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Nystatin-induced lipid vesicles permeabilization is strongly dependent on sterol structure

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Abstract

The selectivity of the antibiotic nystatin towards ergosterol compared to cholesterol is believed to be a crucial factor in its specificity for fungi. In order to define the structural features of sterols that control this effect, nystatin interaction with ergosterol-, cholesterol-, brassicasterol- and 7-dehydrocholesterol-containing palmitoyloleoylphosphocholine vesicles was studied by fluorescence spectroscopy. Variations in sterol structure were correlated with their effect on nystatin photophysical and activity properties. Substitution of cholesterol by either 7-dehydrocholesterol or brassicasterol enhance nystatin ability to dissipate a transmembrane K^+ gradient, showing that the presence of additional double bonds in these sterols–carbon C7 and C22, plus an additional methyl group on C-24, respectively–as compared to cholesterol, is fundamental for nystatin–sterol interaction. However, both modifications of the cholesterol molecule, like in the fungal sterol ergosterol, are critical for the formation of very compact nystatin oligomers in the lipid bilayer that present a long mean fluorescence lifetime and induce a very fast transmembrane dissipation. These observations are relevant to the molecular mechanism underlying the high selectivity presented by nystatin towards fungal cells (with ergosterol) as compared to mammalian cells (with cholesterol).

Keywords: Nystatin; Sterol structure; Membrane permeabilization; Nystatin–sterol complex; Antifungal agent; Fluorescence

1. Introduction

Polyene macrolide antibiotics are a family of naturally occurring compounds, mostly produced by *Streptomyces* species, characterized by the presence of a large lactone ring containing several double bonds [1]. AmB (an heptaene) and nys (a tetraene linked to a diene) (Fig. 1) are the most important members of this family because they are commonly used to treat systemic and superficial fungal infections, respectively, despite their important side effects [2].

The plasma membrane of the antibiotic-sensitive organisms is the target for antifungal polyene drugs like nys, where they form ion channels [1,3,4]. These channels disrupt membrane function by causing uncontrolled cation transport through it that eventually leads to cell death. The higher selectivity of these antibiotics towards fungal cells compared to mammalian cells has often been correlated with the type of sterol present in the cell membrane, erg and chol (Fig. 1), respectively [5]. However, the underlying molecular mechanism that controls the sterol selectivity of these antibiotics is still controversial. Several studies consider that sterols are an integral part of the aqueous channels formed by the antibiotics [3,6], whereas others proposed that chol only helps in the packing of the antibiotic molecules by rigidifying the membrane [7]. Additionally, some authors emphasize the absolute requirement for sterol presence in order for transmembrane aqueous pores to be formed in the lipid membranes [3,8], whereas others state the opposite [9–11].

Abbreviations: 7-DHC, 7-Dehydrocholesterol; AmB, Amphotericin B; bras, brassicasterol; BrPC, 1-Palmitoyl-2-stearoyl (11–12)dibromo-*sn*-glycero-3-phosphocholine; chol, cholesterol; erg, ergosterol; FCCP, (carbonyl cyanide *p*-(trifluormethoxy)phenylhydrazone); LUV, large unilamellar vesicle; nys, nystatin; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Pyranine, (8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt); TMA-DPH, (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate); val, valinomycin

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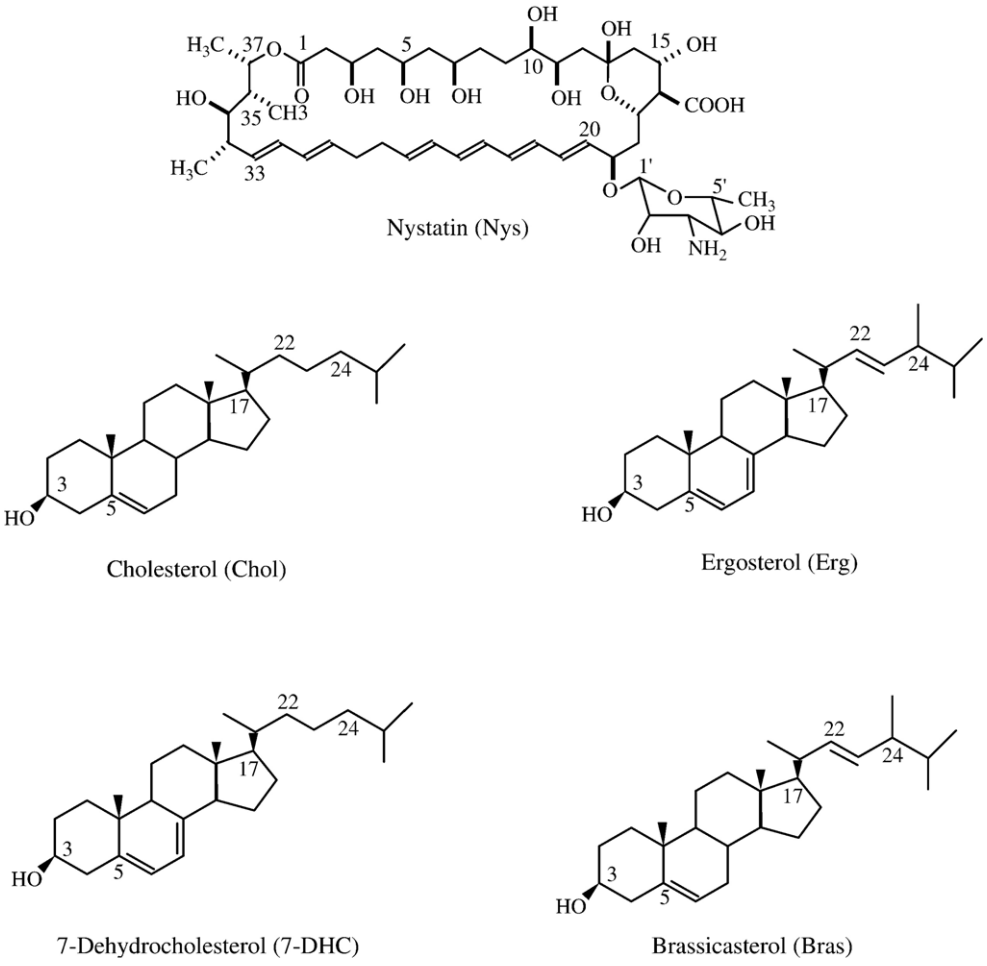

Fig. 1. Chemical structures of the polyene antibiotic nystatin and of the sterols used in this study. Brassicasterol presents the ring system of cholesterol and the aliphatic side chain of ergosterol and the reverse applies to 7-dehydrocholesterol.

In a recent work [12], we proposed that nys mode of action might be distinct in erg- and chol- containing lipid vesicles. In the first case, it was possible to monitor nys self-assembly by following the increase in its mean fluorescence lifetime, which was concomitant with the appearance of transmembrane aqueous pores. These strongly fluorescent species were not detected in POPC-only vesicles and chol-containing liposomes.

The aim of this work was to clarify the mechanism of action of nys, particularly to examine how the structural modifications in sterols can influence nys-sterol interaction. The sterols studied were chol, erg, 7-DHC and bras (Fig. 1). These sterols all have a C5–C6 unsaturation on ring B. Additionally, chol presents a C_8 side chain, whereas erg bears a methyl group on C-24. Erg also contains two extra unsaturations on the C7–C8 bond on ring B and on the C22–C23 bond of its aliphatic tail. In order to determine the influence of the sterol ring structure versus side chain on the spectroscopic and activity properties of nys, 7-DHC and bras were chosen because their structure is a combination of chol and erg, i.e., they have the ring system of one sterol with the side chain of the other and vice-versa (Fig. 1). Furthermore, 7-DHC is a biosynthetic precursor of chol and it has been suggested that the polyene antibiotic AmB presents higher affinity toward this sterol as compared to chol (e.g., [13]). Accordingly to these findings, Feigin [14] has recently reported on a new application of AmB related to cancer therapy: the author has proposed a way to replace chol by its precursor in hepatomas. Since the malignant hepatic cells are not able to regulate chol biosynthesis, 7-DHC would accumulate only in these cells. Due to the higher affinity of AmB for this sterol, the antibiotic would interact preferentially with the malignant cells, leading to their destruction.

Our results show that nys is able to permeabilize all type of sterol-containing vesicles. However, the kinetics and the structures formed are different: (i) in chol-containing vesicles the initial rate of pore formation was very slow and higher nys amounts were required to complete dissipate K^+ gradient; (ii) in the presence of both 7-DHC and bras, the kinetics and extent of K^+ efflux was increased, compared to chol; (iii) only erg was able to induce the formation of strongly fluorescent antibiotic aggregates together with an instantaneous gradient dissipation. These findings imply that the presence of extra double bonds either in the sterol ring (like 7-DHC) or side-chain (like bras) promote the formation of nys channels, but the two features (like in erg) are required for the formation of antibiotic-sterol

aggregates tightly packed and, consequently, that present a long mean fluorescence lifetime.

2. Materials and methods

2.1. Materials

Nys, chol, and the ionophores valinomycin and FCCP were from Sigma (St. Louis, MO). The phospholipids POPC and BrPC were obtained from Avanti Polar Lipids (Alabaster, AL) and the sterols erg and 7-DHC were from Fluka Chemika (Buchs, CH) whereas bras was purchased from Steraloids, Inc. (Newport, RI).). Pyranine and TMA-DPH were from Molecular Probes (Eugene, OR). All organic solvents were Uvasol grade from Merck.

2.2. Preparation of liposomes

POPC and sterol-containing POPC LUV with a mean diameter of 100 nm were prepared by the extrusion method [15] as previously described [16]. The resulting stock solutions were stored at 4 °C until used. The final phospholipid concentration was determined by phosphate analysis [17].

2.3. Nys partitioning experiments

Partition coefficients of nys were determined by a centrifugation method using brominated lipids [18]. The total and free nys concentrations were assayed by absorption spectrophotometry using $\varepsilon_{304}=7.4\times10^4$ M^{-1} cm^{-1} [19].

The mole-fraction partition coefficient, K_p, of nys [20] was calculated and used to compute the mean number of antibiotic molecules associated with a lipid vesicle, <A>, [21]:

$$\langle A\rangle = \frac{\mu x_i [N]_t}{[L]_t} \quad (1)$$

where, μ is the average number of lipid molecules per lipid vesicle, and $[N]_t$ and $[L]_t$ are the overall antibiotic and lipid concentration present in the sample.

2.4. Nys activity assays

H^+/K^+ exchange assays across the membrane bilayer were conducted as described previously to indirectly evaluate the effect of nys on the potassium permeability of the lipid vesicles [12]. Briefly, the decrease in the fluorescence intensity of the entrapped pH sensitive pyranine was used to follow the acidification of the intravesicular space of the liposomes prepared with a variable lipid composition. The activity assays were started by submitting the liposomes to a transmembrane K^+ gradient of 3.3:1 $[K^+]_{in}$:$[K^+]_{out}$. After 120 s, nys (5–25 μM final concentration) was added to the lipid suspension. The ionophore valinomycin was added to the liposomes after 300 s.

All the data were analyzed according to the formalism described in [12]. The percentage of potassium gradient dissipation was calculated from:

$$\%K^+\text{dissipation} = \left(\frac{pH_{in}^0 - pH_{in}^{300}}{pH_{in}^0 - pH_{in}^{val}}\right)\times 100 \quad (2)$$

where pH_{in}^0 and pH_{in}^{300} are the pH inside the lipid vesicles at times $t=0$ s and $t=300$ s and pH_{in}^{val} is the pH obtained after the addition of valinomycin.

The apparent initial rate of pore formation by nys in the liposomes, v_o, was determined from the initial slope of the function $p'(t)$, which is the apparent average number of pores per lipid vesicle that had been formed from $t=0$ to t [22–24]:

$$p'(t) = -\ln R(t) = -\ln\left(\frac{pH_{in}(t) - pH_{in}^{300}}{pH_{in}^0 - pH_{in}^{val}}\right) \quad (3)$$

where $R(t)$ is the retention function of the liposome population and $pH_{in}(t)$ is the time course of the pH decrease of the inner vesicular space of the liposomes, which is followed by the variation of fluorescence intensity of pyranine (see Results).

2.5. Absorption and fluorescence measurements

Absorption spectra were recorded at room temperature using a Jasco V-560 and steady-state fluorescence measurements were done in a SLM-Aminco 8100 Series 2 spectrofluorometer [16]. Nys and pyranine fluorescence measurements were carried out with $\lambda_{exc}=320$ nm and $\lambda_{em}=410$ nm and $\lambda_{exc}=460$ nm and $\lambda_{em}=510$ nm, respectively. Steady-state fluorescence anisotropy measurements of TMA-DPH ($\lambda_{ecx}=355$ nm and $\lambda_{em}=428$ nm) were obtained at a probe to phospholipid molar ratio of 1:100.

The time-correlated single-photon timing method was used to obtain the fluorescence intensity decays of nys. The instrumentation used and data analysis was described in detail elsewhere [25]. The samples were excited at 320 nm and the emission (at 410 nm) was detected at the magic-angle (54.7°) relative to the vertically polarized excitation beam. The fluorescence intensity decay curves were analyzed as a sum of exponential terms and the mean fluorescence lifetime was calculated as:

$$\langle\tau\rangle = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i \quad (4)$$

were α_i and τ_i are the normalized amplitude and lifetime of component i.

3. Results

3.1. Partition experiments

Lipid vesicles prepared with different sterols may influence the partitioning behavior of the polyene antibiotic nys either directly through the establishment of specific interactions between the antibiotic and the sterols present in the membrane, or indirectly by altering the bulk physical properties of the lipid bilayer. In order to address this issue, the partitioning of nys towards 5 and 10 mol% of the several sterols-containing lipid vesicles was measured. It was found that the partition of nys into the lipid vesicles was largely insensitive to the structure and sterol content of the liposomes, always presenting a K_p value of $(1.7\pm0.2)\times10^4$ ($n=8$) (Table 1).

3.2. Nys self-association in the membranes

It has been previously shown that the main factor controlling the fluorescence spectra and emission kinetics of nys is its oligomeric state and packing in the membrane [19,21]. These two properties were used here to monitor the formation of those supramolecular aggregates by nys in 5 and 10 mol% bras- and 7-DHC containing POPC LUV. The excitation and emission spectra of nys in lipid vesicles with 10 mol% of these sterols

Table 1
Mole-fraction partition coefficients of nystatin, K_p, for vesicles with different lipid composition (sterol-free and sterol-containing POPC LUV)

Sterol	mol%	K_p ($\times10^4$)
Cholesterol	5	1.5±0.2
	10	1.7±0.5
Brassicasterol	5	1.8±0.4
	10	1.8±0.5
7-Dehydrocholesterol	5	1.9±0.3
	10	1.9±0.7
Ergosterol	5	1.2±0.2
	10	1.5±0.4
POPC-only vesicles	–	1.4±0.2

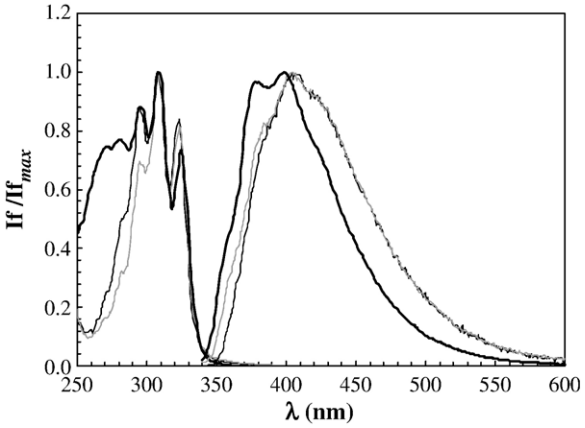

Fig. 2. Normalized excitation (λ_{em}=410 nm) and emission (λ_{exc}=320 nm) spectra of nystatin (20 μM) in the presence of (thick black) 10 mol% ergosterol-, (thin black) brassicasterol- and (gray) 7-dehydrocholesterol-containing POPC LUV.

were very similar to the ones formerly obtained with chol-rich lipid vesicles (Fig. 2). The emission spectra of the antibiotic presented a single emission band centered at 404 nm, whereas its excitation spectra exhibited the typical vibrational progression of the monomeric antibiotic molecule [21]. It was also found that both the sterol content of the lipid vesicles (5 and 10 mol%) and the antibiotic overall concentration in solution (between 2.5 and 25 μM) did not alter nys fluorescence spectra in these conditions. On the other hand, nys behavior in 5 and 10 mol% erg-containing POPC LUV was different: its emission spectra was blue-shifted with an emission maximum at 398 nm and its excitation spectrum now presented a vibrational progression typical of excitonic coupling [26]. In addition, both spectra were dependent on the erg molar fraction and nys concentration used, confirming that the nys-to-erg ratio in the membranes influenced the interaction between the two molecules, as previously shown [12]. Note that the transition dipolar moments of the cromophores must be close and parallel to each other in order to lead to excitonic coupling. Since excitonic coupling was only observed when erg was present in the vesicles, and was dependent both on sterol content and antibiotic concentration, it clearly shows a specific antibiotic–erg interaction.

Regarding nys photophysics, four-exponentials were always required to describe the fluorescence intensity decay curves of nys in interaction with sterol-containing liposomes. The best-fitting parameters (pre-exponentials and lifetimes) were antibiotic-concentration independent for chol-, bras- and 7-DHC-containing vesicles: nys presented two very fast (50–80 ps and 340–480 ps, respectively) and two slow (2.0–2.5 ns and 7.1–7.4 ns, respectively) fluorescence lifetimes in these POPC/sterol mixed bilayers, resulting in an intensity-weighted mean fluorescence lifetime (Eq. (4)) of 5.0±0.3 ns. In contrast, there was a sharp increase in nys mean fluorescence lifetime from approximately 4 ns to 20 and 37 ns upon increasing the antibiotic overall concentration in solution from 2.5 to 25 μM in the presence of 5 and 10 mol% erg-containing POPC LUV, respectively, in agreement with what was previously reported for this lipid vesicles [16]. The appearance of these long-lived antibiotic fluorescent species has been formerly ascribed to nys oligomerization in the membranes, which produces a strong immobilization of its polyene chains and a concomitant decrease of its non-radiative decay rates. These oligomers are attributed to the transmembrane aqueous pores formed by nys [12].

To further check for eventual changes undergone by the aggregation state of nys partitioned into the lipid vesicles, the steady-state fluorescence anisotropy of the antibiotic was also measured in the presence of 5 and 10 mol% of the several sterol-containing POPC LUV (Table 2). It was found that the anisotropy of nys was essentially invariant and independent of the type and sterol content of the lipid vesicles used for antibiotic 15, 20 and 25 μM. From its high value in all the types of vesicles ($\langle r\rangle$=0.28±0.01), it can be concluded that the antibiotic is, in all cases, strongly immobilized in the membrane, and from this parameter, no specific information on the interactions between nys and sterols is obtained. Furthermore, the steady-state fluorescence anisotropy of TMA-DPH, a fluorescent probe that is sensitive to surface alterations in the membrane [27], also did not change with the lipid vesicle composition within the antibiotic concentrations used ($\langle r\rangle$=0.196±0.002, Table 2). This is further evidencing that membrane-induced alterations are localized and no long range perturbations are occurring upon nys partition to the membrane.

3.3. Nys activity assays

The polyene antibiotic nys interacts with the plasma membrane of the antibiotic-sensitive organisms causing monovalent cation leakage [1]. Its effect on the permeability properties of the lipid bilayers can be used to monitor the formation of functional antibiotic species, which can be correlated with spectroscopic changes undergone by the antibiotic molecules. The variation of the fluorescence intensity of the pyranine dye entrapped inside the lipid vesicles has been used as a reliable spectroscopic method for measuring the activity of this antibiotic. As shown in Fig. 3A, in all POPC/sterol mixed bilayers, nys was able to induce a complete

Table 2
Effect of membrane sterol content on the steady-state fluorescence anisotropy of nystatin, $\langle r\rangle_{nys}$ (λ_{exc}=320 nm and λ_{em}=410 nm), and TMA-DPH, $\langle r\rangle_{TMA\text{-}DPH}$ (λ_{exc}=355 nm and λ_{em}=428 nm) in interaction with 1.4 mM sterol-containing POPC LUV

Sterol	mol%	$\langle r\rangle_{nys}$ (±0.01)	$\langle r\rangle_{TMA\text{-}DPH}$ (±0.001)
Cholesterol	10	0.28	–
Brassicasterol	5	0.28	0.194
	10	0.29	0.198
7-Dehydrocholesterol	5	0.29	0.197
	10	0.28	0.195
Ergosterol	10	0.30	–

The errors represent the standard deviation of 8 independent measurements carried out with antibiotic concentrations between 0–25 μM and 3 independent measurements carried out with antibiotic concentrations of 15, 20 and 25 μM for TMA-DPH and nystatin fluorescence anisotropy measurements, respectively.

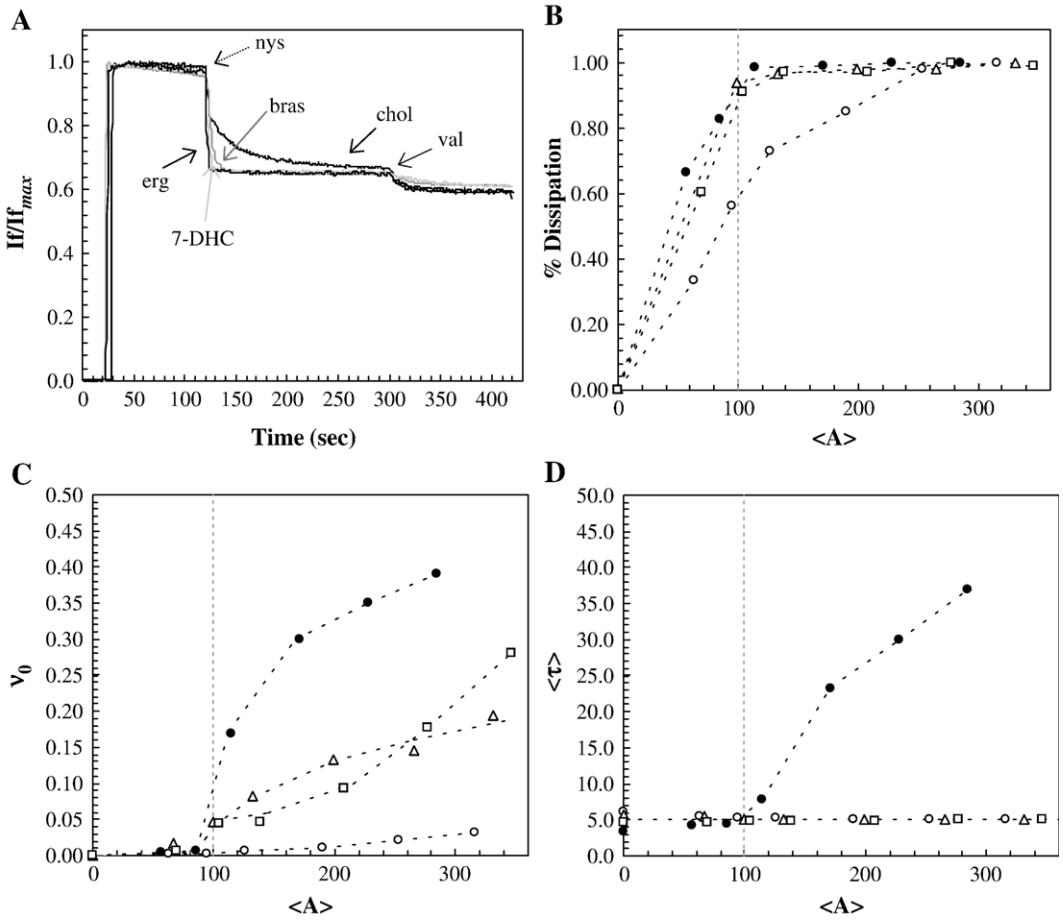

Fig. 3. (A) Pyranine fluorescence intensity variation upon addition of nys to 10 mol% sterol-containing POPC lipid vesicles (see Materials and methods for further details). Dependence of (B) the percentage of transmembrane K^+ gradient dissipated 300 s after the beginning of each assay (before val addition), (C) the apparent initial rate of pore formation, v_o, and (D) the intensity-weighted mean fluorescence lifetime of the antibiotic, $<\tau>$, on the mean number of membrane bound antibiotic molecules per liposome, $<A>$. 10 mol% cholesterol- (o), ergosterol- (●), brassicasterol- (△) and 7-dehydrocholesterol- (□) containing POPC were used in these experiments. The dashed vertical line at $<A>=100$ signals the onset of the sharp increase simultaneously detected in the initial rate of pore formation and in the mean fluorescence lifetime of the antibiotic in the presence of 10 mol% erg-containing liposomes.

dissipation of K^+ gradient. This was further verified upon the addition of Val, an ionophore that is able to complete dissipate the K^+ gradient. The small variation observed on the gradient after adding Val is only due to the effect of the solvent (see, e.g., [12]), showing that total gradient dissipation was already attained. However, the kinetics of dissipation was dependent on the type of sterol: whereas the addition of antibiotic to erg-containing vesicles lead to an instantaneous and total efflux of K^+, total gradient dissipation was only achieved at higher time in chol-containing vesicles. Substitution of chol by either 7-DHC or bras increased in a similar way the rate of K^+ efflux, but was still slower compared to erg. In order to make a quantitative comparison of nys activity in liposomes containing the different sterols tested, the percentage of transmembrane K^+ gradient dissipated 300 s after the beginning of each assay (Eq. (2)) (Fig. 3B), the apparent initial rate of pore formation (Eq. (3)) (Fig. 3C), and the intensity-weighted mean fluorescence lifetime of the antibiotic (Eq. (4)) (Fig. 3D), were plotted as a function of the mean number of membrane bound antibiotic molecules per liposome, $<A>$. Fig. 3 shows that: (i) membrane permeabilization occurs in all type of vesicles with different kinetics, and (ii) the long-lived antibiotic species are formed only in the presence of erg.

4. Discussion

The aim of this study was to investigate the influence of sterol side chain and nucleus structure on nys binding to the lipid vesicles and formation of active antibiotic species. In order to understand what are the sterols structural features that stabilize nys oligomers and increase their mean fluorescence lifetime, we studied nys interaction with POPC liposomes containing 5 and 10 mol% of bras and 7-DHC, which have the side chain of erg and the sterol nucleus of chol and vice-versa, respectively (Fig. 1), and in erg- and chol-containing lipid vesicles.

The first issue addressed was the influence that sterols exerted on nys partitioning to the lipid vesicles, since different types of sterols might induce different bulk membrane properties [28,29]. Chol and erg are known to order the phospholipid acyl chains in the membrane leading to phase separation [30,31] and sterols that similarly to erg also present

an extra unsaturation in the ring system, as 7-DHC, have been shown to have an even higher ability to induce this effect [29,32]. For the range of sterol concentration used in this study, the bulk membrane properties of chol- and erg-containing lipid vesicles were shown to be similar [30,31,33]. Nevertheless, it was essential to determine the partition of nys to the different sterol-loaded lipid vesicles, since specific interactions and not the type of phase could be the ruling factor determining eventual K_p variations. Our data showed that the partition of the polyene antibiotic nys was insensitive to both the type of sterol and its content on the lipid vesicles (5 and 10 mol%) (Table 1). It can then be concluded that the surface antibiotic concentrations reached in each type of lipid vesicles are very similar when the experiments are carried out with the same total lipid concentration in solution. Hence, the influence of this factor on the final extent of nys oligomerization in the lipid vesicles can be ruled out as a possible explanation for the variable spectroscopic properties presented by the antibiotic. The same applies regarding the observed differences in activity.

The fluorescence behavior of nys in the presence of bras- and 7-DHC POPC LUV was analogous to the one observed earlier with chol-containing lipid vesicles [12,16], namely both its excitation and emission spectra (Fig. 2) and its intensity-weighted mean fluorescence lifetime were independent on antibiotic concentration and sterol content. However, the activity assays revealed that when in the presence of 7-DHC and bras, nys was more efficient in dissipating the transmembrane potassium gradient (Fig. 3A, B and C): the initial rate of pore formation and extent of gradient dissipation was increased compared to chol/POPC mixed bilayers. These results raise questions about the nature of the active species formed by the antibiotic in each type of phospholipid vesicles. When in the presence of chol-containing vesicles, nys was still able to dissipate K^+ gradient, but at a much slower rate. Even at high antibiotic concentration, the initial rate of pore formation was small, suggesting that in this situation initial membrane permeabilization is occurring by a non-specific mechanism. Nevertheless, comparing with vesicles lacking sterol in its composition [12], ion efflux was higher showing that chol accelerates the permeabilization process, as also reported for AmB [34,35]. In fact, several studies using AmB suggest that, independently of its aggregation state, this antibiotic is able to permeabilize erg-containing lipid vesicles, but permeabilization of chol-containing or sterol-free lipid vesicles occurred only when in the aggregated or highly aggregated form, respectively [11,36,37]. Moreover, it was also suggested [36] that in these vesicles, AmB molecules would initially accumulated in the surface or inside the membrane originating defects. Subsequent association of antibiotic molecules within the membrane would result in the enlargement of the pre-existing defects, ultimately leading to pore formation. Regarding our data, is likely that a similar mechanism is taking place in chol/POPC vesicles: low nys concentration cause defects in the membrane which leads to a moderate leakage of K^+; at high antibiotic concentration, the initial efflux of ions is still slow, but total gradient dissipation occurs, suggesting that nys molecules are assembling. This is further supported by recent results showing that in ternary chol/erg/POPC liposomes chol competes with erg for the binding to nys and for antibiotic–sterol complex formation [33].

In the presence of erg, at all antibiotic concentrations gradient dissipation is total, and the process of pore formation is fast after a threshold nys concentration is reached. We proposed [12] a two-stage model for membrane permeabilization by nys in this type of vesicles. When the mean number of membrane-bound nys molecules per lipid vesicle is low ($<A>^* < 100$) the antibiotic molecules transiently induce structural defects in the membrane, perturbing its selective permeability. After reaching this threshold, the classical model of transmembrane channel formation occurs, leading to instantaneous and total gradient dissipation. Note that the definition of $<A>$ takes into account both the nys molecules that are adsorbed and inserted into the bilayer [21]. Identical antibiotic concentrations have similar effects in the gradient dissipation at 300 s (Fig. 3B) when 7-DHC and bras are present in the vesicles, but the initial rate of pore formation is slower (Fig. 3C). The formation of very compact and stable nys aggregates occurs only in the presence of erg, suggesting a direct participation in the formation of channel complex [7,35].

The fine details of the molecular arrangement adopted by the antibiotic molecules in the aggregates formed vary considerably, depending on the molecular structure of the sterol incorporated in the membrane. Apparently, all the chemical features that distinguish erg from chol, both in the ring and in the tail, are essential requisites for the formation of strongly and long-lived fluorescent oligomers by nys, presenting spectral alterations typical of excitonic coupling. The formation of pores with different sizes cannot be completely rule out, however, the size of the aggregates should not interfere in the photophysical properties of the antibiotic, since: (i) excitonic coupling can only be attributed to the close proximity and parallel arrangement between the transition moments of the chromophores [26]; (ii) the long fluorescence lifetime is due to the decrease of the non-radiative excited state deactivation upon the increase of Nys rigidity. In addition, since nys partition is independent both on sterol type and content, formation of more pores in erg-containing vesicles is not a likely process. If the sterol molecules facilitate the assembly of the channel by acting as a "glue" that links the nys monomers together [38], then the increased planarity, rigidity and polarizability of ring B with a 5,7-diene, as in erg and 7-DHC, is expected to promote a tighter packing with the macrolide polyene antibiotic by an enhancement of van der Waals forces, rigidifying the polyene chains. Therefore, the non-radiative deactivation pathways of the antibiotic decrease and, consequently, nys mean fluorescence lifetime rises from around 5 to 37 ns. The importance of these structural features of sterols for nys activity is supported by experiments showing that nys toxicity was highly dependent on the presence of sterols carrying a Δ5,7-dienic structure [39]. Other studies have also emphasized the importance of an extra double bond in the sterol structure relatively to chol for an increased strengthen of the hydrophobic interactions between the polyene antibiotics and the sterols. However, there is no consensus regarding its optimum positions: (i) some authors point that an extra double bond in the carbon C7 of the ring

system, as in the case of erg and 7-DHC, is of fundamental importance (e.g., [13,37]), whereas others (ii) suggest that the additional unsaturation should be present at the sterol side chain in carbon C22, like in erg and bras [40,41]. The present study, however, demonstrates that the appropriate conjugated system of the sterol nucleus is not enough, and an adequate sterol tail is also necessary to stabilize nys oligomers because 7-DHC, which lacks a methyl group at position 24 but has a double bond configuration of the B ring identical to that of erg, failed to induce the formation of long-lived fluorescent antibiotic aggregates. The simultaneous introduction of a double bond at position 22 and the addition of an alkyl group at position 24 make the sterol side chain of erg bulkier and stiffer, characteristics that seem to help to stabilize the interaction between nys and the sterol. Therefore, the sterol–antibiotic complexes formed are very compact, and also putatively more stable [6,8]. However, regarding the activity of nys, it should be stressed that the single introduction of alterations either in the ring or in the tail increases the antibiotic ability to permeabilize the membranes showing that they are essential for nys-sterol interaction. In addition, it was clearly shown in the present study that 7-DHC-containing liposomes present higher sensitivity to the antibiotic action, as compared to chol-rich vesicles, which makes 7-DHC suitable for replacing chol in hepatomas, as suggested by Feigin [14], for a new application of polyene antibiotics in cancer therapy.

Our findings provide an example of how slight structural alterations of sterols can affect the architecture of the channel structures formed by nys, which in turn must be responsible for its antifungal action and drug toxicity in mammalian cells.

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