently in fungal cells, when the ergosterol content of the involved membranes is not impaired (*17–21*). Ergosterol is also required, for example for signalling functions (*22*) as well as for homotypic vacuole fusion (*23,24*), and for special types of autophagosomal reactions (*25*). Many of these physiological processes are vital and, in principle, they all could be targets of polyene antimycotics. To our knowledge, this has been tested so far only in a vacuolar fusion assay, where indeed filipin, nystatin and amphotericin B strongly inhibited homotypic vacuolar fusion (*23*). This has recently been confirmed also for natamycin (*26*).

Although it cannot be excluded that crucial intracellular processes requiring ergosterol may be the targets for the toxic action of polyenes, it is more likely that polyenes preferentially interact with the sterols that are abundant in the PM and thus are the first ones which face and sequester the drug added to the medium. The observation that the presence or absence of Pdr proteins, responsible for active export of toxins, does not affect the sensitivity towards polyenes, certainly strengthens this assumption (André Goffeau, personal communication). This is furthermore supported by recent findings of te Welscher *et al*. (*15*). The authors performed a transcriptome analysis with germinating conidia of *Aspergillus niger* in the presence or absence of natamycin. Natamycin treatment markedly increased the expression of 17 out of 20 transcripts for sugar transport proteins and 18 out of 30 for amino acid transporters; in 8 h the overall increase of the transcripts for the two transporter groups amounted to 6- and 2.6-fold, respectively (*15*). Next, they showed that this upregulation was elicited to compensate for impaired protein functions caused by the polyene. The effect of natamycin was tested on several plasma membrane transport systems in *S. cerevisiae*. Indeed,

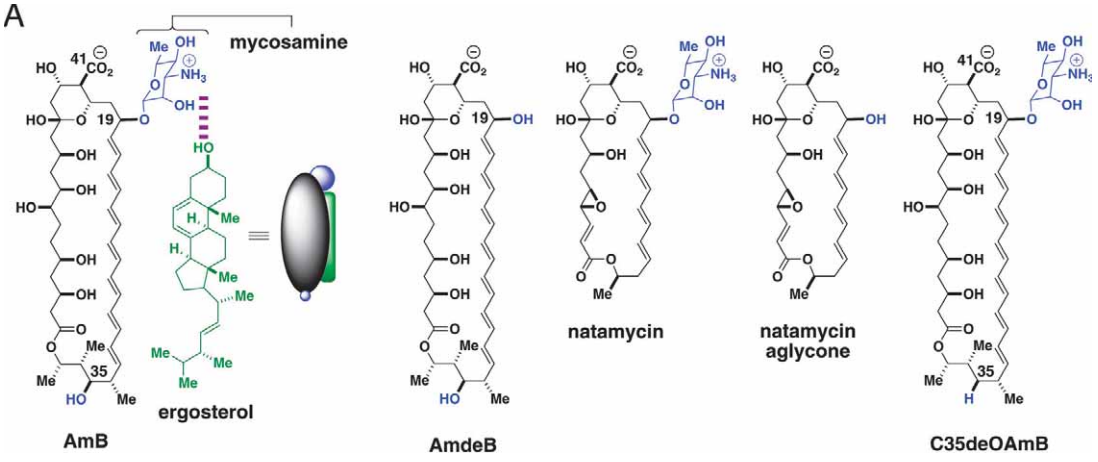

Fig. 1. Chemistry and biology of polyene macrolides. Molecular structures of the polyene macrolide natural products AmB, natamycin, and a series of their functional group-deficient derivatives prepared *via* chemical synthesis (taken from ref. *14*, Fig. 1A)

natamycin directly inhibited the transport of arginine, proline and glucose (*15*). These data suggest that the natamycin binding to ergosterol executes a direct effect on transporters and consequently on the expression of this set of vital plasma membrane proteins. On the bases of previous independent studies (see below), te Welscher *et al*. (*15*) propose that the same primary mechanism holds for other polyene antimycotics.

For all that, plasma membrane transporters should be the focus for elucidating the detailed mode of action of polyene antimycotics. Studies describing an effect of nystatin on membrane transport processes have appeared since the seventies (*10*,*11*,*27–29*). Proton-coupled transport systems for amino acids and sugars can accumulate substrate analogues up to more than thousand fold (Table 1 and *27–29*,*31*,*32*). It was shown that their uptake was inhibited by uncouplers like 2,4-dinitrophenol (DNP), carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), or carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) as well as by other metabolic inhibitors. Unexpected and not really understood mechanistically until today, however, was the observation that accumulated substrate analogues were retained within the cells when the uncouplers were added (*27–29*,*31*,*32*). Conversely, the addition of nystatin caused their fast release, which documented that substrate analogues were present in cells in a free form. This finding was interpreted according to the explanation prevalent at the time that polyenes form pores and that substrates leak out through these pores (*27–29*).

Only in two instances an alternative interpretation, namely, that the substrate efflux following the addition of antimycotics is mediated by the transport protein, has been tested: the sugar/proton symporter of *Chlorella* HUP1 (*10*) and the arginine/proton symporter Can1 of *S. cerevisiae* (*11*). Both Can1 and the heterologously expressed HUP1 protein localize in the so-called MCC patches (**M**embrane **C**ompartment of **C**an1), yeast plasma membrane microdomains (*33*,*34*, reviewed in *35*) that are enriched in ergosterol (*36* and Fig. 2). These two transporters, therefore, offer ideal models to study mechanistic details of polyene interference with transport activities.

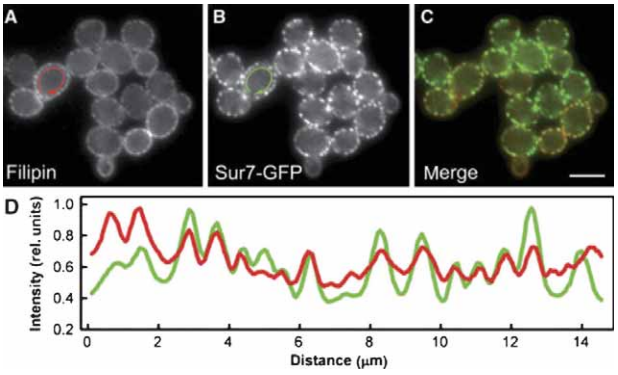

Fig. 2. Sites of sterol accumulation in plasma membrane colocalize with MCC. Simultaneous localization of filipin-stained sterols (A; red in C and D) and an MCC marker Sur7GFP (B; green in C and D) was performed in living GYS48 cells. Wide-field fluorescence micrographs (A–C) and the fluorescence intensity profiles along the cell surface (D; outside the arrows in A and B) are presented; bar: 5 μm (taken from ref. *36*)

Coincidentally, it was the glucose transporter HUP1 from an alga where for the first time a direct effect of nystatin on a plasma membrane protein was postulated (*10*). The HUP1 transport protein requires sterol for full activity and when purified to homogeneity from transgenic yeast, the protein retains 2–3 ergosterols bound per protein molecule (*37* and Table 1). When expressed

Table 1. HUP1, hexose-H^+ symporter of *Chlorella*: heterologous expression, membrane localization, activity, properties

Expressed in	Properties/Localization		Reference
S. cerevisiae	Active/localized in MCC patches together with ergosterol (Fig. 2)		(*34*,*36*)
Lipids purified with the protein/(mol/mol)	Ergosterol 2–3, PE 2 and PC 2		(*37*)
S. pombe	Active/localized in terminal caps together with ergosterol		(*34*)
E. coli	Highly expressed; inactive *in vivo*		(*38*)
HUP1 from *E. coli* reconstituted in vesicles	Active in the presence of ergosterol		(*38*)
Chlorella kessleri	HUP1 localized in PM patches, as well as sterols stained with filipin		(*34*, and G. Grossmann, unpublished data)
	In the presence of nystatin, HUP1 acts as facilitator; overshoot experiment is possible (see Fig. 2)		
	Control	+ Nystatin	
Accumulation of 6-dG	>1000-fold	1.2-fold	(*10*,*39*)
K_m(influx$_{6\text{-dG}}$)/mM	0.21	>10	
K_m(efflux$_{6\text{-dG}}$)/mM	21	20	

The referred studies present the following findings relevant to the topic: (*i*) the HUP1 protein colocalizes with sterols as shown for *S. cerevisiae*, *S. pombe*, and *Chlorella*; (*ii*) the HUP1 transporter is inactive when expressed in bacteria lacking sterols (*E. coli*); (*iii*) in the presence of nystatin the properties of the transporter are dramatically changed, see accumulation of 6-dG in *Chlorella* as well as the K_m value for influx. Abbreviations: PE=phosphatidylethanolamine, PC=phosphatidylcholine, 6-dG=6-deoxylucose

in *E. coli*, HUP1 is inactive except when reconstituted in vesicles supplemented with ergosterol (*37*). As mentioned above, the non-metabolizable glucose analogue 6-deoxyglucose is accumulated over 1000-fold by HUP1 in *Chlorella* and is not released into the medium by treatment with uncouplers or metabolic poisons; only the addition of nystatin caused the release of the analogue (*10*). The classical overshoot experiment, proving the existence of facilitator activity and refuting transport through pores (*40*), clearly showed that the sterol-requiring and strictly energy-coupled HUP1 protein was transformed to a facilitator by nystatin (Fig. 3, and ref. *10*). Similar results were obtained by *in vitro* experiments when Can1 activity was tested in vesicles (*11*). The uptake of arginine into vesicles was driven by proton motive force generated by cytochrome c oxidase. Addition of nystatin to vesicles containing ergosterol caused fast release of the

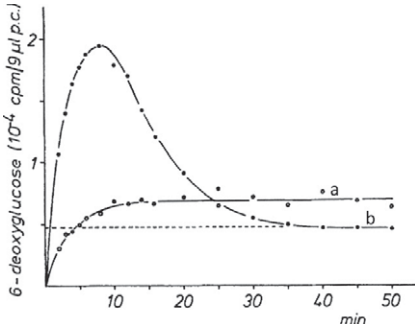

Fig. 3. Active transporter HUP1 changes to facilitator by the action of nystatin. Overshoot experiment: *Chlorella* cells in sodium-phosphate buffer were preloaded with 100 mM 6-deoxyglucose in the presence of nystatin (12 µg/mL). The preloaded cells (b) were washed and resuspended in the buffer containing 10 µM ^{3}H-6-deoxyglucose, 50 µM FCCP and nystatin. Control: not preloaded cells (a) were resuspended in the same buffer with the additions as above. The radioactivity in the cells was determined at the times indicated. In the absence of nystatin, FCCP inhibits influx even before the concentration equilibrium and the overshoot in radioactivity cannot be observed. The broken line indicates the concentration equilibrium of the radioactive sugar (taken from ref. *10*)

accumulated arginine, although a membrane potential of –80 mV (proton and potassium gradient) was maintained, proving that no pores were formed. Last year, te Welscher *et al*. (*15*) showed that natamycin inhibited ^{14}C-arginine uptake in *S. cerevisiae*, but did not lead to its efflux once accumulated. This discrepancy with the *in vitro* experiment is understandable since in living cells, rapid incorporation of arginine into proteins or its sequestration to the vacuole proceeds. On the other hand, there may be a difference in the mechanism between nystatin and natamycin.

The studies carried out with the proton symporters HUP1 and Can1 indicate that either these proteins require ergosterol for their coupling to energy or that they convert to facilitators once their interaction with sterols is disturbed or the sterols are replaced by polyenes. Förster resonance energy transfer studies *in vivo* showed that in the presence of ergosterol, nystatin is located much closer to membrane proteins than in its absence (*30*). In *S. cerevisiae*, the hexose transporters HXT1-13 facilitate energy-independent transport of glucose but, all the same, they are inhibited by natamycin (*15*). Therefore, a mechanism different from the uncoupling of energy may have to be postulated in addition.

Conclusion

The new mode of action established for polyenes will have two major consequences for future investigations. First, the elucidation of the precise molecular role that membrane sterols play while interacting with specific membrane proteins will be greatly stimulated. Secondly, knowledge of the actual fungal targets for toxicity as well as the interacting group of polyenes with sterols (*14*) will give rise to the possibility to improve the specificity of their interaction with ergosterol as compared to that with cholesterol, and thus eventually help to increase the therapeutic index for human patients.

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References

1. A. Butts, D.J. Krysan, Antifungal drug discovery: Something old and something new, *PLOS Pathogens, 8* (2012) e1002870.
2. M.C. Fisher, D.A. Henk, C.J. Briggs, J.S. Brownstein, L.C. Madoff, S.L. McCraw *et al.*, Emerging fungal threats to animal, plant and ecosystem health, *Nature, 484* (2012) 186–194.
3. M.A. Ghannoum, L.B. Rice, Antifungal agents: Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial mechanisms of resistance: A review, *Clin. Microbiol. Rev. 12* (1999) 501–517.
4. J. Bolard, How do the polyene macrolide antibiotics affect the cellular membrane properties?: A review, *Biochim. Biophys. Acta, 864* (1986) 257–304.
5. D. Gotlieb, H. Carter, J. Sloneker, A. Ammand, Protection of fungi against polyene antibiotics by sterols, *Science, 128* (1958) 361.
6. B. de Kruijff, R.A. Demel, Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. III. Molecular structure of the polyene antibiotic-cholesterol complexes, *Biochim. Biophys. Acta, 339* (1974) 57–70.
7. Y.M. te Welscher, H.H. ten Napel, M.M. Balague, C.M. Souza, H. Riezman, B. de Kruijff, E. Breukink, Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane, *J. Biol. Chem. 283* (2008) 6393–6401.
8. B.E. Cohen, Amphotericin B membrane action: Role for two types of ion channels in eliciting cell survival and lethal effects: A review, *J. Membrane Biol. 238* (2010) 1–20.
9. C.C. HsuChen, D.S. Feingold, Two types of resistance to polyene antibiotics in *Candida albicans*, *Nature, 251* (1974) 656–659.
10. B. Komor, E. Komor, W. Tanner, Transformation of a strictly coupled active transport system into a facilitated diffusion system by nystatin, *J. Membrane Biol. 17* (1974) 231–238.

11. M. Opekarová, W. Tanner, Nystatin changes the properties of transporters for arginine and sugars. An *in vitro* study, *FEBS Lett. 350* (1994) 46–50.

12. F.C. Odds, A.J.P. Brown, N.A.R. Gow, Antifungal agents: Mechanisms of action, *Trends Microbiol. 11* (2003) 272–279.

13. D.S. Palacios, I. Dailey, D.M. Siebert, B.C. Wilcock, M.D. Burke, Synthesis-enabled functional group deletions reveal key underpinnings of amphotericin B ion channel and antifugal activities, *Proc. Natl. Acad. Sci. USA, 108* (2011) 6733–6738.

14. K.C. Gray, D.S. Palacios, I. Dailey, M.M. Endo, B.E. Uno, B.C. Wilcock, M.D. Burke, Amphotericin primarily kills yeast by simply binding ergosterol, *Proc. Natl. Acad. Sci. USA, 109* (2012) 2234–2239.

15. Y.M. te Welscher, M.R. van Leeuwen, B. de Kruijff, J. Dijksterhuis, E. Breukink, Polyene antibiotic that inhibits membrane transport proteins, *Proc. Natl. Acad. Sci. USA, 109* (2012) 11156–11159.

16. A.M. Szpilman, J.M. Manthorpe, E.M. Carreira, Synthesis and biological studies of 35-deoxy amphotericin B methyl ester, *Angew. Chem. Int. Edit. 47* (2008) 4339–4342.

17. C.M. Souza, H. Pichler, Lipid requirements for endocytosis in yeast: A review, *Biochim. Biophys. Acta, 1771* (2007) 442–454.

18. T.J. Proszynski, R.W. Klemm, M. Gravet, P.P. Hsu, Y. Gloor, J. Wagner *et al.*, A genome-wide visual screen reveals a role for sphingolipids and ergosterol in cell surface delivery in yeast, *Proc. Natl. Acad. Sci. USA, 102* (2005) 17981–17986.

19. T. Iwaki, H. Iefuji, Y. Hiraga, A. Hosomi, T. Morita, Y. Giga-Hama, K. Takegawa, Multiple functions of ergosterol in the fission yeast *Schizosaccharomyces pombe*, *Microbiology, 154* (2008) 830–841.

20. D. Salaun, D.J. James, L.H. Chamberlain, Lipid rafts and the regulation of exocytosis: A review, *Traffic, 5* (2004) 255–264.

21. H. Pichler, H. Riezman, Where sterols are required for endocytosis: A review, *Biochim. Biophys. Acta, 1666* (2004) 51–61.

22. C.J. Mousley, P. Yuan, N.A. Gaur, K.D. Trettin, A.H. Nile, S.J. Deminoff *et al.*, A Sterol-binding protein integrates endosomal lipid metabolism with TOR signaling and nitrogen sensing, *Cell, 148* (2012) 702–715.

23. M. Kato, W. Wickner, Ergosterol is required for the Sec18/ATP dependent priming step of homotypic vacuole fusion, *EMBO J. 20* (2001) 4035–4040.

24. C. Hongay, N. Jia, M. Bard, F. Wiston, Mot 3 is a transcriptional repressor of ergosterol biosynthetic genes and is required for normal vacuolar function in *S. cerevisiae*, *EMBO J. 21* (2002) 4114–4124.

25. S. Yamashita, M. Oku, Y. Sakai, Functions of PI4P and sterol glucoside are necessary for the synthesis of a nascent membrane structure during pexophagy, *Autophagy, 3* (2007) 35–37.

26. Y.M. te Welscher, L. Jones, M.R. van Leeuwen, J. Dijksterhuis, B. de Kruijff, G. Eitzen, E. Breukink, Natamycin inhibits vacuole fusion at the priming phase *via* a specific interaction with ergosterol, *Antimicrob. Agents Chemother. 54* (2010) 2618–2625.

27. A. Kotyk, L. Rihova, Transport of α-aminoisobutyric acid in *Saccharomyces cerevisiae*, Feedback control, *Biochim. Biophys. Acta, 188* (1972) 380–389.

28. R. Sumrada, T.G. Cooper, Allantoin transport in *Saccharomyces cerevisiae*, *J. Bacteriol. 131* (1977) 839–847.

29. J. McKelvey, R. Rai, T.G. Cooper, GABA Transport in *Saccharomyces cerevisiae*, *Yeast*, *6* (1990) 263–270.

30. M. Opekarová, P. Urbanová, I Konopásek, P. Kvasnicka, K. Strzalka, K. Sigler, E. Amler, Possible nystatin-protein interaction in yeast plasma membrane vesicles in the presence of ergosterol. A Förster energy transfer study, *FEBS Lett. 386* (1996) 181–184.

31. M.L. Greth, M.R. Chevallier, F. Lacroute, Ureidosuccinic acid permeation in *Saccharomyces cerevisiae*, *Biochim. Biophys. Acta, 465* (1977) 138–151.

32. M. Höfer, A. Kotyk, Tight coupling of monosaccharide transport and metabolism in *Rhodotorula gracilis*, *Folia Microbiol. 13* (1968) 197–204.

33. K. Malinská, J. Malinský, M. Opekarová, W. Tanner, Visualization of protein compartmentation within the plasma membrane of living yeast cells, *Mol. Biol. Cell*, *14* (2003) 4427–4436.

34. G. Grossmann, M. Opekarová, L. Novakova, J. Stolz, W. Tanner, Lipid raft-based membrane compartmentation of a plant transport protein expressed in *Saccharomyces cerevisiae*, *Eukaryot. Cell, 5* (2006) 945–953.

35. J. Malinský, M. Opekarová, G. Grossmann, W. Tanner, Membrane microdomains, rafts, and detergent-resistant membranes in plants and fungi: A review, *Annu. Rev. Plant Biol. 64* (2013) 501–529.

36. G. Grossmann, M. Opekarová, J. Malinský, I. Weig-Meckl, W. Tanner, Membrane potential governs lateral segregation of plasma membrane proteins and lipids in yeast, *EMBO J. 26* (2007) 1–8.

37. I. Robl, R. Graßl, W. Tanner, M. Opekarová, Properties of a reconstituted eukaryotic hexose/proton symporter solubilized by structurally related non-ionic detergents: Specific requirement of phosphatidylcholine for permease stability, *Biochim. Biophys. Acta, 1463* (2000) 407–418.

38. M. Opekarová, I. Robl, R. Grassl, W. Tanner, Expression of eukaryotic plasma membrane transporter HUP1 from *Chlorella kessleri* in *Escherichia coli*, *FEMS Microbiol. Lett. 174* (1999) 65–72.

39. E. Komor, D. Haass, B. Komor, W. Tanner, The active hexose-uptake system of *Chlorella vulgaris*; K_m-values for 6-deoxyglucose influx and efflux and their contribution to sugar accumulation, *Eur. J. Biochem. 39* (1973) 193–200.

40. T. Rosenberg, W. Wilbrandt, Uphill transport induced by counterflow, *J. Gen. Physiol. 41* (1957) 289–296.