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EFFECT OF THE LIPID COMPOSITION OF *MYCOPLASMA MYCOIDES* SUBSPECIES *CAPRI* AND PHOSPHATIDYLCHOLINE VESICLES UPON THE ACTION OF POLYENE ANTIBIOTICS

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SUMMARY

(1) The effects of filipin and amphotericin methyl ester upon the K^+ efflux from *Mycoplasma mycoides* subsp. *capri* and egg lecithin sonicated vesicles were investigated. Both the nature of the sterol and the composition of the membranes affected the sensitivity to each polyene antibiotic.

(2) *M. mycoides* subsp. *capri* containing ergosterol was much more sensitive to amphotericin methyl ester than cells containing cholesterol. Cholesterol-containing cells were about twice as sensitive to filipin as the ergosterol-containing cells. These results were confirmed with phosphatidylcholine vesicles.

(3) At 2 °C the filipin sensitivity of *M. mycoides* subsp. *capri* was independent of the membrane cholesterol content and the sensitivity towards amphotericin methyl ester decreased when the membrane cholesterol content was increased, in contrast to the results at 20 °C.

(4) At 2 °C, sterol-free egg lecithin vesicles became very sensitive to both filipin and amphotericin methyl ester and the presence of cholesterol in the vesicles did not increase the sensitivity further. At high concentrations of cholesterol (> 30 mol %), the polyene antibiotic sensitivity, particularly towards amphotericin methyl ester, was greatly reduced.

INTRODUCTION

This study was designed to correlate polyene antibiotic sensitivity of *M. mycoides* subsp. *capri* (*Mycoplasma* PG3) with its membrane lipid composition, and growth of this organism in the presence of ergosterol or cholesterol was chosen as a model membrane system for fungal and animal cells, respectively. The sterol requirement for polyene antibiotic sensitivity in mycoplasmas and acholeplasmas has been reported [1–4], but the exact membrane lipid compositions of these organisms were not determined. A detailed lipid analysis of *Mycoplasma* PG3 grown under the conditions used in this study has already been published [5].

Artificial lipid membranes have been used in many studies with polyene antibiotics, and investigations of the K^+ efflux from unsonicated liposomes has demonstrated the importance of sterol in egg lecithin liposomes for polyene sensitivity [4, 6]. However, in multilamellar systems it seems that only the sterol in the outermost layer of the liposomes interacts with the polyene antibiotic [4]. Sonicated vesicles are considered good systems for studying the lipid bilayer [7] and its interaction with polyene antibiotics, and so were used in this study in preference to multilamellar systems.

METHODS

Materials

The sources of amphotericin methyl ester and filipin have been reported [8]. The filipin used in this study is more correctly referred to as the filipin complex, although at the concentrations used in this work the stringent cholesterol requirement of the major component (Filipin III) will predominate [9]. Egg lecithin was prepared and purified as described by Bangham et al. [10].

The organism

The source, growth and lipid analysis of *Mycoplasma* PG3 has been described previously [5]. In this work, the organism was grown in a modified Edward medium [11] supplemented with 1 mg fatty acid-free bovine serum albumin (Sigma) ml^{-1}, 0.025 mM sodium oleate, 0.025 mM sodium palmitate and sterol at the required concentration. For studies of K^+ efflux, the cells were harvested at the late exponential phase of growth by centrifugation on an MSE 18 high speed centrifuge for 15 min at $15\,000 \times g$, washed three times in 0.02 M Tris · HCl (pH 7.0) containing 0.01 M $MgCl_2 \cdot 6\,H_2O$ and then twice in 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose, before being resuspended in 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose at a concentration of 0.5 mg dry wt./ml.

Sonicated vesicles

Egg lecithin or egg lecithin and sterol at the required molar ratio was added to a flask as a solution in chloroform, and the solvent removed by rotary evaporation. 0.15 M KCl was added so that the final lipid dispersion contained 1 % lipid by weight. The lipid was resuspended by vortex mixing for 1–2 min in the presence of a few glass beads. The liposome suspension was then placed in a sonic bath (Dawe sonicleaner type 6441A; Dawe Instruments Ltd., London) operating at 50 kHz and 80 W. Sonication was continued for 1 h under N_2 at 20 °C. A decrease in absorbance occurred during this period but no further decrease occurred after 1 h, when electron microscopy of negatively stained preparations demonstrated that a preparation of microvesicles (diameter $< 0.05\ \mu m$) of comparable uniformity to Fraction II described by Huang [12] was present.

After sonication, the vesicle preparation was separated from exogenous KCl by passage down a 25×2 cm column of Sephadex G-50 equilibrated with 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose.

Estimation of K^+ efflux

K^+ was estimated using a K^+-sensitive electrode (type BH 115, Electronic

Instruments Ltd., Richmond, Surrey) connected to a measuring unit, Model C33B-2 and Vibron Electrometer, Model 33B-2 (Electronic Instruments Ltd.) recording on a potentiometeric recorder (Servoscribe RE 511.20, Smith Industries Ltd., Wembley, Middlesex). The salt bridge, connecting the cell suspensions to the K^+ electrode, contained 0.1 M Tris · HCl (pH 7.5). The equipment was calibrated to allow determinations of rates of K^+ efflux, and the total K^+ released.

Suspensions of washed cells (0.5 mg · ml^{-1}) recorded K^+ concentrations less than 10^{-6} M and did not leak K^+ under these conditions, at 20 °C or 2 °C. All vesicle preparations (0.1 %, by wt.) were stable at 20 °C and were only slightly leaky at 2 °C (this basal leakage rate was subtracted from observed rates in the presence of polyene antibiotics). Filipin was added from a 1 mg · ml^{-1} solution in 96 % ethanol, and amphotericin methyl ester from 1 mg · ml^{-1} solution in dimethylsulphoxide. Ethanol and dimethylsulphoxide alone, at these concentrations, did not result in K^+ efflux from cells or vesicles.

K^+ efflux from *Mycoplasma* PG3 commenced immediately after the addition of polyene antibiotic and continued at a linear rate until the intracellular K^+ became depleted. The K^+ efflux from cells is expressed as nequiv K^+ released · min^{-1} · mg^{-1} dry wt. of cells. The K^+ efflux from vesicles occurred immediately after addition of the antibiotic. The K^+ efflux induced by amphotericin methyl ester continued at this linear rate until the internal K^+ became depleted, but when induced by filipin the initial rate decreased after about 2 min. The K^+ efflux from vesicles was expressed as the percentage of the total K^+ enclosed by the vesicles in the 20 ml incubation volume which was released min^{-1}. The total K^+ enclosed by a vesicle preparation was determined by incubating 0.1 ml of the preparation with 0.1 ml 10 % (v/v) Triton X-100 at 100 °C for 0.5 min. This was then cooled and the sample assayed for K^+ using the K^+ electrode.

RESULTS

K^+ efflux from Mycoplasma PG3 in the presence of polyene antibiotics

The K^+ efflux from *Mycoplasma* PG3 grown in medium supplemented with 0.08 mM, 0.04 mM and 0.01 mM cholesterol (Fig. 1) demonstrates that cells with a high cholesterol content were more sensitive (at 20 °C) to both filipin and amphotericin methyl ester than cells with a low cholesterol content. This was also true for the cells grown in the presence of ergosterol (Figs. 2 and 3). The effect on membrane fluidity of an alteration in sterol content is opposed by a change in the ratio of saturated to unsaturated fatty acids [5, 13]. It is apparent, though, that the polyene sensitivity of *Mycoplasma* PG3 depends on both the sterol present in the plasma membrane and the particular polyene antibiotic. At 20 °C, cholesterol-containing *Mycoplasma* PG3 was approximately as sensitive to filipin as it was to amphotericin methyl ester, but growth in the presence of ergosterol distinguished the actions of these antibiotics. Ergosterol-grown cells were about 20 times more sensitive to amphotericin methyl ester than to filipin. Also by comparison with the cholesterol-grown *Mycoplasma* PG3, the ergosterol-containing cells were about 10 times more sensitive to amphotericin methyl ester but about half as sensitive to filipin.

At 2 °C, the sensitivities of *Mycoplasma* PG3 grown in the presence of cholesterol to both filipin and amphotericin methyl ester were different from those at 20 °C

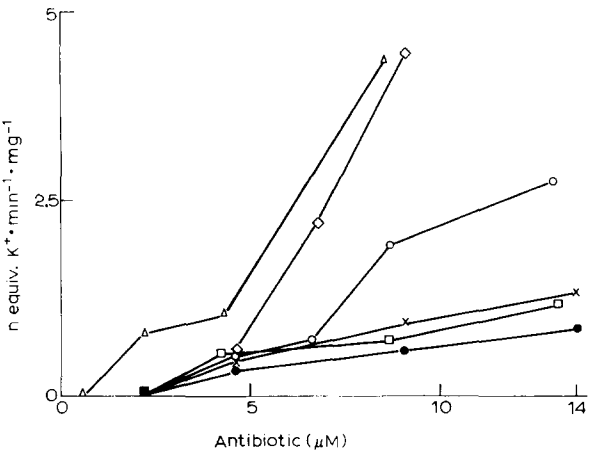
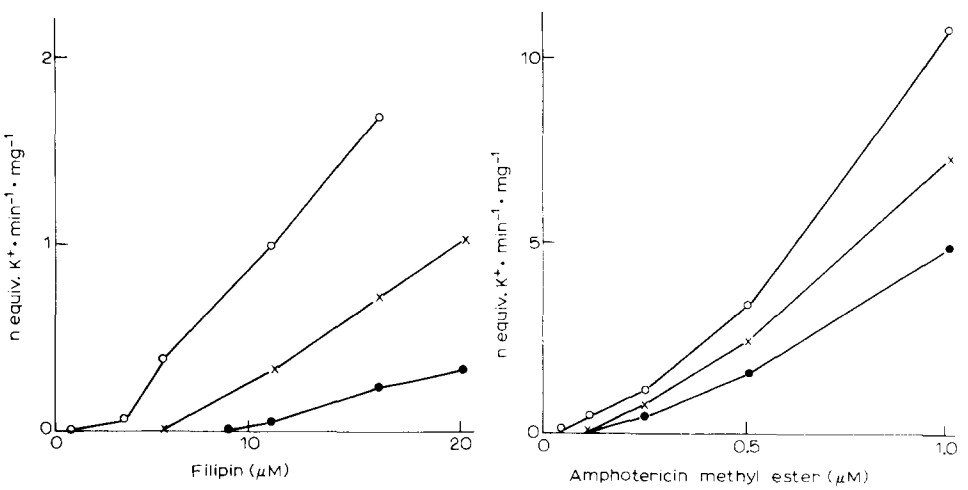


Fig. 1. K^+ efflux from cholesterol-containing *Mycoplasma* PG3 in the presence of filipin and amphotericin methyl ester at 20 °C. Cells were grown in Edward medium containing 0.08 mM, 0.04 mM and 0.01 mM cholesterol. The cells were harvested at the late logarithmic stage of growth and washed three times in 0.02 M Tris · HCl (pH 7.0) containing 0.01 M $MgCl_2 \cdot 6\ H_2O$ and twice in 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose before being resuspended at a concentration of 0.5 mg · ml^{-1} of the final washing buffer at 20 °C. Filipin: ◇, 0.08 mM cholesterol; ×, 0.04 mM cholesterol; ●, 0.01 mM cholesterol. Amphotericin methyl ester: △, 0.08 mM cholesterol; ○, 0.04 mM cholesterol; □, 0.01 mM cholesterol.



Figs. 2 and 3. K^+ efflux from ergosterol-containing *Mycoplasma* PG3 in the presence of filipin (Fig. 2) and amphotericin methyl ester (Fig. 3) at 20 °C. Cells were grown in Edward medium containing 0.08 mM, 0.04 mM and 0.01 mM ergosterol. The cells were harvested at the late logarithmic stage of growth and washed three times in 0.02 M Tris · HCl (pH 7.0) containing 0.01 M $MgCl_2$, 6 H_2O and twice in 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose. The cells were then suspended at a concentration of 0.5 mg · ml^{-1} of the final washing buffer at 20 °C. Ergosterol in medium: ○, 0.08 mM; ×, 0.04 mM; ●, 0.01 mM.

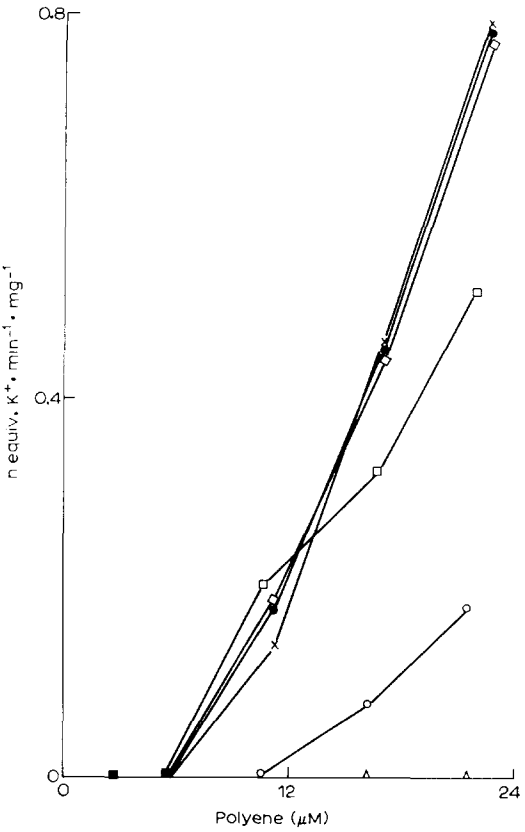
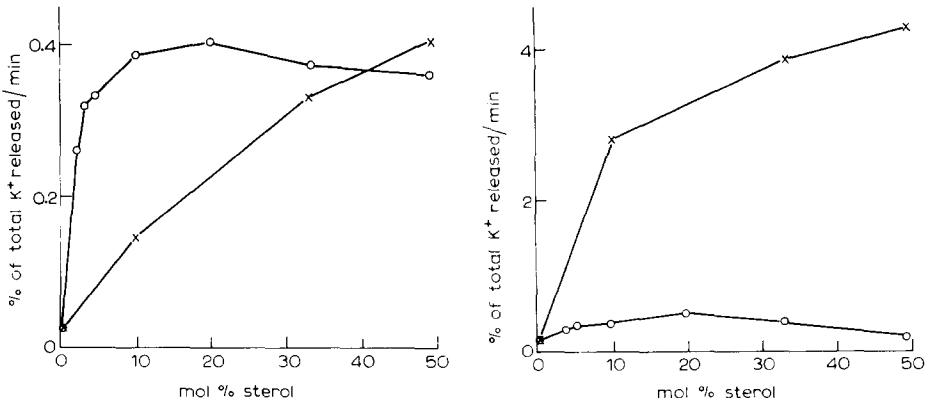


Fig. 4. The K^+ efflux from cholesterol-containing *Mycoplasma* PG3 in the presence of filipin and amphotericin methyl ester at 2 °C. Cells were grown in Edward medium containing 0.08 mM, 0.04 mM and 0.01 mM cholesterol. The cells were harvested at the late logarithmic stage of growth and washed three times in 0.02 M Tris · HCl (pH 7.0) containing 0.01 M $MgCl_2 \cdot 6\ H_2O$ and then twice in 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose. The cells were then suspended at a concentration of 0.5 mg · ml^{-1} of the final washing buffer at 2 °C. Plus filipin: ( ), 0.08 mM cholesterol; ×, 0.04 mM cholesterol; ●, 0.01 mM cholesterol. Plus amphotericin methyl ester: △, 0.08 mM cholesterol; ○, 0.04 mM cholesterol; □, 0.01 mM cholesterol.

(Fig. 4). All K^+ efflux values were reduced in comparison to the values at 20 °C, which probably reflects the reduced solubilities of both filipin and amphotericin methyl ester at low temperatures. At 2 °C, *Mycoplasma* PG3 was sensitive to filipin irrespective of the lipid cholesterol content and, in direct contrast to the results at 20 °C, the sensitivity of the cells to amphotericin methyl ester decreased with increasing cholesterol content of the cells. This situation is not comparable with the effect of temperature on the sensitivity of *Acholeplasma laidlawii* to filipin [1]. A loss of filipin sensitivity at 37 °C was prevented by incubation of *A. laidlawii* at 2 °C. This was probably the case because at 37 °C a metabolic conversion of membrane-located cholesterol to a glucoside derivative occurred, a function which *Mycoplasma* PG3 is incapable of.

K^+ efflux from lipid vesicles in the presence of polyene antibiotics

The effect of filipin at a concentration of 10 μg · ml^{-1} and amphotericin methyl



Figs. 5 and 6. The K^+ efflux from egg lecithin sonicated vesicles containing cholesterol and ergosterol in the presence of 10 μg filipin/ml (Fig. 5) and 0.5 μg amphotericin methyl ester/ml (Fig. 6) at 20 °C. The vesicles were prepared by dispersing egg lecithin plus sterol (from 0–50 mol %) in 0.015 M KCl at 20 °C and sonicating under N_2 for 1 h. The exogenous KCl was removed by gel filtration on Sephadex G-50 and the vesicles were suspended in 0.05 M Tris · HCl (pH 8.0) containing 0.125 M sucrose at a concentration of 0.1 % lipid (by wt.) at 20 °C. The K^+ efflux in the presence of polyene antibiotic is expressed as % of total K^+ enclosed by the vesicles in the 20 ml incubation volume which was released per min. The total K^+ enclosed was determined by incubating 0.1 ml of the vesicle suspension with 0.1 ml 10 % (v/v) Triton X-100 at 100 °C for 0.5 min. This was then cooled and assayed for K^+ using the K^+ electrode. ○, cholesterol; ×, ergosterol.

ester at a concentration of 0.5 $\mu g \cdot ml^{-1}$ on the K^+ efflux from egg lecithin vesicles containing different amounts of different sterols at 20 °C is illustrated in Figs. 5 and 6, respectively. Pure phosphatidylcholine vesicles were only slightly sensitive to either filipin or amphotericin methyl ester at this temperature, and incorporation of sterol into the vesicles increased their sensitivity to polyene antibiotics. It is interesting to note that under these conditions the incorporation of cholesterol into the vesicles beyond 10–20 mol % did not further increase their polyene sensitivity, which did not occur with ergosterol-containing vesicles. As was found for whole cells of *Mycoplasma* PG3, cholesterol conferred on the vesicles a greater sensitivity to filipin than to amphotericin methyl ester, and vice versa for ergosterol. The difference between the sensitivities towards filipin and amphotericin methyl ester was more marked in ergosterol-containing vesicles than cholesterol-containing ones.

At 2 °C, the sensitivity of sterol-free egg lecithin vesicles to both filipin and amphotericin methyl ester increased dramatically (Table I) and the presence of cholesterol did not increase the sensitivity significantly. Sensitivity towards amphotericin methyl ester was greatly reduced in vesicles with high cholesterol contents (> 30 mol %). The sensitivity of sterol-free vesicles is all the more remarkable since an overall decrease in sensitivity was expected because of the reduced solubility of both polyene antibiotics at 2 °C.

Work with thin lipid membranes has shown that an increase in temperature decreases ion conductance in the presence of nystatin [14, 15]. Although the K^+ efflux from sterol-free vesicles in the presence of filipin and amphotericin methyl ester was greater at 2 °C than at 37 °C, the opposite effect has been observed with sterol-containing vesicles and *Mycoplasma* PG3.

TABLE I

The K^+ efflux from egg lecithin sonicated vesicles containing cholesterol in the presence of filipin and amphotericin methyl ester at 20 °C and 2 °C. The K^+ efflux in the presence of polyene antibiotic is expressed as the % of the total K^+ enclosed by the vesicles in the 20 ml incubation volume which was released per min. In the absence of polyene antibiotic there was no basal leakage of K^+ from the vesicles at 20 °C but the efflux values from vesicles at 2 °C have been corrected for the small degree of K^+ efflux in the absence of polyene antibiotic.

Mol % cholesterol	% Total K^+ released/min							
	20 °C				2 °C			
	0	10	33.3	50	0	10	33.3	50
Filipin ($\mu g \cdot ml^{-1}$)								
0.5	0.012	0.146	0.061	0.059				
1	0.021	0.177	0.132	0.124	0.005	0.040	0.034	0.050
2	0.022	0.220	0.144	0.121	0.035	0.084	0.049	0.050
5	0.025	0.252	0.232	0.229	0.111	0.144	0.116	0.081
10	0.025	0.398	0.345	0.348	0.164	0.156	0.150	0.114
15	0.031	0.596	0.480	0.488	0.184	0.186	0.187	0.116
Amphotericin methyl ester ($\mu g \cdot ml^{-1}$)								
0.1	0.036	0.058		0.059				
0.15		0.117	0.122	0.087				
0.2	0.054	0.152	0.195		0.079	0.060		
0.5	0.104	0.364	0.343	0.195	0.121	0.161	0.139	
0.75			0.488	0.240	0.191	0.232		
1	0.153	0.916	1.08	0.432	0.314	0.272	0.193	0.10
2					0.703	0.61	0.258	0.031
5				1.37			0.800	0.091
10								0.214
15								0.353

DISCUSSION

The membrane sterol composition of *Mycoplasma* PG3 does not alter in isolation when grown in media containing sterol at different concentrations [5], though it is clear from this work that both the nature and the amount of sterol in a membrane are important in determining the sensitivity to a particular polyene antibiotic, supporting proposals which have been made previously by a number of authors [4, 8, 16–20]. However, at a temperature that approaches the lipid transition temperatures for both *Mycoplasma* PG3 and egg lecithin vesicles, the sterol dependance of the polyene action was not apparent. It has been suggested that cholesterol decreases the amphotericin B sensitivity of artificial phosphatidylcholine liposomes at a temperature below the transition temperature of the phosphatidylcholine [21]. 2 °C approaches the transition temperatures in this study, and it is clear that lipid fluidity has a profound effect upon the sensitivity to polyene antibiotics, and particularly to amphotericin methyl ester. At 2 °C *Mycoplasma* PG3 was not osmotically fragile and did not leak K^+ in the absence of polyene antibiotics and at this temperature the vesicles were only slightly leaky so it is unlikely that these results can be explained by osmotic swelling and breakage of the cells or vesicles as has been suggested [6].

The insensitivity of *Mycoplasma* PG3 and *Candida albicans* [8] to amphotericin methyl ester at reduced temperatures is in contrast to the increased sensitivity of *A. laidlawii* to amphotericin B at reduced temperatures [6]. It must be remembered, though, that the sterol content of the membrane lipid of *A. laidlawii* [22] is low in comparison to that of *C. albicans* [23] and *Mycoplasma* PG3 [5]. The role of sterol in acholeplasmas may be different from that in mycoplasmas as the cholesterol content of *A. laidlawii* did not affect the fatty acid composition [22] or glycolipid content [24] of the membrane lipid. Organisms lacking membrane sterol (or with very little membrane sterol) may regulate their membrane fluidity differently from organisms with a high membrane sterol content [25], so the results with *Mycoplasma* PG3 may not be incompatible with those obtained with *A. laidlawii*. The importance of both membrane sterol and membrane fluidity for polyene antibiotic sensitivity is clear, although it may not be sufficient to invoke the role of sterol as merely providing an ordered region of membrane lipid because a direct interaction of polyene antibiotic with membrane sterol has previously been shown [6, 26].

The modes of action of filipin and amphotericin methyl ester have been clearly distinguished. It has been proposed that filipin forms large aggregates with sterol within a membrane leading to membrane disruption whereas amphotericin B (and amphotericin methyl ester) associates with membrane sterol to form pores [27–29]. Although the value for the dissociation constant of the dehydroergosterol-amphotericin methyl ester complex was erroneous [30], the reversible nature of this association was demonstrated and other polyene-sterol associations have been shown to have different affinities [19] and be reversible [31]. Amphotericin methyl ester is far more active against ergosterol-containing membranes than against cholesterol-containing ones and a mechanism to account for this has been proposed [30]. Filipin has a greater affinity for cholesterol than for ergosterol [19] but has to remove cholesterol from a tight association with the membrane phospholipids [32], whereas, although the affinity of filipin for ergosterol is lower [19], so is the affinity of ergosterol for membrane phospholipids [32]. Thus, although ergosterol-containing membranes are far more sensitive to amphotericin methyl ester than are cholesterol-containing ones, the sensitivities towards filipin would not be expected to be very different.

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