EFFECTS OF POLYENE MACROLIDE ANTIBIOTICS ON NORMAL AND PROTOPLAST TYPE L-FORM CELLS OF *ESCHERICHIA COLI* W1655F+

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The action of the polyene macrolide antibiotics mycotrienin, pimaricin, lucensomycin, tetramycin, rimocidin, nystatin, filipin, lagosin, pentaene antibiotic 2814P, flavomycoin, flavofungin, hexaene antibiotic 5001P, and candicidin, including perhydro derivatives of them, on wall-less stable protoplast type L-form and normal rod form cells of *E. coli* W1655F+ was studied. No inhibition of the normal rod form cells was detected. In contrast to these results the growth of the L-form cells was inhibited by all of the substances tested, with the exception of pimaricin.

Further experiments have shown that the differences in sensitivity of normal and L-form cells cannot be explained by differences in sterol content, the target site of polyene antibiotics in sensitive eukaryotic cells. According to our results it is obvious that the cell wall of the normal cells functions as a penetration barrier to polyene antibiotics.

Polyene macrolide antibiotics are active against yeasts, fungi and other eukaryotic cells, but, with a few exceptions, are inactive against bacteria[1]. Results given by other authors indicate that polyene antibiotics produce lethal permeability alterations of the cytoplasmatic membrane in microorganisms containing sterols in their membranes. The resistance of bacteria is generally explained by the absence of sterols in these microorganisms[2,3].

In contrast to these results we found that polyene antibiotics and perhydro derivatives of them exhibit an antibacterial activity, too, if the wall-less stable protoplast type L-form of *E. coli* W1655F+ is used as a test organism[4]. The results are discussed with respect to sterol content of the normal rod form and the L-form cells, as well as with respect to permeation experiments with EDTA-pretreated normal rod form cells of the same strain.

Materials and Methods

Antibiotics

The polyene antibiotics employed were kindly supplied by the following authors: mycotrienin (Dr. CORONELLI, Lepetit, Milan, Italy); pimaricin (Dr. HOOGLAND, Gist-Brocades, N.V. research and development, Delft, Holland); tetramycin (DORNBERGER *et al.*[5]); lucensomycin (Dr. CASSINELLI, Farmitalia, Italy); rimocidin (Dr. FALKOWSKI, Polytechnical University, Gdansk, Poland); nystatin (our production); antibiotic 2814P (THRUM and DCHO[6]); filipin (Dr. WHITFIELD, Upjohn Company, Kalamazoo, USA); lagosin (Dr. HERRMANN, Glaxo Research Ltd., Greenford, England); antibiotic 5001P (Dr. KLEINWÄCHTER, ZIMET, Jena, GDR); candicidin (our production); flavofungin (Dr. POZSGAY, Pharmaceutical Works, Debrecen, Hungary); flavomycoin (SCHLEGEL and THRUM[7]). The antibiotics were dissolved in methanol (2 μg/ml) immediately before use. Perhydro derivatives of polyene antibiotics were prepared by catalytic hydrogenation in acetic acid with platinum dioxide as catalyst. The absence of the polyene systems was controlled spectrophotometrically.

Microorganisms and culture media

Experiments were performed with the normal and L-form of *E. coli* W1655F^{+}[8,9]. As the L-form does not grow in synthetic media, both, the L-form and normal cells, were cultured in nutrient broth (pH 7.2) extracted, prior to use, with ether (rest sterol content 0.027 μg/ml). In the experiments with EDTA the normal cells were grown in a synthetic medium containing 15 g $Na_2HPO_4 \cdot 12H_2O$, 3 g KH_2PO_4, 1 g NH_4Cl, 246 $MgSO_4 \cdot 7H_2O$, 0.27 mg $FeCl_3 \cdot 6H_2O$, 22 mg $CaCl_2 \cdot 6H_2O$, 10 g glucose, and 20 mg methionine in 1,000 ml aqua dest.

In all experiments the bacterial cells were grown in 25 ml medium in 100 ml flasks at 37°C on a rotary shaker as described previously[11]. After inoculation with over-night cultures the medium contained about 1×10^7 normal cells per ml and about $1 \sim 2 \times 10^7$ L-form cells per ml. Growth was measured with the spectral photometer "Spekol" (VEB Carl Zeiss, Jena) at 640 nm in cuvettes of 0.5 ml.

For the treatment with EDTA normal cells of *E. coli* W1655F^{+} were harvested by centrifugation and washed twice with 0.12 M Tris-HCl buffer pH 8.0. The washed cells were resuspended in the same buffer with EDTA (final concentration 10^{-4} M) and shaken for one minute. The exposure to EDTA was terminated by tenfold dilution with the above mentioned synthetic medium[10]. Immediately after dilution the antibiotics were added at the concentrations indicated.

Assay of sterol content

The L-form and normal cells of *E. coli* W1655F^{+} were cultured on nutrient broth extracted with ether (rest sterol content 0.027 μg/ml). In addition to this the normal cells were also grown on a synthetic medium without sterols. The cells were collected by centrifugation in the cold and dried by lyophilization. After saponification of the cell material with methanolic KOH, the methanol was evaporated and the resulting solution extracted with ether, five times. The sterol-containing extracts were combined, washed with water, dried with sodium-sulfate, and concentrated *in vacuo*. Separation was carried out on alumina using a benzene-hexane gradient of 0~100 % benzene, followed by a gradient of 0~2 % methanol in benzene. The identification and quantitative determination of the sterols were performed by gaschromatography with a Varian 2740 instrument, using a 2.5 m × 3 mm I.D. glass column packed with 2 % XE 60 on gaschrom Q (80~100 mesh). The carrier gas was argon. The column temperature was 200°C, the injector temperature 235°C and the detector temperature (FID) 235°C.

Assay of phospholipidphosphate

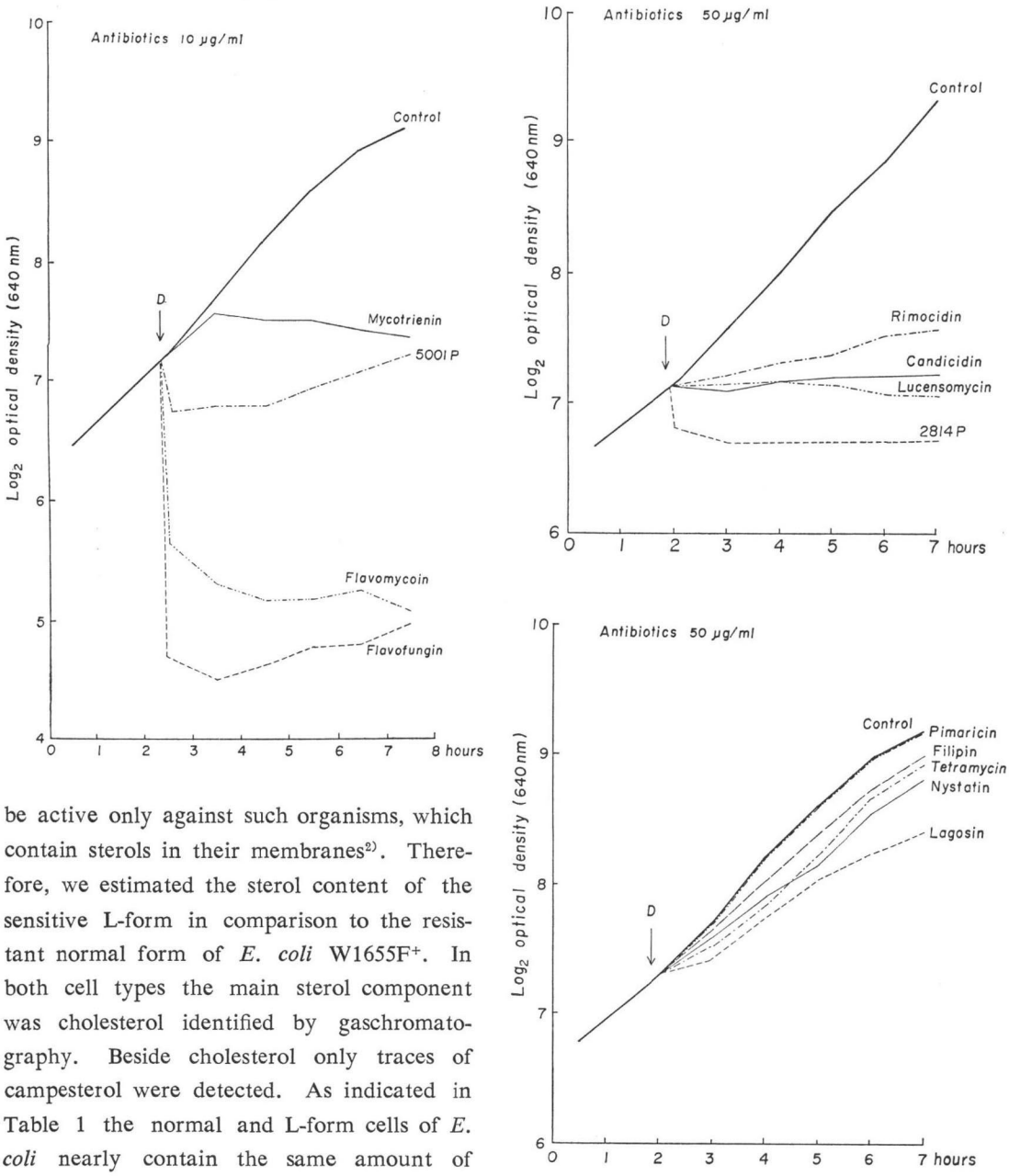
Aliquotes of the lyophilized cells were extracted with a mixture of chloroform-methanol (2:1). The extracts were dried *in vacuo*. After digestion in a perchloric-sulphuric acid mixture (1:1) the phosphate was determined in the residue as described by GERLACH and DEUTICKE[12].

Results

In Fig. 1 the effects of 13 polyene antibiotics on exponentially growing cultures of L-form cells of *E. coli* W1655F^{+} are shown. All of the polyene antibiotics tested, except pimaricin, inhibited the growth of L-form cells, while none of them influenced the growth of normal cells. Mycotrienin, hexaene antibiotic 5001P, flavofungin, and flavomycoin belong to the group with the strongest effects. Within this group flavomycoin and flavofungin cause a strong lysis of the L-form cells immediately after drug application. The pentaene antibiotic 2814P, lucensomycin, candicidin, and rimocidin induce growth inhibition of L-form cultures to an extent of 80 to 100 % at a concentration of 50 μg/ml. The antibiotics with the lowest effects are filipin, tetramycin, nystatin, and lagosin.

According to results of studies with eukaryotic organisms polyene antibiotics are said to

Fig. 1. Effect of polyene antibiotics on the growth of the wall-less L-form cells of *E. coli* W1655F+. *D*=drug application.



be active only against such organisms, which contain sterols in their membranes[2]. Therefore, we estimated the sterol content of the sensitive L-form in comparison to the resistant normal form of *E. coli* W1655F+. In both cell types the main sterol component was cholesterol identified by gaschromatography. Beside cholesterol only traces of campesterol were detected. As indicated in Table 1 the normal and L-form cells of *E. coli* nearly contain the same amount of sterols, which is very low in comparison to the sterol content of eukaryotic organisms sensitive to polyene antibiotics. This means that the differences in sensitivity of normal and L-form cells cannot be explained by differences in sterol content.

Further experiments have shown that the resistant normal rod form cells of this *E. coli* strain may become sensitive, too, if the permeability of the cell wall of these cells is raised by treatment with EDTA. This is demonstrated in Fig. 2 for the polyene antibiotics mycot-

rienin, antibiotic 5001P, and flavofungin. The inhibition of the EDTA pretreated cells by polyene antibiotics is very strong immediately after drug application. After one hour the antibiotic effects decrease because the cells repair the EDTA defect of their permeability

Table 1. Sterol content in normal and L-form cells of *E. coli* W1655F+.

E. coli	Growth medium (μg sterol/experiment)	Dry weight (g)	Phospho-lipid-phosphate (mg)	Sterol content		
				μg	% of dry weight	% of phospho-lipidphosphate
L-Form	Nutrient broth, extracted with ether (34.0)	1.3	24.5	12.5	0.00096	0.046
Normal form	Nutrient broth, extracted with ether (14.8)	1.3	13.4	6.2	0.00047	0.051
Normal form	Synthetic medium (0)	1.3	18.4	13.0	0.0010	0.071

Fig. 2. Effect of polyene antibiotics on the growth of EDTA-pretreated normal cells of *E. coli* W1655F+.
Drug application at point zero.

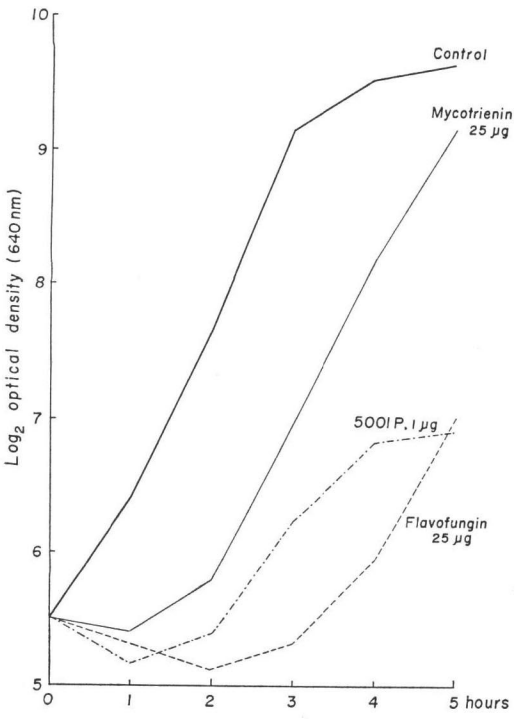
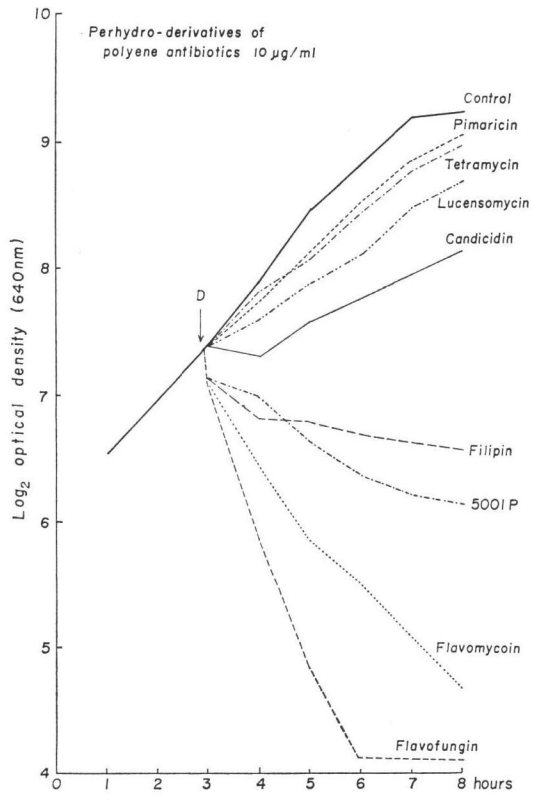


Fig. 3. Effect of perhydro derivatives of polyene antibiotics on the growth of the wall-less L-form cells of *E. coli* W1655F+.
D=Drug application.



barrier[10] and regain insensitivity to polyene antibiotics.

Our experiments with the perhydro derivatives of pimaricin, lucensomycin, tetramycin, filipin, flavomycoin, flavofungin, antibiotic 5001P, and candicidin have shown that the

L-form cells of *E. coli* W1655F$^+$ are sensitive to these substances, too (Fig. 3). The perhydro derivatives of flavofungin, flavomycoin, antibiotic 5001P, and filipin had the strongest effects on the growth of the L-form cells, comparable to those of the original antibiotics. Already 10 μg/ml of the perhydro derivatives of flavomycoin, flavofungin, the hexaene 5001P, and filipin cause lysis of the L-form cells. It is remarkable that perhydrofilipin proved more effective than filipin itself. The perhydro derivatives of pimaricin, tetramycin, lucensomycin, and candicidin show in contrast to the group mentioned above only moderate inhibition effects on L-form cells at concentrations of 10 μg/ml. But higher concentrations (25 μg/ml) of such antibiotics *e.g.* lucensomycin and candicidin also caused cell lysis.

Discussion

The resistance of bacteria against polyene antibiotics is generally explained by the absence of sterols in these organisms. However, in our experiments we could demonstrate that polyene antibiotics have an antibacterial activity against the stable wall-less L-form of *E. coli* W1655F$^+$, though these cells contain sterols in traces only. Both, the L-form and the normal rod form cells of *E. coli* W1655F$^+$ have similar sterol contents comparable to those described for *E. coli* by SCHUBERT.[13,14] Therefore, the different behaviour of both cell types against polyene antibiotics cannot be sufficiently explained by differences in the sterol content.

The sensitive L-form cells differ from the corresponding normal cells by the absence of the cell wall[8,9]. For that reason, we conclude that the cell wall functions as a penetration barrier and is responsible for the resistance of the normal form against polyene antibiotics. Further hints for this assumption are the experiments with normal cells of *E. coli* W1655F$^+$ treated with EDTA. These cells became sensitive to polyene antibiotics for a limited time, when the permeability of the cell wall was raised by EDTA treatment.

The EDTA experiments were performed with normal cells of *E. coli* W1655F$^+$ grown in a synthetic sterol free medium. Therefore, the sensitivity of these cells to polyene antibiotics cannot be explained by a sensibilisation of sterols in the growth medium. We must assume that cells of *E. coli* contain target sites for polyene antibiotics on principle. Considering the sensitivity of *E. coli* with its low sterol content the question arises again, whether sterols are really a necessary prerequisit for sensitivity to polyene antibiotics or not. In model systems SESSA and WEISSMAN[15] have shown that polyene antibiotics may also interact with sterol-free phospholipids.

Our experiments with several perhydro derivatives of polyene antibiotics demonstrate that these substances inhibit the L-form cells of *E. coli*, too. This means that the hydrogenation of the polyene system does not cause decrease of antibacterial activity against L-form cells. Opposite results are described with perhydrofilipin in model systems using cholesterol monolayers[16] or erythrocytes[17].

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