SENSITIVITY AND PERMEABILITY OF THE ANTHRAMYCIN PRODUCING ORGANISM *STREPTOMYCES REFUINEUS* TO ANTHRAMYCIN AND STRUCTURALLY RELATED ANTIBIOTICS

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Streptomyces refuineus, the microorganism which produces the DNA reactive antibiotic anthramycin, has been shown to possess a quite specific mechanism to survive and grow in the presence of this antibiotic. Stationary phase cells are insensitive to anthramycin since the antibiotic is prevented from entering these cells. However, cells in early log phase are inhibited by concentrations of anthramycin that are later produced by these same cells. Significantly, sibiromycin, a closely related antibiotic, is taken up by cells of *S. refuineus* independent of the age of the culture. Anthramycin reacts *in vitro* equally as well with DNA isolated from *S. refuineus* and other procaryotic and eucaryotic cells. When *S. refuineus* has reached the production phase the anthramycin is probably biosynthesized outside the cell membrane which also becomes specifically impermeable to anthramycin.

Anthramycin is a DNA reactive[2] antibiotic which is produced by *S. refuineus* var. *thermotolerans*[1]. How antibiotic producing microorganisms can survive in the presence of the potent compounds they produce is an intriguing problem[3]. In a few cases the mechanism for resistance has been elucidated. For example, thiostrepton, siomycin and sporangiomycin[4] are cases in which the producing microorganism methylates the 23S ribosomal RNA, thereby modifying the target structure, or in the case of the lipogenic drug cerulenin[5], the target enzyme is rendered insensitive. To our knowledge reports have not appeared on how a microorganism which produces an antibiotic which covalently binds to DNA, like anthramycin, defends itself from this class of compound. In the case of actinomycin, a DNA intercalating agent, a change in culture sensitivity occurs during transition from growth to production phase[6], which is most probably related to a chance in cell permeability to actinomycin[7].

Materials and Methods

Growth of Microorganism

S. refuineus was grown on a medium containing 2% Casitone, 1% galactose and 0.3% yeast extract. This medium enabled growth to be measured by following OD_{650}, and anthramycin was also produced in this medium.

Uptake of Radiolabeled Antibiotics

(15-³H)Anthramycin (specific activity=68.8 μCi/μmole) and (14, 15-³H)sibiromycin (specific activity=12.9 μCi/μmole) were biosynthetically prepared as described previously[8,9] and added in the amount of 0.96 μg and 0.43 μg respectively to 10 ml of a *S. refuineus* cell suspension with an OD_{650} of 11.8 in 0.1 M sodium phosphate buffer pH 7.0. One ml samples of cells were withdrawn, washed with 0.1 M sodium phosphate buffer pH 7.0 and prepared for radioactivity assay by incubating with 0.5 ml, 0.2 M SDS and 0.5 ml, 0.1 M NaOH for 2 hours at 90°C and then counted in 20 ml Aquasure (New England Nuclear). The percentage uptake compared to that at zero time was calculated.

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Results

Anthramycin (**I**), tomaymycin (**II**) and sibiromycin (**III**) are members of the pyrrolo(1,4)benzo-diazepine antitumor antibiotic group[1] (Fig. 1), all being closely related structurally, biosynthetically[10], and in the manner in which they react with DNA[11]. *S. refuineus* was tested for sensitivity to all three antibiotics and was found to be sensitive to tomaymycin and sibiromycin, but not to anthramycin

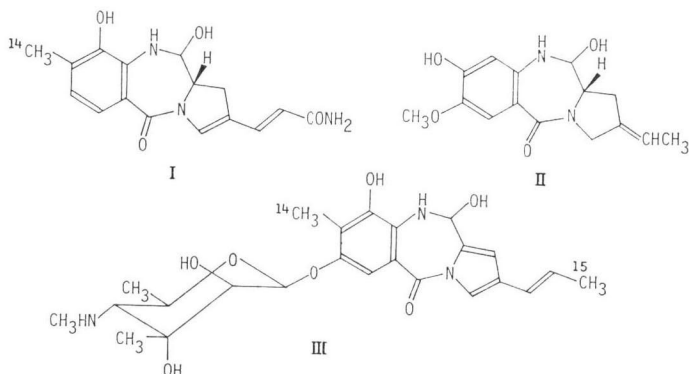Fig. 1. Structures of anthramycin (**I**), tomaymycin (**II**) and sibiromycin (**III**).

Fig. 2. Inhibition of growth of *S. refuineus* by anthramycin added at various stages of growth.

Stationary cells transferred to fresh medium containing no anthramycin (1), same volume of methanol as for 100 μg/ml of anthramycin (2), 50 μg/ml anthramycin (3), and 100 μg/ml anthramycin (4). Cells grown for 3 hours were transferred to fresh medium containing 100 μg/ml anthramycin (5).
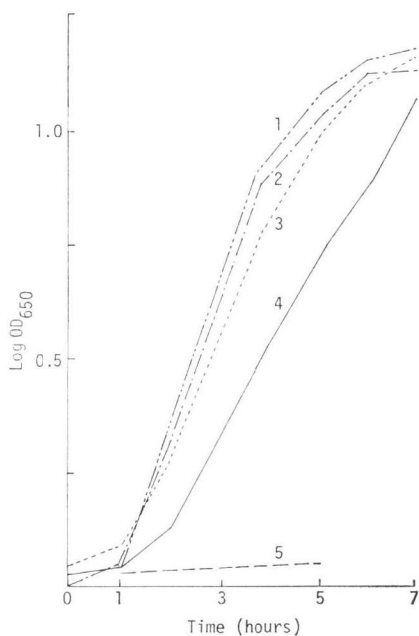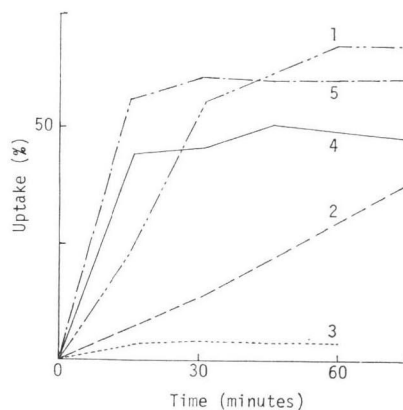

Fig. 3. Uptake of (15-³H)anthramycin and (14, 15-³H)sibiromycin by *S. refuineus* cells of various ages.

Uptake of (15-³H)anthramycin by 3 hours cells (1), 15 hours cells (2), 18 hours cells (3). Uptake of (14, 15-³H)sibiromycin by 3 hours cells (4) and 15 hours cells (5).

(results not shown).

When *S. refuineus* cells of various ages were tested for sensitivity towards anthramycin it was found (Fig. 2) that while stationary phase cells were not affected by concentrations of anthramycin up to 100 μg/ml (the strain produces 150~200 μg/ml), when early log phase cells (3 hours cells) were incubated with the same

concentration growth was prevented.

(14,15-^{3}H)Sibiromycin and (15-^{3}H)anthramycin were used to measure uptake of the antibiotics by *S. refuineus* cells of various ages (Fig. 3). While the effective uptake of (14,15-^{3}H)sibiromycin was independent of the age of the cells, the uptake of (15-^{3}H)anthramycin into *S. refuineus* cells was strictly age dependent; *i.e.* anthramycin was effectively taken up by young cells but not by older, production phase cells.

Discussion

Since anthramycin, tomaymycin and sibiromycin have similar mechanisms of action and compete with each other for binding sites on DNA[12], it would therefore seem probable that there exists either a permeability barrier, or a selective enzymatic inactivation process that is very specific for anthramycin, because *S. refuineus* is sensitive to tomaymycin and sibiromycin.

As can be seen from Fig. 3 a quite specific permeability change occurs as the cells reach the production phase and consequently after that time anthramycin is unable to gain access to DNA, the demonstrated biological target within the cell[13].

The results in which *S. refuineus* is shown to be sensitive to sibiromycin and tomaymycin, but not to anthramycin, also exclude other mechanisms of resistance such as modification of DNA or efficient DNA repair, since if one or more of these defense systems had been operable, *S. refuineus* should show equal resistance to all three antibiotics. Modification of DNA to prevent anthramycin binding was further excluded by experiments in which it was shown that *in vitro* DNA isolated from *S. refuineus*, *Clostridium perfringens* and calf thymus, bound anthramycin to approximately equal degrees of saturation (1: 16, 1: 13.5, 1: 12.5 anthramycin to base ratios, respectively). Evidence against biotransformation of anthramycin by *S. refuineus* has been obtained from experiments in which anthramycin is biosynthetically labelled *in vivo* (unpublished experiments).

When lysozyme or penicillin were added to growing cultures of *S. refuineus* (results not shown) they did not inhibit growth but the production of anthramycin was drastically reduced, indicating that an intact cell wall is required for the biosynthesis of anthramycin. We therefore propose that the cell wall and periplasmic space are the probable sites for anthramycin biosynthesis, so that the antibiotic is prevented from direct contact with the cellular DNA. Complete compartmentalization of the anthramycin is assured as the cell membrane becomes impermeable to anthramycin at the onset of production phase.

Acknowledgements

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