REVERSAL OF EFFLUX PUMP-MEDIATED MULTIDRUG RESISTANCE IN BACTERIA AND TUMOUR CELLS

Ph.D. Thesis

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INTRODUCTION

Since the introduction of penicillin into the clinical practice, the antibiotic era had undergone big change. It means that the originally effective antibiotics had become step by step ineffective against certain bacteria of clinically importance, which leads to therapeutic failures. The antibiotic resistance can be due to the very widespread and inappropriate use of antimicrobials and also to the marvellous adaptable ability of bacteria to the changed environment, which ability can be due to their very plastic genetic stock.

The genetic of resistance

Not all bacteria are intrinsically sensitive to all antibiotics. This type of "resistance" is called **intrinsic resistance** or bacteria with this phenomenon are termed insensitive. The so-called **acquired resistance** is occurred among bacteria which are originally sensitive to a certain antibiotic: one type of this resistance is the *mutational resistance*, where in a large population of bacterial cells a very few individual cells may spontaneously become resistant to one certain drug and the long-term administration of this drug can select the resistant cells. In *transmissible resistance*, genes conferring antibiotic resistance are transferred from a resistant *strain* to a sensitive one. These genes can occur on bacterial chromosome or on mobile genetic elements, like plasmids, transposons or integrons. Their transfer mechanisms are the followed:
In the course of **transformation** DNA fragments from a dead degraded bacterium or from artificially origin are taken up by the competent recipient bacterial cell. The **transduction** is DNA transfer between bacteria carried out by bacteriophages: after the transfection the bacteria, integrated phage-DNA cut out of bacterial chromosome, some host genes can be withcut and will be a part of phage genome and by a next phage infection these genes of non-phage origin can be horizontally transferred into a new host bacterial cell. The **conjugation** is transmission of DNA from a living donor bacterium to a recipient bacterium by the means of plasmids. During this process direct contact is needed between the two bacteria, which can occur by the formation of plasma bridge. The importance of this mechanism is, that the resistance plasmids can horizontally spread by this transfer process.

Mechanisms of antibiotic resistance

The mechanisms of the antibiotic resistance are very diverse. They comprise the **enzymatic inactivation** of antibiotics, **enzymatic modification** of antibiotics. Both results the ineffective form of the drug molecule. When the **drug target alters,** its original function does not destroy, however will be unavailable for the antibiotic. During the **bypass mechanism** a biochemical process to be originally inhibited by antibiotics goes through in an alternative way, and antibiotics won't be able to influence it. With the **changing of permeability** the number of porin-channels decreases or their structure alters. These porins are situated in the outer membrane of gram-negative bacteria, and ensure the enter of antibiotics into the cell. During the **active efflux** the drug molecules are pumped out of the cells through the working of bacterial drug transporters.
Some transporters mediate the extrusion of only a given drug or class of drugs In contrast to these specific drug transporters, the so-called multidrug transporters can handle a wide variety of structurally unrelated compounds and can be divided into two major classes.

Secondary multidrug transporters utilize the transmembrane electrochemical gradient of protons or sodium ions to drive the extrusion of drugs from the cell, **ATP-binding cassette** (ABC-type) multidrug transporters use the free energy of ATP hydrolysis to pump drugs out of the cell.

Secondary multidrug transporters

AcrAB-TolC efflux pump system

One of the main representatives of the **secondary multidrug transporters** and one of the main participant of this thesis is the so-called **AcrAB-TolC** efflux pump system in *E.coli.* This efflux pump belongs to the biggest group of the secondary multidrug transporters, namely to the RND family (resistance nodulation cell division), and has the typical structure phenomenal for this family: Acr B is the RND-type transporter which uses proton motive forces (PMF) for the active efflux of the compounds. AcrA is the periplasmic membrane fusion linker protein, which links the inner membrane and the outer membrane channel protein, TolC, together. AcrAB system pumps out an extraordinary wide variety of antibiotics, chemotherapeutic agents, detergents and dyes. As far as the characteristics of the substrates are considered it is evidence, that AcrAB system can produce significant resistance to large hydrophobic agents e.g. erythromycin which was studied in the experiments involved in this thesis as well. Most of the natural antibiotics belong to this class and their range has traditionally been considered to be limited to gram-positive bacteria. The intrinsic "gram-negative-resistance" is often caused by the efflux pumps of the whole RND family.

ATP-binding cassette (ABC-type) multidrug transporters

All ATP-dependent drug efflux proteins known to date are members of the ABC (ATP-binding cassette) superfamily. ABC transporters require four distinct domains: two highly hydrophobic (N-terminal) membrane domains, which usually consist of six putative transmembrane α-helices each, and two hydrophilic (C-terminal) nucleotide binding domains (NBDs),

One of the bacterial ABC multidrug transporters is LmrA found in *Lactococcus lactis.* The other, namely HlyB transporter of *E. coli* is involved in transport of bacterial haemolysin. Both proteins are homologous to each of the two halves of the human multidrug transporter **P-glycoprotein** (P-gp), which responsible for the multidrug resistance of many type of cancer cells. This homology indicates, that this type of multidrug transporter is conserved from bacteria to humans.

Possibilities for overcoming drug resistance in bacteria and tumour cells

The main aim of this work is to demonstrate some practical solutions to overcome resistant in bacteria and tumour cells as well. Theoretically decreasing the resistance can be achieved by different ways: in bacteria eliminating the extrachromosomal genetic elements (e.g. plasmids), inhibiting their intercellular transfer in the population and enhancing the uptake of antibiotics, exerting direct effects on drug accumulation of microorganisms and cancer cells by inhibition of their efflux transporter proteins.

AIMS OF THE THESIS

For the rapidly increasing antibiotic resistance urging solutions are needed: either by the introduction of new antibiotics, or by combinations of old antibiotics with resistance modifier agents. In earlier works the reversal of antibiotic resistance meant the elimination of plasmids which confer genes for resistance. Since elimination never occurs in the whole population a more effective mechanism was found for increasing the accumulation of drugs in each cell of the population, namely the inhibition of proton- (in bacteria) and ABC- (in tumours) pumps -extruding wide range of structurally unrelated compounds or xenobiotics out of the bacteria and tumour cells- by different chemical agents.

1. The antibacterial effect of the resistance modifiers such as trifluoro-methylketones, as known proton pump inhibitors (e.g. Omeprasol), other known resistance modifiers e.g. verapamil, a phenotiazine e.g. promethazine, were determined on *E. coli* AG100 (operating with proton pump) resistant strain and 100A (proton pump defective) sensitive strain.
2. The most potent trifluoro-methylketones were chosen and examined for interaction in antibacterial effect with promethazine and verapamil in checkerboard microplate method on *E. coli* AG100 and AG100A.
3. A clinically important model was constructed, when *E. coli* AG100 and AG100A were transformed with pBR322 plasmid. These bacteria were considered as clinical model of resistant bacteria. The elimination of pBR322 plasmid by promethazine from the two E. *coli* strains can provide clinical relevance.
4. The most potent trifluoro-methylketone, 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18), was examined in checkerboard method with some representatives of antibiotics (tetracycline, ampicillin, erythromycin) on *E. coli* AG100 and AG100A for inhibitory effect.
5. Since 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) was found to be a potent proton pump inhibitor and resistance modifier, other known proton pump inhibitor (Omeprasol), and 1-(2-benzoxazolyl)-2-propanone (TF51) -a structurally close derivative of TF18- were studied in combination with erythromycin on *E. coli* AG100 and AG100A .
6. Known resistance modifier (promethazine, verapamil) were applied in combination with erythromycin and ampicillin on both *E. coli* strains.
7. As direct evidence for interaction of trifluoro-methylketones with proton motive forces the antimotility action of the compounds was examined on the bacterial motion treating *P. vulgaris* cells.
8. The correlation between the chemical structure of trifluoro-methylketones and their antibacterial, antimotility and proton pump inhibitory effects were studied.
9. In cancer cells, for the possible multidrug resistance reversal effect, the drug accumulation was tested by the Rhodamine-123 extrusion assay. For these experiments acridine derivatives: aza mono, bi- and tricyclic compounds and benzonaphthyridine-5-one derivatives were studied
10. The relationship between the chemical structure of effective agents and the biological effect was evaluated with particular respect to the type of aromatic moiety, and the structure of the side chains (substituents and size).

MATERIALS AND METHODS

Culture media for bacteria

MTY / tryptone-yeast extract media /: liquid media was used for the cultivation of *E. coli* AG 100, AG100A and *P. vulgaris* bacterial strains

MTY agar: was used for the determination of live cell counts of *E. coli* AG100, AG100A and P. vulgaris culture. It was used for the maintenance of the mentioned bacterial strains.

YTB / Yeast extract –Tryptone Broth /: liquid media was used for the cultivation of *E. coli* AG100, AG100A strains to be transformed with pBR322 plasmid and was used for the dilution of bacteria during transformation procedure.

YTB agar /supplemented with tetracycline and ampicillin / was used for the plating, cultivation and selection of *E. coli* AG100 pBR322 and AG100A pBR322 strains.

Media for cell line cultivation

McCoy's 5A medium modified (GIBCO BRL)

PAR: Streptomycin, Nystatin, 200 mM L-glutamine, 10 % heat inactivated horse serum

MDR: Streptomycin, Nystatin, 200 mM L-glutamine, 10 % heat inactivated horse serum

Stock solution for preparation of competent cells

Calcium chloride ($CaCl_2$) 1.0 M, Magnesium chloride ($MgCl_2$) 1.0 M, potassium chloride (KCl) 1.0 M

Dye

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma)

Strains and cell lines

Bacterial strains

Laboratory strains: *E. coli* AG 100, *E. coli* AG 100A were constructed and kindly provided by Professor Nikaido (University of California, USA), *E. coli* AG100 pBR322 and *E. coli* AG 100A pBR322: the former two strains transformed with pBR322 plasmid respectively

Clinical isolate: *P. vulgaris* (derived from Institute of Clinical Microbiology, University of Szeged),

Tumour cell lines

(provided by Professor Adorján Aszalós, FDA, Washington, USA)

L5178Y mouse T cell lymphoma cell line (PAR)

L5178 mouse T cell lymphoma MDR 1 / A retrovirus transfected cell line (MDR)

Compounds studied

Ca channel inhibitors

Promethazine (Pipolphen®) (EGIS Pharmaceutical Company, Budapest, Hungary), verapamil (Verapamil®) (Chinoin, Budapest, Hungary)

.Proton pump inhibitor

Omeprazol (AstraZeneca)

Antibiotics

Ampicillin (Penbritin®), erythromycin (Erythromycin lactobionate), tetracycline (Tetracycline®) (Chinoin, Budapest, Hungary)

Trifluoro-methylketones (TF compounds)

TF5: 3-trifluoroacetylindole (obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan), TF6: 2-trifluoroacetylbenzoxazole], TF10: 4,4,4-trifluoro-1-phenyl-1,3-butanedione, TF11: 1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone], TF18: 1-(2-benzoxazolyl)-3,3,3-

trifluoro-2-propanone, TF19: 3-(2-benzothiazolyl)-1,1,1-trifluoro-2-propanone], TF20: 3-(2-benzimidazolyl)-1,1,1-trifluoro-2-propanone], TF50: 3,3,3-trifluoro-2-hydroxypropyl phenyl ketone, TF51: 1-(2-benzoxazolyl)-2-propanone
All compounds were dissolved in DMSO
Acridine derivatives: aza mono, bi- and tricyclic compounds
4-[(2'-Diethylaminoethyl)amino]-pyridine (2), 4-[(2'-Diethylaminoethyl) methylamino]-pyridine (3), 7-Chloro-4-[(2'-diethylaminoethyl)amino]-quinolone (4), 7-Chloro-4-[(2'diethylaminoethyl)-methylamino]-quinolone(5), 6-Chloro-2-methoxy-9-[(2'-diethylaminoethyl)amino]-acridine (6), 6-Chloro-2-methoxy-9-[(2'-diethylaminoethyl)methylamino]acridine (7), 2,8,10-Trimethyl-4,6-bis[N-(2'-diethylaminoethyl)-N-methylamino) ethoxy] pyrido-[3,2-g] quinoline (21) (2,8,10-Trimethyl-bis-4,6-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (24),2,8,10-Trimethyl-6-phenoxy-4-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (25)
All compounds were dissolved in DMSO
Benzo[b]-1,8-naphthyridine-5-one derivatives
Benzonaphthyridine-5-one 10-(2'-dimethylaminoethyl) (1), Benzonaphthyridine-5-one 10-(3'-dimethylaminopropyl) (2), Benzonaphthyridine 5-one 10-(2'-diethylaminoethyl) (3), Benzonaphthyridine-5-one 10-(2'-diisopropylaminoethyl) (4), Benzonaphthyridine-5-one 10-(2'-pyrrolidonoethyl) (5), Benzonaphthyridine-5-one 10-(2'-(N-methyl)-pyrrolidinoethyl) (6), Benzonaphthyridine-5-one 10-(2'-piperidinoethyl) (7), Benzonaphthyridine-5-one 10-(3'-piperidinopropyl) (8),
Benzonaphthyridine-5-one 10-(2'morpholinoethyl) (10).
All derivatives were tested as the hydrochloride salt and dissolved in PBS

METHODS

Determination of minimum inhibitory concentration (MIC)

Dispensing and diluting of bacteria / compounds / reagents to a 96 well microplate were conducted with the aid of an 8 multi barrel pipette. First the half dilution series of the compounds was prepared on the plate in sterile physiological saline. An overnight preculture of the bacterial strain was diluted to 10^{-4} in 2x MTY broth (about 1.0 McF), and 50 µl of this bacteria suspension was added to the each 50 µl of the dilutions of the compounds on the microplate. The microplates were incubated at 37 °C for 24 hours. 10 µl of the solution of MTT (5 mg/ml in phosphate buffered saline (PBS)) was added to each well in order to evaluate the rate of bacterial growth. In this manner, mitochondrial dehydrogenase of living cells reduces MTT resulting in a blue coloured formazan salt. The plates were further incubated for 4 h at 37 °C, and then the MIC values of the compounds against the bacterial strain was determined.

Checkerboard microplate method for evaluating the combined effect of two drugs

The effect on bacteria of two drugs in combination can also be studied. The half dilution of drug 'A' is made by the above mentioned method. No drug 'A' was put into 12th column (control of drug 'B'). The half dilution of drug 'B' is prepared in selected glasses and 50µl of each dilution was added to each well per row. The concentration of solutions decreases in the direction from up to down. No drug 'B' was added into H row (control of drug ' A'). The bacterial strains were cultured and diluted the same way mentioned above but 100µl of 10^{-4}

diluted bacterial suspension was added into each well. The plates were incubated for 24 hours at 37°C. MTT (10 µl) was added to the wells and incubated further 4 hours at 37°C. According to the colour of MTT the further rates can be determined: MIC values of drug 'A' and drug 'B' separately and MIC values of drug 'A' in combination and MIC values of drug 'B' in combination. According to the further rates the effect of drug combination can be determined.

$$FIC_A = MIC_A comb / MIC_A alone \qquad FIC_B = MIC_B comb / MIC_B alone$$

$$FIX = FIC_A + FIC_B$$

$0{,}51 < FIX < 1 \rightarrow$ additive effect; $FIX < 0{,}51 \rightarrow$ synergism; $1 < FIX < 2 \rightarrow$indifferent effect; $FIX > 2 \rightarrow$ antagonism

Transformation of bacteria with pBR 322 plasmid

1 ml of an overnight YTB culture of an ampicillin and tetracycline sensitive bacterial strain was added to 100 ml YTB broth and incubated at 37°C until an optical density (OD) of 0,25-0,30 at 600 nm was reached. The culture was transferred to an ice bath for 10 minutes, centrifuged at 4500 rpm for 10 minutes at 4 °C, then supernatant was removed and pellet re-suspended in ice cold 50 ml 0.1 M $MgSO_4$, centrifuged in cold tube at 4500 rpm for 10 minutes, the supernatant removed and the pellet was re-suspended in ice-cold 3.3 ml 0,1 M $CaCl_2$ and incubated for 1 hour in an ice bath. 200 µl from these cultures containing the competent cells were transferred into tubes containing 1µl of pBR322 (plasmid carrying the genes for ampicillin and tetracycline resistance) and the tubes kept in an ice bath for 30 minutes and then rapidly transferred to 42°C for 1 minute in order that the cells be "shocked". One ml of YTB broth was added to the "shocked" cells and the tubes incubated for 1 hour at 37 °C. These cells were centrifuged in an Eppendorf centrifuge for a few seconds and 800 µl of supernatant removed. The cells were re-suspended in the remaining supernatant and an aliquot of 200 µl was plated onto YTB agar supplemented with tetracycline and ampicillin. Colonies present on these plates were indicative of containing the plasmid that bestowed them with resistance to ampicillin and tetracycline.

Method for elimination of pBR322 plasmid from *E. coli* AG100 and AG100A strains with replica plating

One colony of bacteria transformed with pBR322 plasmid was added to MTY broth media (5 ml) supplemented with glucose and $MgSO_4$ and incubated for 24 h at 37 °C. From a 10^{-4} dilution of this overnight initial culture 2 ml was transferred to tubes containing 200 ml of MTY broth media, mixed, and distributed in 5 ml aliquots in test tubes. Different concentrations of curing compounds (compounds known to cause the elimination of plasmids) such as promethazine were added to the cells which were then incubated for 24 h at 37°C. 10^4 and 10^5 dilution of these cell suspensions were made and aliquots of 100 µl plated onto YTB agar and the plates incubated for 24 h at 37 °C. The colonies present on these Master plates were transferred by velvet replica plating technique onto YTB agar containing ampicillin and tetracycline (Replica plate). The plates were further incubated at 37 °C 24 h after which time the distribution of colonies present in each of the respective plates was compared by simple over-laying methods. Colonies present on replica plates contain the pBR 322 plasmid which codes for resistance to ampicillin and tetracycline. Colonies present on the master plate and which do not grow on the Replica plate provide

evidence of plasmid elimination promoted by promethazine or any other compound tested. Comparing the number of colonies on both plates provides an estimate of percent plasmid elimination (cure index) produced by a given compound.

Method for determining antimotility effect of drugs on *P.vulgaris*

From the overnight MTY culture of bacteria 100 µl was added to 900 µl of PBS which contains the drug in subinhibitory (sub MIC) concentration: 10, 90 and 50 percent of the MIC values of certain drugs were administered. PBS with no drug was used as a negative control and promethazine applied in the sub MIC concentration mentioned above was used as a positive control. The samples in Eppendorf tubes were incubated for 15 min at 37 °C. One drop of sample was placed on a microscopic slide and covered with 18 mm square coverslip. The preparation was examined with a phase contrast Zeiss microscope with 63x water objective in the case of *P. vulgaris.* 200-300 cells of *P.vulgaris* were totally counted from 4-6 fields using hand-tally counter. The motile (running), the non-motile and the vibrating (tumbling) cells were counted separately. The ratio of cells with different moving was expressed in percentage of untreated control.

Multidrug resistance reversal effect by decreasing Rhodamine 123 efflux

The L51478 mouse T cell lymphoma cell line was infected with the mdr1/A retrovirus. mdr1 expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintaine expression of the mdr phenotype. The L5178 MDR cell line , and the L5178Y PAR cell line were grown McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine (1 ml/100ml), streptomycin (1 ml/100ml) and nystatin (0.1 ml/100ml). The cells were adjusted to a concentration of $2x10^6$ / ml and resuspended in serum free McCoy's 5A medium and the cells were distributed into 0.5 ml aliquots to Eppendorf centrifuge tubes. Then the tested compounds were added in various concentrations of the 1.0 mg/ml stock solutions, and the samples were incubated for 10 min at room temperature. Then 10 µl (5.2 µM final concentration) indicator R123 was added to the samples, and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 ml PBS for analysis. The fluorescence of cell population was measured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the R123 exclusion experiments. The percentage of the control mean fluorescence intensity was calculated for PAR and MDR cell lines as compared to untreated cells. An activity ratio was calculated by the following equation on the basis of measured fluorescence values:

$$FAR= (MDR_{treated} / MDR_{control}) / (PAR_{treated} / PAR_{control})$$

RESULTS

Experiments on bacteria having proton pump transporter with resistance modifier

The interaction of Ca-channel blockers (promethazine, (±)verapamil) and potential proton pump inhibitor trifluoro-methylketones on *E. coli* AG100 and AG100A strains

In preliminary experiments, the interaction of promethazine of potent antimicrobial effect and newly synthesised trifluoromethyl-ketones was evaluated on both strains respectively in agar diffusion method to use sterile filter paper strips containing 50 µg promethazine and 200 µg TF compounds. The strips containing promethazine and a representative of TF compounds were put at right angles to each other on the MTY agar containing the bacterial culture. After incubation the type of interaction was evaluated. TF

50, 19, 20 were ineffective, TF 5 had very slight , TF 10 and 11 had strong and TF 18 had very strong synergistic effect with promethazine on both strain. TF 11 had slight antibacterial effect on both strain, TF 10 exerted stronger inhibition only on mutant strain however TF 18 inhibited the growth of both strain strongly.

Based on the preliminary experiments the three most effective TF compounds (TF 10, 11 and 18) were examined in checkerboard experiments with promethazine. Synergistic effect between TF compounds and promethazine was exhibited only by the combination of TF 18 with the Ca-calmodulin channel blocker promethazine just on the proton pump expressing *E. coli* strain. TF 10, 11 showed moderate combined effect on both *E. coli* strain. According to the experiments the most effective compound (TF 18) was examined further by the combination with the Ca-channel blocker (±)verapamil on both *E. coli* strain. Interestingly on the mutant strain the combination was additive however on the proton pump expressing wild strain an antagonism was demonstrated.

Elimination of pBR 322 plasmid from *E. coli* AG100 and AG100A strains by promethazine

The calmodulin inhibitory promethazine is long known to have antiplasmid effect. We were wonder whether the existence of proton pump in the bacterial membrane can influence this phenomenon. The plasmid curing ability of promethazine was evaluated with the use of the two *E. coli* strains transfected with pBR322 plasmid (tet^r, amp^r). Promethazine promotes the elimination of plasmids from the wild type *E. coli* strain in a concentration dependent manner such that the maximum curing effect takes place at a concentration just below the MIC of the compound (120 mg/l). In contrast to the wild type strain, promethazine causes elimination of plasmids from the proton pump-deleted mutant at lower concentration of the compound (80 vs. 140 mg/l).

The interaction of potential proton pump inhibitor TF 18 and some representatives of antibiotic groups on *E. coli* AG100 and AG100A strains

The further step was to study whether TF 18 can influence the antibiotic sensitivity of both *E. coli* strain. It is well known, that the AcrAB-TolC proton efflux pump has its antibiotic substrate of large hydrophobic characteristic e.g. erythromycin and tetracycline. Hydrophilic ampicillin was also employed as a control. According to the results of the checkerboard technique TF 18 at a concentration well below its MIC and in combination of tetracycline, erythromycin each of which is also well below its MIC, have a synergistic effect on the inhibition of both strain, however the lower FIX index in the case of proton-pump-expressing wild strain indicates the stronger synergism than that of in the case of proton-pump-deleted mutant strain. Remarkable that the MIC value of erythromycin which is proved to be the substrate of AcrAB-TolC proton efflux pump decreased 32 fold in the presence of TF 18, in the proton-pump-expressing strain (from 80 mg/l to 2,5 mg/l), however this decreasing was only 4 fold in the mutant strain. Expectedly the MIC values of ampicillin did not change significantly in combination with TF 18 in both strain.

Comparison of the interaction of erythromycin plus known proton pump inhibitor Omeprasol with that of erythromycin plus the newly synthesised TF 18 – a potential proton pump inhibitor-and with its structurally close derivative TF 51 on *E. coli* AG100 and AG100A strains.

To determine the specificity of proton pump inhibitory effect, a known and two newly synthesised TF compounds were compared in the combination experiment with erythromycin on both *E. coli* strain.

Omeprasol can influence the erythromycin sensitivity nor in the case of *E. coli* AG100, neither in proton pump-deleted mutant. A very marginal inhibitory effect (additive effect,

FIX-index= 1.0) takes place in *E. coli* operating with proton efflux pump. Exactly the same what is to explain about TF 51, however the structural differences between TF 51 and 18 are only a terminal H_3 and F_3 group in the side chain.

Combinations of antibiotics (erythromycin and ampicillin) with known resistance modifiers (promethazine, verapamil) respectively on *E. coli* AG100 and AG100A strains

After a newly synthesised potent resistance modifier trifluoro-methylketones (TF18) was found to be successful in reversing antibiotic resistance caused by a proton efflux pump, known resistance modifiers, the Ca-calmodulin-antagonists promethazine, and the Ca-antagonists verapamil, were employed in combination with erythromycin (substrate of AcrAB efflux pump due to its hydrophobic characteristic) and with ampicillin (surely not substrate of the Acr AB efflux pump due to its hydrophilic phenomenon) in the proton efflux pump-deleted and in the wild *E. coli* strains. In the wild type *E. coli* AG100 strain promethazine only slightly (in additive way), verapamil did not influence the sensitivity of erythromycin which is substrate of the pump of the bacteria. In the proton pump deficient bacteria the Ca-calmodulin antagonist promethazine caused a strong additive effect but not synergy, however the Ca-antagonist verapamil decreased the MIC of erythromycin to the same rate like promethazine did, but promethazine needed its 25% of MIC value for this interaction, however verapamil was able to reach the same only with its 5 % of MIC value, namely the verapamil + erythromycin interaction is synergistic in the proton-pump deficient strain. The sensitivity of ampicillin wasn't influenced by both Ca-antagonists on wild strain, however interestingly promethazine and verapamil stood in antagonism with ampicillin on the mutant strain.

Inhibition of motility by trifluoro-methylketones on a clinical isolates of *P. vulgaris*

The movement of bacterial flagellae is strongly energized by proton motive forces, so the fuel for movement comes from the energy of proton gradient). This fact was used to study TF compounds further, whether really having an effect on proton pumps and so being able to inhibit the movement of bacteria. Two types of movement were examined: the running and the tumbling and of course the non-motile cells. Since the drugs were used in subinhibitory concentrations, first the MIC values of TF compounds were determined. Ren et al suggested that phenotiazines e.g. promethazine can inhibit the movement of bacteria by reversible inhibition on the proton pump of the bacterium. Using this hypothesis promethazine was used as a positive control also in subinhibitory concentration.

Note, that the treatment with the sub-MIC concentration of TF 18, in a short period, does not influence the growth of bacteria.

In the antimotility experiment the average distribution of untreated *Proteus* movement was the following: 64.5± 10 % running, 28.9±8 % tumbling and 6.6± 3 % non-motile, respectively. Compounds **5, 19, 20** at 10 % of their MIC partially inhibited the motility: the ratio of running cells decreased, however one of the tumbling cells significantly increased. The number of non-motile cells increased somewhat only in the sample treated with **TF5** at 10 % of MIC. However **TF 5, 19, 20** had solubility problems and formed slight opalescence which could inhibit the bacterial motility. **TF 6, 10, 11, 18 and 51** could exert real inhibition of moving: the ratio of non-moving cells treated with these compounds increased significantly. In the case of **TF 6** and **18** the number of tumbling cells also changed however **TF10, 11** and **51** didn't alter them significantly. The correlation between concentrations applied and antimotility effect was significant in the case of **TF 6, 18** and **51**. Less correlation was observed with **TF 11** and no correlation could be established with **TF 10**. **TF 18** exerted the most remarkable inhibition of movement: the ratio of non-moving cells

increased significantly in a concentration- dependent manner. Already at 50% of its MIC no cells were running and at 90 % of its MIC 90,9 % of bacteria were totally non-motile and only 9,1 % of the cells showed tumbling without running. As it was expectable among eight TF compounds **TF 18** was the most promising drug, with a strong antimotility effect on *P. vulgaris* reinforcing its proton pump inhibitory action supposed in earlier interaction studies.

Experiments with tumour cells having ABC transporter and resistance modifier agents

Reversal of multidrug resistance in mouse lymphoma cells by mono, bi and tricyclic acridine derivatives

Acridine compounds and other tricyclic derivatives have already been tested and several derivatives have interesting anti MDR activity (Hever et al). The aim of these examinations was to clarify the functional groups, which actually interact with the P-gp 170 protein. Several variations have been made concerning: 1. the aromatic moiety e.g. pyridine (2,3), quinoline (4,5), acridine (6,7) and pyridoquinoline (21, 24, 25) derivatives have been investigated, 2. the side chain has been modified e.g. X atom nature (O, N or S) and / or size of the side chain.

In the Rhodamine 123 accumulation assay on the L5178 mouse lymphoma cell line the follow were found: the two examined pyridine derivatives namely **2** and **3** are inactive. Quinolines **4** and **5** are as active as positive control verapamil. High activity is observed for tricyclic derivatives. Acridine derivatives **6** and **7** present a similar activity at low concentration but the non-substituted compound, **6** could be toxic at high doses. Among pyridoquinolines **24, 25** exerted the best activity.

The most active compounds **–6, 7, 21, 24** and **25** were examined at 0,2 and 2 mg/l concentration as well. **6, 7** and **21** had no anti MDR activity at 0,2 mg/l concentration, however **24** and **25** exerted the strongest anti MDR effect among tricyclic derivatives even at 0,2 mg/l concentration. The lower fluorescence activity ratio values in higher doses can be due to potential toxicity.

Reversal of multidrug resistance in mouse lymphoma cells by benzo [b]-1,8-naphthyridine derivatives

Because of its structural similarity with acridines, benzonaphthyridine nucleus was selected to study the efflux pump inhibitory effect.

Among compounds tested **1** had very weak anti MDR effect at both concentrations.**2, 4, 7** exerted strong reversing activity only at the higher 20 mg/l concentration.**5, 6** and **8** showed the most promising MDR-reversing activity at lower concentration as well in comparison with reference drug verapamil. **5** being the most active of the set and was probably toxic in higher doses. It is important that all of these derivatives (namely **5, 6** and **8**) are substituted with an azaheterocyclic group.

DISCUSSION

In the clinical practice with the more and more frequently appearing multiresistant bacterial strains, causing lethal infections due to therapeutic failure, there has been urging need to solve this world-wide problem. The solution can be the introduction of new antibiotics, but the inhibition of resistance mechanisms by known and newly synthesised resistance modifier agents might be cheaper and more effective.

Earlier it was found, that well-known psychotropic drugs, e.g. phenotiazines have antibacterial and antiplasmid effect. Since the elimination of plasmids never occurs in the whole bacterial population, a more effective mechanism was chosen for reversing drug resistance: the inhibition of drug efflux pumps by chemical agents.
The newly synthesised trifluoro-methylketones have already been shown to have a strong inhibitory effect against *Helicobacter pylori* compared with clinically used metronidazole and clarithromycin]. Taking further this evidence we examined whether they have antibacterial effect on other bacteria and their resistance reversal ability by inhibition of proton efflux pump was also examined.
Among the nine TF compounds, TF 18 (1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone) derivative exerted the strongest antibacterial activity on the proton pump-bearing and – mutant *E. coli* strains as well. Since TF10, 11 and 51 derivatives have also quite low MIC values on both strain, these four compounds were examined further in combination experiments. Only TF 18 showed some effect on the wild type *E. coli* strains combined with known resistance modifier promethazine and verapamil respectively, namely it potentiated the antibacterial action of promethazine indicating that this phenothiazine can be a target of the efflux pump, however with verapamil the combination was an antagonism suggesting, that TF 18 probably makes difference between the calmodulin-antagonistic and Ca-channel blocker property and the proton pump has some role on these effects.
After transformation of the two *E. coli* strains by pBR322 plasmid, practically two strains were observed considered clinical resistant strain. Elimination of the plasmid by promethazine from both strain showed that promethazine could be the substrate of the AcrAB efflux pump, since the maximal elimination rate was significantly higher in wild type than mutant one, and that's why the presence of this efflux pump could influence the plasmid elimination with promethazine. Considering the evidence, that the erythromycin is the substrate of AcrAB efflux pump due to its large hydrophobic characteristics, this antibiotic and the similar tetracyclin were combined with TF 18 on both *E. coli* strain. The hydrophilic ampicillin (with this phenomenon not substrate of AcrAB pump) was also employed as a control. TF 18 decreased the MIC value of erythromycin 32 fold in wild strain, and only 4 fold in mutant strain. Tetracycline and TF 18 also showed a synergism, however -as it was expected- the MIC value of ampicillin wasn't influenced by TF 18 significantly. These results indicate that TF 18 really can have a special effect on inhibiting this proton efflux pump. The slighter synergy of TF 18 and erythromycin on proton pump-deleted mutant strain can be explained only by the fact that TF 18 itself also has a strong antibacterial effect. Comparison of the effect of TF 18 with other proton pump inhibitors e.g. omeprasol and the structurally related TF 51 it was observed, that omeprasol and TF 51 had only additive effect with erythromycin on the wild strains, indicating having no or very slight proton pump inhibitory effect on the AcrAB efflux system. However the structural differences between TF 18 and TF 51 are only a terminal F_3 and H_3 groups in the side chains of the benzoxazole ring. Considering these results an evidence is suggested that the AcrAB efflux pump needs a certain structural requirement in the group of proton pump inhibitors for its successful inhibition.
Finally two known resistance modifier agents were examined in combination with the former antibiotics on the two *E. coli* strains. Neither of them could exert similar decreasing in MIC values of erythromycin on proton pump positive strain what TF 18 could. Interestingly the Ca-channel blocker verapamil was synergistic with erythromycin on the proton pump-deficient strain. This phenomenon can be explained by a changing effect of

verapamil in the membrane permeability, which cannot be specific for its Ca-antagonistic property, since the Ca-calmodulin antagonistic promethazine did not show similar effect. Interestingly the ampicillin with both Ca-antagonists stood in antagonism in mutant strain namely the Ca-channel blockers somehow could decrease the membrane permeability. Moreover the effect of verapamil on mutant strain can be in correlation with the properties of the antibiotics combined with. With hydrophobic erythromycin synergism, with hydrophilic ampicillin antagonism effect was found indicating the difference membrane permeability requirements of the two different type of antibiotics.

Comparing first the chemical structure of the trifluoro-methylketones examined for antibacterial effect it is suggested that the replacement of the benzoxazol residue of the most potent TF18 with other aromatic or heteroaromatic rings with intact side chain (resulting TF19,20) caused strong decrease in antibacterial action indicating that the benzazole derivatives were more active than either benzene or azole derivatives. Next to intact rings the side chain could have also role in antibacterial effect, since removal of the terminal F_3 from TF 18 to H_3 resulting TF51 causes approximately 40 fold increasing in MIC values of both *E. coli* strain. Removal the methylene from the side chain of TF 18 resulting TF6 directs to 160 fold increasing in MIC of both strain. Summarized the structure of the benzoxazole ring and the side chain found in TF18 are together similarly important for the antibacterial action of trifluoro-methylketones. Considering the results of combination experiments, it is sure that the side chain with its terminal F_3 group is essential for proton pump inhibition but the benzoxazol ring has to be also some role in drug reversary action, since TF11 wasn't effective in combination experiments. Further evidences were obtained between chemical structure of trifluoro-methylketones and their proton pump inhibitory effect in antimotility experiments.

For reinforcing the finding, that some TF compounds exert their resistance modifier effect by inhibition of proton pump, the antimotility effect of these drugs was studied on a clinical isolates of a *P. vulgaris* strain.

It is well known that the fuel for rotation of bacterial flagellae is the membrane gradient of protons. The flagellar motor can rotate counterclockwise (CCW), where the several filaments on a cell join in a bundle and drive the cell forward resulting the running movement. In the case of clockwise (CW) rotation of flagellae the filaments bundle are disrupted causing a somersaulting movement or tumbling. Cells can direct their movement by regulating switching between CW and CCW. The supposed proton pump inhibitor TF compounds were tested whether having antimotility effect. With this phenomenon the reversal of antibiotic resistance could be attributed to proton pump inhibitory effect.

Earlier it was shown that the known resistance modifier promethazine has antimotility effect. After studying the newly synthesized TF compounds with possible resistance reversary effect, some connections were established. TF 18 and 51 showed significant antimotile effect in concentration-dependent manner. TF 6 worked also in concentration-dependent manner however only the number of tumbling cells increased significantly with decreasing of running ones. Taking as a basic of molecular structure of most effective TF 18, it is suggested that the benzoxazol ring is essential for antimotile effect as ring substitution next to intact side chain strongly decreased this efficacy. Among molecules having the original ring complex (TF 6, TF 51) with substituted side chains antimotility effect was more expressed. However in the side chain when terminal trifluorid was substituted with H_3 group (resulting TF 51) antimotility effect was weaker. When the molecule of TF 18 was modified by extraction of the methylene group from the side chain (resulting TF 6), only the number

of tumbling cells increased, the number of non-motile cells didn't change significantly. It indicates that this structure may have a role in intervention into the CCW/CW switching and the methyl group may be important for the effective proton pump inhibition. Interestingly a progressivity is phenomenal for the antimotility effect of TF18: with the increasing of the drug concentration first the running cells become tumbling and later, the tumbling ones become non-motile. It is evidence that the inhibition of motility can be by proton pump inhibition and / or decreasing proton motive forces. However it is not closed that the CCW / CW switching and its influence comprises operation of proton pumps / proton motive forces. Taking these suggestions TF 18 can be considered a drug having real proton pump inhibitory effect reinforcing the mechanisms by which TF 18 works as an antibiotic resistance reversing agents. Other importance of the finding is the inhibition of motility – as a potent virulence factor –itself.

Experiments with tumour cells having ABC transporter and with resistance modifier agents

In the clinical practise to avoid the selection of resistant tumour cells combined chemotherapy has to be employed. Theoretically the co-administration of cytostatic drugs with the proper resistant modifier agent could be also a possible and cheaper way to overcoming multidrug resistance and the therapeutic failure.

A plenty of structurally unrelated resistance modifier drugs were found which can inhibit drug efflux pumps of tumour cells. In general the chemosensitizers that block P-gp drug extrusion are lipid-soluble at physiological pH, possess a basic nitrogen atom and at least two co-planar rings, have aromatic moieties, but the presence of electron donor groups is a more general feature.

According to our study the anti MDR activity of mono, bi and tricyclic **acridine derivatives** depends on the aromatic moiety, the bonded X heteroatom and nature of the side chain. A better activity is found: when the aromatic moiety is acridine or pyridoquinoline compared with quinoline and pyridine If we consider the compound activity related to the number and length of the side chain -keeping the same X atom on the ring- activities are in the following increasing order: when X=O 15< 21, when X=N 20 < 24 < 25, that is 2 side 'short' chains<one side 'short' chain or two side 'long' chains. Considering more than 100 compounds already tested, activities have been correlated with the presence of two or three hydrogen bond acceptor (HBA) groups with a spatial separation of 2,5 or 4,6 Å. For the products studied here, possible HBA groups are tertiary amino groups, heterocyclic nitrogen. When X= N e.g. in the case of 24 and 25, the heterocyclic nitrogen potency is increased by a push-pull effect. In addition to its nitrogen HBA group, the heterocycle can interact with P-gp by aromatic hydrophobic interaction. The lack of activity of pyridine and quinoline derivatives 2-5 seems to indicate that hydrophobic interactions are rather involved. Differences in the observed activities of the compounds can be attributed to the spatial position of the amino HBA groups and aromatic rings. The results display that two long chains allow the best interactions between compound pharmacophoric groups and P-gp binding sites.

Tricyclic derivatives including phenotiazines and acridines are among compounds showing anti MDR activity. Unfortunately, most of these agents suffer clinically from their intrinsic toxicity or from side-effects at concentrations which neutralise P-gp. So to design new inhibitors active at low concentrations and free of serious side-effects is vital. **Benzonaphthyridine** nucleus was selected because of its structural similarity with acridines, the more so as compounds in this chemical series are usually less toxic than acridines.

According to the data received there is clear evidence that benzonaphthyridine derivatives increased accumulation of Rhodamine-123 in MDR cells more efficiently than phenotiazines and acridine derivatives, or the positive control verapamil did. Since the hydrophobic moiety could be essential in the activity of MDR modulators, benzonaphthyridine derivatives with the polycyclic nucleus modified in various ways were tested. The activity of benzonaphthyridine derivatives with various side-chain was compared with the aim of determining the effect of the nature of this chain on the MDR reversion. Compounds 5, 6, 8 and 10, which bore saturated aza heterocyclic moieties (pyrrolidine, morpholine, piperidine), were the most active at the lowest concentration. In addition, the presence of the amino group protonatable in the physiological conditions seemed to be necessary for the reversion of MDR in the case of tricyclic derivatives. This is clearly demonstrated by comparing 3 to 11. Indeed, there is the same lipophilic moiety in both compounds but there is an acetamido group in the side-chain of 11 whilst the amino group is protonatable in 3. Similar results were obtained by Hever et al for acridine derivatives.

CONCLUSION / NEW STATEMENTS OF THE THESIS

1. Among trifluoro-methylketones, 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TF10), 1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone (TF11) and 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) has the strongest antibacterial effect on *E. coli* AG100 wild and on *E.coli* AG100A proton pump deleted mutant strain as well.

2.a.) 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TF10) and 1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone (TF11) showed moderate combined effect on both *E. coli* strain in combination with Ca-calmodulin antagonistic promethazine. 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) exerted synergy with promethazine only on the wild *E. coli* strain indicating that this phenotiazine can be a target of the AcrAB efflux pump.

b.) The most effective trifluoro-methylketone, 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) was studied in interaction with Ca-channel blocker verapamil. On mutant strain the combination was additive, however on the wild strain an antagonism was found. It suggests that TF 18 probably makes differences between the calmodulin-antagonistic and Ca-channel blocker property, and the proton pump has some role on these effects.

3. Promethazine eliminated the pBR322 plasmid at a maximal rate from mutant strain at lower concentration than from the wild type indicating that the presence of this efflux pump can influence the plasmid elimination with promethazine.

4. 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) decreased 32 fold the MIC value of erythromycin in wild, and 4 fold in mutant strains respectively. The sensitivity of ampicillin did not change significantly in combination with TF 18 in both strain. It indicates that TF 18 does have a special effect on inhibiting this efflux pump. The weaker synergism of TF18 and ER in mutant strain can be explained by the strong antibacterial effect of TF18.

5. Different proton pump inhibitors were compared in combination with erythromycin on both strains. Neither Omeprasol and nor 1-(2-benzoxazolyl)-2-propanone (TF51) decreased the sensitivity of erythromycin on both strain indicating that there is a certain structural requirements for the inhibition of AcrAB efflux pump.

6. Known resistance modifiers were combined with antibiotics on both *E. coli* strains.

a.) Erythromycin with promethazine was additive on wild and mutant strain as well. Erythromycin with verapamil was indifferent on wild strain, however on mutant strain this combination was synergystic. The latter phenomenon is explained by a changing effect of

verapamil in the membrane permeability, which cannot be specific for its Ca-antagonistic property, since the Ca-calmodulin antagonistic promethazine did not show similar effect.

b.) The ampicillin with both Ca-antagonists stood in antagonism in mutant strain, namely the Ca-channel blockers somehow could decrease the membrane permeability in this strain. The effect of verapamil on mutant strain depends on the property of antibiotics combined with VP: with hydrophobic erythromycin synergism, with hydrophilic ampicillin antagonism were found, indicating the difference membrane permeability requirements of the two antibiotics.

7. Among trifluoro-methylketones the motility of *P. vulgaris* was inhibited by the strongest way by -(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) reinforcing its proton pump inhibitory action supposed in the interaction studies. Furthermore a progressivity is phenomenal for the antimotility effect of TF18: first the running cells become tumbling and with increasing of the drug concentration, the tumbling ones become non-motile.

8. For the strong antibacterial, antimotility and so for the proton pump inhibitory effect of the trifluoro-methylketones, the structural requirements are the follows: 1.presence of the benzoxazol ring, since benzazole derivatives were more active than benzene or azole ones next to intact side chain. Next to the presence of benzoxazol ring 2. presence of side chain found in TF 18 is essential, since removal of terminal F_3 group, methylene group resulted drastic decreasing the antibacterial and antimotility action as well.

9. The MDR reversal activity of acridine derivatives depends on the aromatic moiety, the bounded heteroatom and nature of the side chain. Better activity is found: when the aromatic moiety is acridine or pyridoquinoline compared with quinoline and pyridine. Keeping the same atom on the ring, derivatives, having two side long chains, are more effective than ones, having one side short chain. Anti MDR activity was correlated with the presence of two or three hydrogen bond acceptor (HBA) groups. Possible HBA groups are tertiary amino groups, heterocyclic nitrogen like in the case of 2,8,10-Trimethyl-bis-4,6-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (24) and 2,8,10-Trimethyl-6-phenoxy-4-[N-(2'-diethylaminoethyl)-N-methylamino] pyrido-[3,2-g] quinoline (25). In addition to its nitrogen HBA group, the heterocycle can interact with P-gp by aromatic hydrophobic interaction.

10. The activity of benzonaphthyridine derivatives with various side-chain was compared with the aim of determining the effect of the nature of this chain on the MDR reversion. Compounds, which bore saturated aza heterocyclic moieties (pyrrolidine, morpholine, piperidine) were the most active. The presence of the amino group protonatable in the physiological conditions seemed to be necessary for the reversion of MDR.

SUMMARY

With the more and more emerging multidrug resistance problem, there is urgent need to research the cause, nature, and spread of the resistance, and to find some solutions for reversing MDR both in bacteria and tumour cells. One of the solutions is to develop new and more effective antibiotics and citostatics, but in a few time the appropriate resistance mechanism will arise to make the drugs ineffective. An other way for preventing the appearance of multidrug resistance is the application of combined chemotherapy, particularly in treatments of long-time duration e.g. certain type of cancers and tuberculosis. However it is better, when the resistance mechanisms are inhibited by some chemical agents. During the treatments of bacterial infections there already have been a method working with reversing of resistance, where the β-lactam antibiotics are combined with β-lactamase-inhibitors e.g. amoxicillin + clavulanic acid, ampicillin + sulbactam or piperacillin + tazobactam. Theoretically the elimination of plasmids carrying the resistance genes could also mean a solutions, however the elimination never occurs in the whole bacterial population. A better solution is to inhibit such a resistance mechanism, where the inhibition exists among all the cells of the population. The inhibition of bacterial and tumour multidrug efflux pumps by some chemical agent can be a practicable plan to overcome MDR. Among Gram-negative bacteria the intrinsic resistance for some typically „Gram-positive antibiotics" e.g. erythromycin and for some unrelated drugs, is on the one hand due to the working of an efflux pump system which uses proton gradient for its operation. The successful inhibition of this proton pump by 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF 18) means the possibility to employ an earlier ineffective antibiotics with a pump inhibitor in combination. The experiments show, that a so-called „Gram-negative resistance" can be overcome by this method. The way of the reversal mechanism, namely the inhibition of proton pump by TF 18, was proved by the successful inhibition of bacterial movement.
The reversal of drug resistance by inhibition of P-glycoprotein 170 efflux pump in mouse lymphoma cells was successful with chemically unrelated agents as well. The relationship found between the biological activity and chemical structures provides information for the further drug design research. The results are derived from model experiments. All the new information received can serve as a building stone in the research field of multidrug resistance reversary. First of all the statements of structure-activity relationship may be important for further drug design, which involves the synthesis of more effective and less toxic compounds and for in vivo experiments in the future.

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REVERSAL OF EFFLUX PUMP-MEDIATED MULTIDRUG RESISTANCE IN BACTERIA AND TUMOUR CELLS

Ph.D. Thesis

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