MODEL EXPERIMENTS FOR REVERSING MULTIDRUG RESISTANCE IN BACTERIA AND EUKARYOTIC CELLS

Ph.D. Thesis

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I dedicate this dissertation to my husband, who stood beside me in every way and who had admirable patience for me.

I would also like to dedicate this dissertation to my late maternal grandmother who taught me humanity and the respect of knowledge.

LIST OF PUBLICATIONS RELATED TO THE THESIS

Articles

I. **Shah A., Nalipara Y., Sureja D., Motohashi N., Kawase M., Miskolci Cs., Szabó D., Molnár J.:** *6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones: synthesis and mdr reversal in tumor cells.* Anticancer Res., 18 (4C): 3001-3004, 1998.
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II. **Motohashi N., Kawase M., Saito S., Miskolci Cs., Berek L., Molnár J.:** *Plasmid elimination and immunmodulation by 3-benzazepines in vitro.* Anticancer Res., 19(6B): 5075-5078, 1999.
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III. **Miskolci Cs., Labádi I., Kurihara T., Motohashi N., Molnár J.:** *Guanine-cytosine rich regions of plasmid DNA can be the target in anti-plasmid effect of phenothiazines.* Int. J. Antimicrob. Agents, 14(3): 243-247, 2000.
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IV. **Motohashi N., Shirataki Y., Kawase M., Tani S., Sakagami H., Satoh K., Kurihara T., Nakashima H., Wolfárd K., Miskolci Cs., Molnár J.:** *Biological activity of kiwifruit peel extracts.* Phytother. Res., 15(4): 337-343, 2001.
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V. **Kawase M., Motohashi N., Sakagami H., Kanamoto T., Nakashima H., Ferenczy L., Wolfárd K., Miskolci Cs., Molnár J.:** *Antimicrobial activity of trifluoromethyl ketones and their synergism with promethazine.* Int. J. Antimicrob. Agents, 18(2): 161-165, 2001.
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Book chapter

VI. **Molnár J., Szabó D., Miskolci Cs., Nacsa J., Kawase M., Saito S., Motohashi N.:** *Effect of some new 3-benzazepines on plasmid DNA, mdr P-glycoprotein and reverse transkriptase of leukaemia.* Non Antibiotics, NISCOM, New Delhi, India, 1998.

Abstracts related to the thesis

VII. **Miskolci Cs., Csúri K.:** *The antiplasmid effect of ant-arrhytmics and neuroleptics.* Proceedings of the 3rd PhD. Conference of the Albert Szent-Györgyi Medical University, May 11-14, 1997, Szeged, Hungary.

VIII. **Miskolci Cs., Labádi I., Csúri K., Molnár J.:** *Plasmid curing by some anti-arrhytmics and neuroleptics.* International Conference on Reversal of Drug Resistance, Junel-3, 1997, Szeged, Hungary.

IX. **Miskolci Cs., Labádi I., Molnár J.:** *Plasmid elimination induced by some psyhopharmacons and anti-arrhytmics.* 4th International Symposium on Cytostatic Drug Resistance, Sept. 25-26, 1997, Charité, Humboldt- Universität zu Berlin, Institute für Pathologie.

X. **Miskolci Cs., Labádi I., Wolfárd K., Molnár J.:** *Data for the molecular mechanism of plasmid elimination.* Proceedings of the XIII. Conference of the Hungarian Association for Chemotherapy, June 2-5, 1998, Debrecen, Hungary.

XI. **Miskolci Cs., Labádi I., Wolfárd K., Molnár J.:** *Examination of plasmid elimination mechanisms in E. coli and S. cerevisiae.* Proceedings of the Conference of the Hungarian Assotiation for Microbiology, August, 24-26, 1998, Miskolc, Hungary.

XII. **Molnár J., Gunics Gy., Miskolci Cs., Szabó D., Pusztai R.:** *Models for reversal of resistance.* Proceedings of the International Congress for Chemotherapy, July 4-7, 1999, Birmigham, UK.

XIII. **Miskolci Cs., Labádi I., Kurihara T., Motohashi J., Molnár J.:** *G-C rich regions of plasmid DNA can be the target in antiplasmid effect of phenothiazines.* 13th International Proceedings of the Congress of the Hungarian Society for Microbiology, August 29-Septembet 1, 1999, Budapest Hungary.

XIV. **Molnár J., Gunics Gy., Miskolci Cs.:** *Models for reversal of resistance in bacteria and fungi.* Proceedings of the 13th International Congress of the Hungarian Society for Microbiology, August 29-Septembet 1, 1999, Budapest Hungary.

XV. **Spengler G., Miskolci Cs., Molnár J.:** *Plasmid elimination experiments on clinical isolates.* Proceedings of the Congress of the Hungarian Society for Microbiology, August 24-26, 2000, Keszthely, Hungary.

XVI. **Molnár J., Gunics Gy., Miskolci Cs., Wolfárd K.:** *Models for reversing antibiotic resistance.* Proceedings of the Congress of the Hungarian Society for Microbiology, August 24-26, 2000, Keszthely, Hungary.

XVII. **Spengler G., Molnár J., Miskolci Cs.:** *Study for the effect of antiplasmid compounds on clinical bacterial isolates.* Proceedings of the Jubilee Congress of the Hungarian Society for Microbiology, October 10-12, 2001, Balatonfüred, Hungary.

To nature's great command.
All human laws are frail and weak.

-George Crabbe, 1807

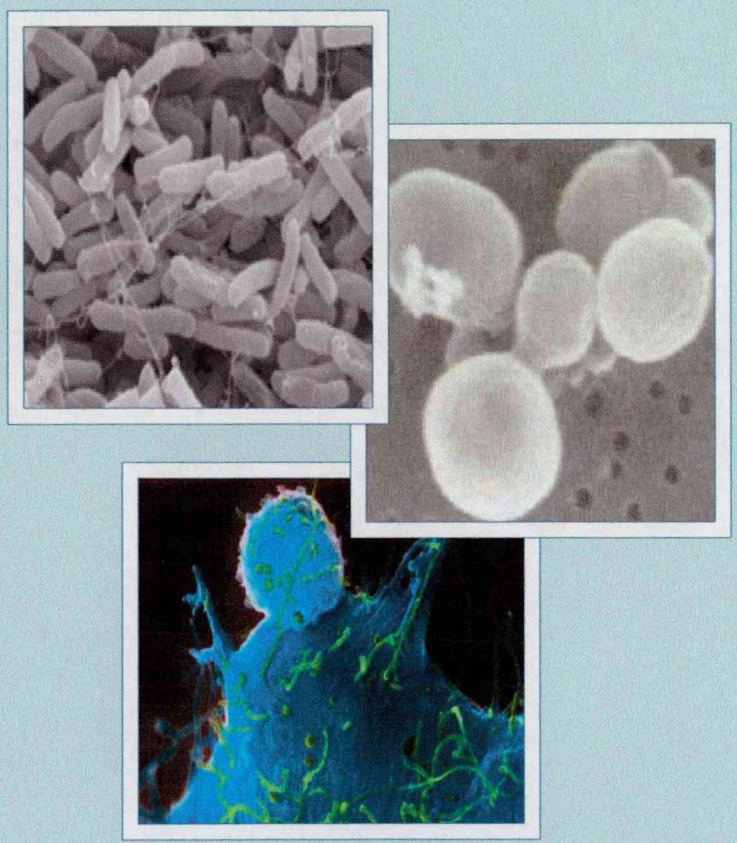
TABLE OF CONTENTS

SECTION **PAGE**

SECTION PAGE

Chapter 1

INTRODUCTION



1. INTRODUCTION

1.1. Antimicrobial Resistance

The first antibiotic, **penicillin**, was discovered in 1929 by *Sir Alexander Fleming*, who observed inhibition of staphylococci on an agar plate contaminated by *a Penicillium* mold. Penicillin became generally available for treatment of bacterial infections, especially those caused by staphylococci and streptococci, about 1946. The period of late 1940s and early 1950s saw the discovery and introduction of **streptomycin, chloramphenicol** and **tetracycline**, and the age of antibacterial chemotherapy came into full being. These antibiotics were effective against the full array of bacterial pathogens including Gram-positive and Gram-negative bacteria, intracellular parasites and tuberculosis bacillus. However, by 1953, during a *Shigella* outbreak in Japan, a strain of dysentery bacillus was isolated which was multiple drug resistant (exhibiting resistance to chloramphenicol, tetracycline, streptomycin, and the sulphanilamide) (1, 2). Bacterial resistance to antibiotics was encountered in clinical isolates soon after the introduction of some of the earliest agents (3). There was also an evidence that bacteria could pass genes for multiple drug resistance between strains and even among species.

Drug-resistant infectious agents are an increasingly important public health concern. Antimicrobial resistance is becoming a factor in virtually all hospital-acquired (nosocomial) infections (3,4). Today, the problem of antibiotic resistance continues to be major factor complicating the use of chemotherapeutic agents and the control of infectious diseases (5).

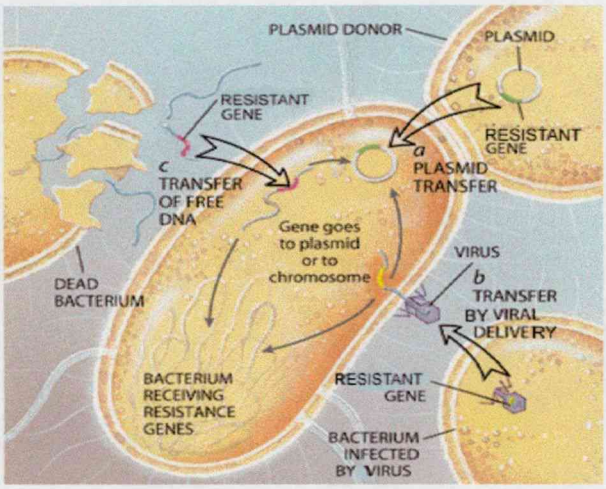
Treating resistant infections often contributes to higher health care costs and often requires the use of more expensive or more toxic drugs and can result in longer hospital stays for infected patients.

Ideal conditions for the emergence of drug-resistant bacteria result when drugs are prescribed for the common cold and other conditions for which they are not indicated or when individuals do not complete their prescribed treatment regimen. Hospitals also provide a fertile environment for drug-resistant pathogens (6).

A key factor in development of antimicrobial resistance is the ability of infectious organisms to adapt quickly to new environmental conditions. Bacteria are unicellular creatures and have a small number of genes. Even a single random gene mutation can have a large impact on their disease-causing properties and since most microbes replicate very fast, they can evolve

rapidly. Thus, a mutation that helps a microbe survive in the presence of an antibiotic drug will quickly become predominant throughout the microbial population (7). Microbes also commonly acquire genes, including those encoding for resistance, by direct transfer from members of their own species or from unrelated microbes.

1.1.1. Bacterial resistance mechanisms



(http://www.sciam.com)

Figure 1. BACTERIA PICK UP RESISTANCE GENES from other bacterial cells in three main ways. They often receive whole plasmids bearing one or more such genes from a donor cell (***a***). Other times, a virus will pick up a resistance gene from one bacterium and inject it into a different bacterial cell (***b***). Alternatively, bacteria sometimes scavenge gene-bearing snippets of DNA from dead cells in their vicinity (*c*). Genes obtained through viruses or from dead cells persist in their new owner if they become incorporated stably into the recipient's chromosome or into a plasmid.

In the past 50 years since antimicrobial agents were first introduced, bacteria have acquired a wide variety of mechanisms, which have enabled them to resist the effect of these drugs (8, 9).

Antibiotic resistance can be either *plasmid* mediated or maintained on bacterial *chromosome.*

The fundamental mechanisms of antimicrobial resistance are:

- *Enzymatic degradation of antibacterial drugs* /Example: cleavage of ß-lactam ring of penicillin by ß-lactamase/.
- *Alteration of bacterial proteins that are antimicrobial targets* /Examples: Erythromycin-resistant organisms have an altered receptor on the 50S subunit of the ribosome that serves as a binding site in susceptible organisms; resistance to some penicillins may be a function of the loss or alteration of PBPs (Penicillin Binding Proteins); etc./.

- *Changes in membrane permeability to antibiotics* /Examples: Streptococci have a natural permeability barrier to aminoglycosides; tetracyclines accumulate in susceptible bacteria but not in resistant bacteria, etc./.
- *Altered metabolic pathway that bypasses the reaction inhibited by the drug* /Example: Some sulphonamide-resistant bacteria do not require extracellular PABA but, like mammalian cells, can utilize performed folic acid/.
- *Development of an altered enzyme that can still perform its metabolic function but its much less affected by the drug than the enzyme in the susceptible organism* /Example: in some sulphonamide-susceptible bacteria, the tetrahydropteroic acid synthetase has a much higher affinity for sulphonamide than for PABA. In sulphonamide-resistant mutants, the opposite is the case/.

**Further information concerning resistance mechanisms outlined above can be found in the references: 10-35.*

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Inherent (Natural) Resistance. Bacteria may be inherently resistant to an antibiotic. For example, a streptomycete has some gene that is responsible for resistance to its own antibiotic; or a Gram-negative bacterium has an outer membrane that establishes a permeability barrier against the antibiotic; or an organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is hit by the antibiotic.

Acquired Resistance. Bacteria can develop resistance to antibiotics, e.g. bacterial populations previously sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria:

a.) *Mutation and selection* (sometimes referred to as vertical evolution)

b.) *Exchange of genes between strains and species* (sometimes called horizontal evolution)

Vertical evolution is strictly a matter of Darwinian evolution driven by principles of natural selection: a spontaneous mutation in bacterial chromosome imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the wild type (non-mutants) is killed and the resistant mutant is allowed to grow and flourish. The mutation rate for most bacterial genes is approximately 10^{-8}.

Horizontal evolution is the acquisition of genes for resistance from another organism. Some bacterium develops genetic resistance through the process of mutation and selection and then

donates these genes to some other bacterium through one of several processes for genetic exchange that exist in bacteria.

Bacteria are able to exchange genes in nature by four processes*: conjugation, transduction transformation* and *transposition. Conjugation* (36, 37) involves cell-to-cell contact as DNA crosses a sex pilus from donor to recipient (38). This is the commonest method by which multidrug resistance spreads among different genera of Gram-negative bacteria. Transfer of resistance plasmids (39, 40) also occurs among some Gram-positive cocci. During *transduction*, a virus transfers the genes between mating bacteria. For example the plasmid carrying the gene for β-lactamase production can be transferred from penicillin resistant to a susceptible *Staphylococcus* if carried by a suitable bacteriophage. Similar transduction occurs in salmonellae. In *transformation*, DNA is acquired directly from the environment, having been released from another cell (42). Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). It is common for DNA to be transferred as *plasmids* between mating bacteria. Since bacteria usually develop their genes for drug resistance on plasmids (called resistance transfer factors; RTF), they are able to spread drug resistance to other strains and species during genetic exchange processes. *Transposition* is a transfer of short DNA sequences (transposons, transposable elements) occurs between one plasmid and another or between a plasmid and a portion of the bacterial chromosome within a bacterial cell (36, 43, 44).

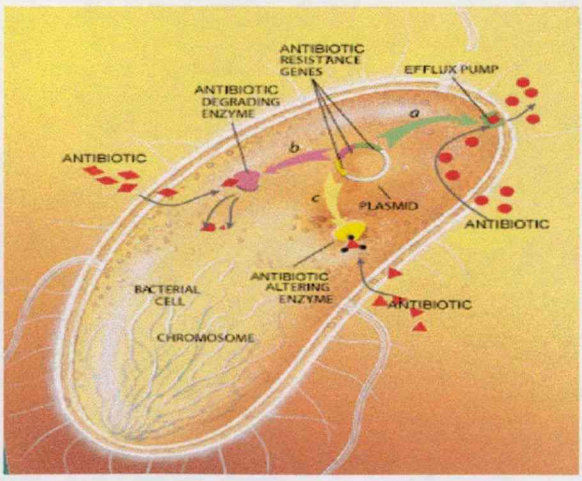


Figure 2. ANTIBIOTIC-RESISTANT BACTERIA owe their drug insensitivity to resistance genes. For example, such genes might code for "efflux" pumps that eject antibiotics from cells (*a*). Or the genes might give rise to enzymes that degrade the antibiotics (*b*) or that chemically alter and inactivate the drugs (*c*). Resistance genes can reside on the bacterial chromosome or, more typically, on small rings of DNA called plasmids. (*http://www.sciam.com)*

1.1.1.1. What are Plasmids?

Fertility (F) and drug resistance (R) factors are plasmids; that is, autonomously replicating entities, or extra chromosomal hereditary determinants (45). Plasmids isolated from bacteria exist as double-stranded DNA circles. Their host cells can usually survive without them. They have relatively small size, generally in the range between 1.5 and 100 million Daltons.

F plasmid: facilites conjugation. F plasmids live in bacterium *E. coli* (46) and were discovered in 1920s.This can give a bacterium new genes that may help it survive in a changing environment. The F plasmid consists of 25 genes that mostly code for production of sex pili. Bacteria that have an F plasmid are F^+ or male. Those that do not have an F plasmid are F^- or female. F plasmid can behave as an *episome*-it means that it can integrate reversibly into the bacterial chromosome. When the F^+ plasmid is integrated within the bacterial chromosome, the cell is called an Hfr cell (high frequency of recombination cell). A cross over event can occur between homologous genes of the Hfr fragment and the F-DNA. The recombinant genome can be passed on to future generations.

R plasmid: R plasmids were first discovered in Japan in 1957 (1). In Japan, dysentery was treated with sulphonamide until about 1950. Then more and more strains of bacteria causing dysentery became resistant to this antibiotic, rapidly rendering it ineffective. Then tetracycline, streptomycin and chloramphenicol were used against these bacteria. By 1957, 2% of the bacteria causing dysentery were resistant to one or more of these drugs, and by 1960, 13% were resistant. Resistance or R plasmids carry genes that confer resistance to certain antibiotics as well as to poisonous metal ions such as arsenic, silver, copper, mercury, lead, zinc and so on (47). The R plasmid usually has two types of genes:

1. R-determinant: resistance genes that code for enzymes that inactivate certain drugs
2. RTF (Resistance Transfer Factor): genes for plasmid replication and conjugation.

The presence of resistance genes, on the other hand, allows for their transcription and translation into enzymes that make the drug inactive. R plasmids can also be transferred by conjugation from one bacterial cell to another.

Colicin plasmids: The colicins of *E. coli* are members of the large and diverse family of antimicrobial toxins known as bacteriocins. The characterised colicins (over 20) can be divided into two major classes according to their mode of action: the enzymatic and the channel-forming colicins (48). Members of both classes of colicins share a number of characteristics, but they share a low level protein sequence similarity (49, 50). Colicin gene clusters consist of three tightly linked genes: a colicin gene; an immunity gene, which

encodes an immunity protein that provides specific protection against the encoded colicin (51); a lysis gene, which encodes a protein that is involved in colicin release from the cell. Colicin gene clusters are encoded on plasmid replicons and the colicin production is SOS mediated (52).

Virulence plasmids: virulence plasmids contain genes that make their bacterial hosts more virulent to "their" hosts. A familiar example involves the bacterium *E. coli*, which inhabits the human large intestine. Certain strains of *E. coli* contain plasmids whose genes make the *E. coli* synthesize toxins (54) that cause diarrhea (53). These "enterotoxogenic strains" of *E. coli* are probably an important cause of diarrhea among travellers (55). More seriously, in developing countries, diarrhoea is one of the principal causes of death among those under five (56).

Metabolic plasmids: metabolic plasmids contain genes that let their bacterial hosts metabolise or degrade otherwise indigestible or toxic chemicals. For example, the bacterium *Pseudomonas putida* is able to grow on a wide range of organic compounds that are toxic to most bacteria, including toluene, octane, camphor, naphthalene and nicotinic acid! It does this with the help of genes contained by metabolic plasmids called TOL, OCT, CAM, NAH, and NIH plasmids.

Other metabolic plasmids allow bacteria to degrade herbicides like 2,4 D, as well as certain detergents!

Cryptic plasmids: cryptic plasmids are plasmids that have no known effect on their hosts.

Cosmids: cosmids are man-made circular loops of DNA containing plasmid DNA together with an arbitrary sequence of up to 45,000 base pairs of DNA. They are constructed by recombinant DNA techniques and then packaged in lambda phage protein coats. They are used to transfer genes to bacteria (57).

Phasmids: phasmids are man-made linear DNA molecules whose ends are sequences taken from the lambda phage, while the middle is a sequence taken from a plasmid, together with a sequence of whatever DNA one wants. Like cosmids, they are constructed by recombinant DNA techniques and packaged in lambda phage protein coats, and used to transfer genes to bacteria. However, both the lamda phage and plasmid replication functions are intact. In particular, they contain the lambda phage genes for "lysis", the process whereby a virus dissolves the cell membrane of its host. Depending on the conditions, the phasmid can act either like a highly defective prophage or a plasmid-hence its name (58, 59, 60).

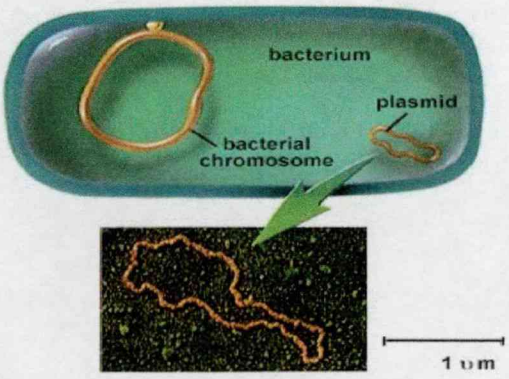


Figure 3. Structure of bacterial plasmid. (*http://www.biosci.uga.*)

1.1.1.2. Multidrug efflux pumps of gram positive bacteria

Major facilitator family (MF) of transporters: contain 12-14 transmembrane helicles and utilize proton-motive force as an energy source. Two classes of multidrug pumps are known within this family:

- *Bmr (Bacillus multidrug resistance)-NorA (norfloxacin) group and the QacA (quaternary ammonium disinfectants/cationic dyes)* (61, 62).
- *Smr (staphylococcal multidrug resistance)* pump-small transporter, contain only four transmembrane helicles. Pumps out quaternary ammonium disinfectants and basic dyes (61, 63).

1.1.1.3. Multidrug efflux pumps of Gram-negative bacteria

Two types of multidrug efflux pumps have been reported:

Emr (mvrC) *pump of E. coli-member of Smr family*. The drugs are exported into periplasmic space. The disruption of the emrE gene causes hyper susceptibility to lipophilic cations (64).

Pumps, which can excrete, drug molecules directly into the medium contain at least three subunits: transporter protein located in the cytoplasmic membrane, an outer membrane channel and a periplasmic "linker" protein that connects the two (61, 64, 65, 66). The transporters of this type of pumps are energized by proton motive force MF family (EmrAB of *E. coli* contains a transporter of the MF family, EmrB (67)). When the emr AB operon is on multicopy plasmid, the susceptibility of *E. coli* to carbonyl cyanides m-chlorophenylhydrazone /CCCP/, and tetrachlorosalicylanilide becomes decreased. It makes *E. coli* more resistant to nalidixic acid and thiolactomycin. Emr A proteins are distantly related to membrane fusion protein of paramyxovirus (68), suggested that they may fuse or bridge the

inner and outer membranes, and proposed to call them membrane fusion proteins (MFP). EmrAB does not appear to be involved in resistance to clinically important antibiotics, or to the resistance-nodulation division (RND) family. These pumps, especially those of RND family, show extremely broad substrate specificity (69).

Efflux across a single membrane layer e.g. **Tet pump** -classical efflux pump, pump out solute molecules across a single cytoplasmic membrane layer, move agents from the cytoplasm into periplasmic space (70). Pumps of this type need to have high throughput, as the spontaneous influx of the drug through the lipid bilayer domains of the cytoplasmic membrane would also be rapid.

A few of Gram-negative multidrug efflux pumps appear to catalyse excretion across the cytoplasmic membrane, e.g. Smr family, which are small proteins that predicted to span the membrane four times. The *emr (mvrC)* gene, whose disruption causes hyper susceptibility to tetraphenylphosphonium, methylviologen and ethidium bromide, is a member of this family found *in E. coli* (64). The multicopy plasmids containing the gene (overproduction of Emr E protein) makes *E. coli* slightly more resistant to tetracycline, erythromycin and sulfanazide (71).

Efflux across a double-membrane system (AcrAB system of *E. coli* (72, 73); MexAB-OprM system of *P. aeruginosa* (74); haemolysin of *E. coli* HlyB-HlyD-TolC (75, 76); proteases B and C of *Erwinia chrysanthemum* PrtD-PrtE-PrtF; and cycloysin of *Bordetella pertussis* CyaB-CyaD-CyaE). In all these systems the transporter protein located in the cytoplasmic membrane is thought to be brought into apposition with an outer membrane channel (TolC, PrtF and CyaE) through the linker protein (HlyD, PrtE, CyaD), which were shown to make up the named MFP (membrane fusion protein) family (68). Transporters in these systems (HlyB, PrtD, CyaB) are all members of ABC (ATP-binding cassette) family in contrast to the transporters of multidrug efflux systems, which are energised by proton motive force.

This type of multidrug efflux pumps exclusively in Gram-negative bacteria shares the same construction. Systems containing transporters of the RND (resistance nodulation division). RND transporters are much larger proteins than MF proteins. The RND transporter contains 12 transmembrane helicles and two large periplasmic domains between the transmembrane helicles 1 and 2, and 7 and 8, in contrast to MF transporters, which do not contain such domains.

Table 1. Multidrug efflux systems in Gram-negative bacteria.

Transporter	Linker	OM pore	Repressor gene	Organism	Substrates
Smr type					
EmrE				*E. coli*	lipophilic cations (e.g. ethidium bromide,methyl-viologen)
MF type					
EmrB	EmrA	TolC	emrR	*E. coli*	CCCP, nalidixic acid, thiolactomycin
RND type					
AcrB	AcrA	TolC	acrR	*E. coli*	Tet, CP, FQ, ß-lactams Nov, Em, FuA, Rif, EB, AF, CV, SDS, DOC
Mex B	MexA	OprM	mexR (nalB)	*P. aeruginosa*	Tet, CP, FQ, ß-Lactams (except carbapenems), Nov, Em, FuA, Rif, TMP, SMZ
Mex D	MexC	OprJ	nfxB	*P. aeruginosa*	Tet, CP, FQ, TMP, 4^{th}–generation cephems (but not conventional ß-lactams or carba-penems)
Mex F	Mex E	OprN	nfxC	*P. aeruginosa*	CP, TM, FQ
MtrD	MtrC	MtrE	mtrR	*N. gonorrhoeae*	Tet, CP, ß-lactams, Em, FuA, Rif, EB, AF, CV, TX, DOC

1.1.2. Antifungal drug resistance

With increased number of immuncompromised patients there is an increase in the number of clinically significant fungal infections. Unfortunately, widespread use of limited numbers of antifungal agents led to the development of drug resistance.

The most common mechanisms for the development of resistance involve changes in the enzymatic pathways, which serve as drug targets. For instance, changes in enzymes responsible for the biosynthesis of ergosterol, the target of azole activity lead to azole resistance. Another common mechanism used by fungi to avoid drug toxicity includes reduced intracellular accumulation of drug through both decreased permeability and energy-dependent efflux pumps.

1. **Primary resistance** occurs without organism ever having been in the presence of drug.
2. **Secondary or acquired resistance** arises only after exposure of fungi to the drug.

Intrinsic resistance exists even all members of species are resistant to a certain drug, such as *Candida crusei* for fluconazole and *Scedosporium prolificans* to all present antifungals.

As summarised by *Rex and co-workers* (77) resistance may be due to either the acquisition of an intrinsically resistant species of fungus, selection of a resistant strain from a population of multiple strains or mutation of an initially susceptible strain, which then becomes resistant.

The development of drug resistance in fungi can occur at several sites within the fungus:

- *uptake mechanism(s) of the drug into the yeast cells*
- *drug target alterations*
- *efflux pumping mechanisms*

1.1.2.1. Antibiotic action and resistance mechanisms in fungi

Polyenes: The first antifungal agent amphotericin B was discovered in 1956 (78). Amphotericin B is a member of the macrolide polyenes, which exhibit fungicidal activity by binding to ergosterol in the cell membrane of fungi, leading to the formation of pores in the fungal cell membrane and subsequent leakage of intracellular molecules (78, 79). Another proposed effect of these drugs includes stimulation of cell proliferation (80). The polyene antifungals display the widest spectrum of antifungal activity of any of classes of antifungal agents and since its discovery amphotericin B. has become the gold standard of antifungal therapy for invasive mycoses (80).

Amphotericin B contains both a hydrophobic chain with double bonds and hydrolated chain, which is hydrophilic. An interaction between the double bond chain of amphotericin B and ergosterol present in the cell membrane of fungus leads to formation of sterol-polyene complex. This interaction thus displaces the ergosterol from its normal phospholipid interactions in the membrane, allowing leakage of protons. Mutant strains of moulds and yeasts, which lack ergosterol in the cell membrane, are therefore likely to be resistant to the polyene antifungals. Mutant strains of *Aspergillus fennellial*, which were resistant to polyenes, were found to have sterols other than ergosterol in cell membrane (81). Likewise a nystatin-resistant strain of *Saccharomyces cerevisiae* was found to have 5,6-dihydroergosterol as its main sterol component (81). The isolation of azole-polyene resistant *Candida albicans* strains from the blood of two leukaemic patients had no ergosterol (82).

It was supposed that the prophylactic use of fluconazole led to the depletion of ergosterol in the cell membrane and thereby loss of an active site for amphotericin B binding, resulting high MICs for the latter drug. Results of the sterol membrane analysis indicated that a specific mutation in the $\Delta^{5,6}$-sterol desaturase enzyme led to the decrease in membrane ergosterol→lack of binding site (82).

Fluoropyrimidines: Flucytosine is taken up by the fungal cells via the enzyme cytosine permease, and then converted to fluorouracil by cytosine deaminase (not present in human cells) with subsequent conversion to fluorouridine triphosphate (FUTP) via several intermediate steps. FUTP is then incorporated into fungal RNA, causing alterations in protein synthesis. Alternatively fluorouracil may also be converted to fluorodeoxyuridine monophosphate (F-dUMP), which acts as a noncompetitive inhibitor of thymidylate synthetase, ultimately interfering with DNA synthesis. *Normark and Schonebeck* (83) have characterised two *C. albicans* resistance phenotypes:

- *Class I.* Isolates display total resistance to even high concentrations of flucytosine,
- *Class II.* Organisms display initial sensitivity with the development of resistance after incubation with the drug (investigation of 442 yeast isolates of *Candida* and *Torulopsis* (84)).

The genetic basis for this resistance to flucytosine has been postulated to be via genetic resistance traits, which are both homozygous and heterozygous to select homozygous resistance traits encoding for secondary resistance.

Resistance in *S. cerevisiae* develops as a result of mutations which encode for the loss of activity of cytosine permease, cytosine deaminase or uridine monophosphate pyrophosphorylase (UMPP) (85). Basically, resistance can result when there is a mutation in enzyme necessary for flucytosine action. In addition, mutations leading to increased de novo

synthesis of pyrimidines can lead to resistance as well. Kerrige and *Whelan* (86) suggest that the UMPP mutation is the most common defect in *C. albicans* resistance.

Azoles: The target enzyme of the azoles is lanosterol 14α demethylase (P450DM), a cytochrome P450-dependent enzyme active in the synthesis of ergosterol. Azoles bind to the iron of P450DM, thereby inhibiting the binding of lanosterol (as well as molecular oxygen) to the P450DM. This subsequently prevents the first hydroxylation reaction of the 14α-methyl group of lanosterol and thereby blocks the formation of ergosterol (81). Further investigations revealed that the resultant changes in the cell membrane also led to abnormal activity of other membrane-bound enzymes such as those active in chitin synthesis (81). More recent studies suggest that azoles may also target, either directly or indirectly 3-ketosteroid reductase, the enzyme which catalyses the synthesis of 4,4-demethylated 14α-methylsterols that may function as surrogates of ergosterol (87).

Since azoles work by interfering with the biosynthesis of ergosterol, alterations in the P450DM and $\Delta^{5,6}$ sterol desaturase enzymes, both active in the biosynthesis of ergosterol, have been shown by several investigators to occur in azole-resistant *Candida* strains (88).

Another proposed mechanism for drug resistance is decreased permeability of the fungal membrane to azoles. The decreased permeability is considered secondary to a variety of mechanisms which change sterol compositions and thereby the permeability of the membrane. Membrane changes are thought to make difficult for azoles to penetrate into the cell (89).

Recently, active efflux mechanisms have been a focus for investigations into antifungal drug resistance and it is assumed that the mayor mechanism for resistance in fungi will actually be associated with energy-dependent pumps (90, 91 92 93, 94, 95).

Thus far, work with *S. cerevisiae* has led to the identification of a number of genes believed to be involved in coding for MDR transporters. Seven pleiotropic drug resistance (PDR) genes have been identified. PDRs gene is believed to encode for an ABC transporter, which could be active in antifungal resistance (96). The other PDR genes are thought to encode transcriptional regulators affecting the expression of such pumps (96). In addition to this ABC-transporter, MFS transporters have also been identified in *S. cerevisiae* (96).

Candida spp. has been found to contain genes, which encode for MDR pumps as well. A gene known as BEN^r has been isolated in *C. albicans* (95) and has been shown to confirm resistance to a wide range of agents including benomyl and methotrexate when incorporated into previously susceptible strains of *S. cerevisiae* (97). Another gene, homologousous to

PDR_5 and identified as CDR1, has also been isolated in *C. albicans*. (92) Sanglard et al. have documented increasing levels of mRNA encoding for both CDR1 and BEN^r genes (90). In fluconazole resistant *C. albicans* isolates from AIDS patients as well as by Albertson and co-workers (91) while working with a fluconazole-resistant *C. albicans* strain, in which they had documented the presence of an energy dependent drug efflux mechanism accounting for the fluconazole resistance. This evidence has led to the speculation that the CDR1 and BEN^r genes do indeed encode for MDR transporters responsible for azole resistance in *Candida* species.

Subsequently researchers were able to demonstrate competition between rhodamine 123 and fluconazole for efflux out of strains of azole-resistant *C. glabrata*, indicating that the same pump in this fungus transported both (93). Furthermore, *Parkinson et al.* (98) documented competition between benomyl and fluconazole for transport out of fluconazole-resistant *C. glabrata* via an energy-dependent mechanism. This same isolate exhibited cross-resistance to ketoconazole and itraconazole. These findings suggested that MDR pumps were indeed, at least in part, responsible for resistance to fluconazole and possibly responsible for azole cross-resistance as well.

1.1.3. Drug resistance in cancer cells

The failure of many metastatic cancers to be treated effectively or cured by chemotherapy has led to a search for the genetic alterations in cells, which are responsible for resistance to cytotoxic drugs. Analysis of the phenotype of these drug-resistant cancer cells has allowed classification into two groups: cells demonstrating single agent resistance with the same mechanism of action, and cells broadly resistant to many chemically diverse anti-cancer drugs with different mechanisms of action. The multidrug resistance (MDR) is more urgent problem in cancer chemotherapy. The way by which cells become multidrug resistant indicates several different resistance mechanisms: interference with programmed cell death such as inactivation of p53 (99) and the expression of energy dependent pump systems, which pump out anticancer drugs from cells (100). Two such pump systems have been detected and their cDNAs have been cloned from human cancer cells:

- ***MDR1*** (the multidrug transporter or 170 kDa P-glycoprotein (101, 102) encoded by MDR1 gene (103).
- ***MRP*** (encoding 190 kDa MDR-associated protein, the glutathione conjugate transporter encoded by 6.5 kb mRNA (104, 105, 106).

Both are ATP-dependent transporters which belong to a larger family of ATP-binding cassette (ABC) proteins and consist of two homologousous halves, each containing six transmembrane regions and one ATP binding/utilization site, but they share less than 15% amino acid identity (104).

MRP was cloned originally from multidrug-resistant small cell lung cancer cell line (130) and has been subsequently found to be overexpressed in number of drug- selected cell lines derived from a variety of different tumour types (104, 105, 106, 107, 108, 109, 110)

The gene encoding Pgp belongs to the MDR multigene family, which consists of two highly homologousous genes MDR1 and MDR2 situated on chromosome 7q21.1 in humans (111).

Pgp is phosphorylated and glycosylated protein 1280 amino acids long, which intersects the plasma membrane and consists of two homologousous halves, each of which contains six hydrophobic domains and hydrophilic nucleotide binding fold (Fig. 5.). The hydrophobic regions represent putative transmembrane domains forming a pore-like structure (112). The two adenosine triphosphate (ATP)-binding folds are located intracellulary and exhibit significant ATPase activity (113). The drug binding sites are localized in the transmembrane domains close to the cytosolic surface (114) (Fig. 5.).

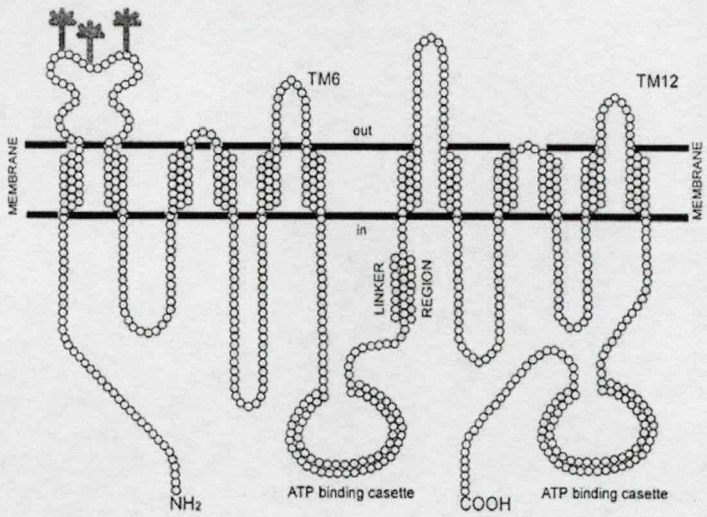


Figure 4. Schematic drawing of Pgp demonstrating two homologousous halves with six transmembrane regions and nucleotide binding domain each connected with a linker region. The N-linked carbohydrates are presented as branched structures on the first extracellular loop. This model of human P-gp was derived from sequence analysis. Source: Adapted from Ref.: 101.

P-glycoprotein expression in normal tissues has been determined by Northern blot analysis of MDR1 RNA and by immunhistochemical analysis with monoclonal antibody (115, 116). In human high levels of P-glycoprotein expression were found in epithelial cells on the lumenal surface of biliary hepatocytes, small and large intestine mucosal cells pancreatic ductules,

proximal tubule of kidney, endothelial cells of brain and testis capillaries (117, 118), in the placenta (118, 119) and in secretory glands of pregnant endometrium (120, 121). The localization of P-glycoprotein in these tissues suggests that it may have a normal physiological role in transporting cytotoxic compounds or metabolites. Pgp situated on the luminal surface of intestinal mucosa may contribute to the elimination of certain drugs via feces and thus limit their bioavailability after oral administration (122).

The binding of drugs to Pgp was first demonstrated using MDR drugs and later extended to include Pgp inhibitory agents. Structure-activity studies of tioxantens (123) and reserpin analogs (124) have described a general pharmacophore for Pgp inhibitors, which contains planar aromatic domains. Drugs that interact with Pgp are frequently large (MW > 400), hydrophobic or amphipatic molecules with a planar ring system and a basic nitrogen side chain (124). They often carry a positive charge at physiological pH but this is not true for colchicine and certain hydrophobic peptides indicating that these substrates engage alternative substrate binding sites (125-128).

The MDR1-encoded P-glycoprotein pump recognizes and transports many different substrates, including most natural product anticancer-drugs such as doxorubicin, daunorubicin, vinblastine, vincristine, and actinomycin D (113). Many non-chemotherapeutic compounds exist that inhibit drug binding to P-glycoprotein and reverse the multidrug resistance phenotype in culture. Such reversing agents include *calcium channel blockers* (e.g. verapamil) (131), *antipsychotics* (phenothiazines), *antihypersensitive agents* (reserpine), *calmodulin antagonists* (trifluoperazine), *non-ionic detergents* (Tween-80) and immunosuppressants (cyclosporine A, FK-506) (130-138), which are presumed to be substrates for P- glycoprotein. These compounds can act competitive with cytotoxic chemotherapeutic agents for binding to the multidrug transporter (Fig. 5. right).

Tiamulin (a diterpenic veterinary drug widely used in swine) is a semi-synthetic antibiotic, which overpasses the capacities of reference products such as verapamil and cyclosporin-A in terms of *in vitro* reversal effect. It is also active *in vivo* without displaying pernicious side effects at doses necessary to reverse the MDR phenotype (129).

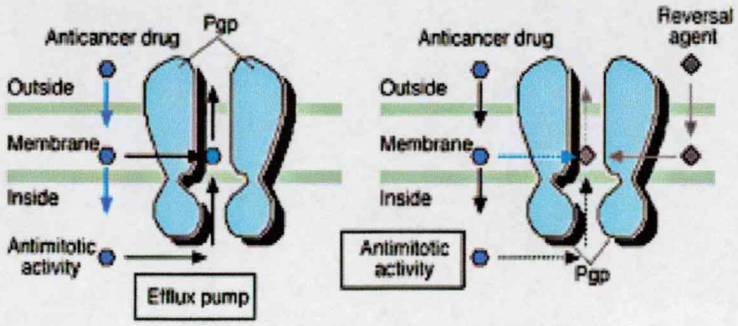


Figure 5. Reversal agents block the efflux of anticancer drugs.

Transport and binding studies suggest that Pgp interacts with substrates in the inner leaflet of the lipid bilayer of the plasma membrane and acts by flipping substrates to the outer leaflet of the bilayer (130,139, 140).

The patterns of expression of the MDR1 gene:

- Untreated cancers derived from normal tissues that express the multidrug transporter, such as carcinomas of the colon, kidney, pancreas, liver etc.
- Increased expression of P-glycoprotein is detected after chemotherapy, including acute leukaemias, brest cancers, ovarian cancers. Alternatively, the limiting dose of cytotoxic drugs applied in therapy may directly induce a cellular genetic response to the cytotoxic insults, including elevated mdr expression and the biochemical changes in the surviving cancer cells.
- MDR1 gene expression may occur in some cancers not derived from MDR1 expressing normal tissues and not previously exposed to drugs (e.g. neuroblastomas, sarcomas, chronic myelogenous leukaemias, non-small cell lung carcinomas) and tumour cell during the chronic phase does not show high MDR RNA levels. The emergence of multidrug resistance might be closely associated with tumour progression and that the MDR1 gene could be turned on as a result of *ras* oncogene activation and p53 tumour suppressor gene inactivation which occur through genetic lesions during tumorogenesis.

Some changes observed in MDR cells:

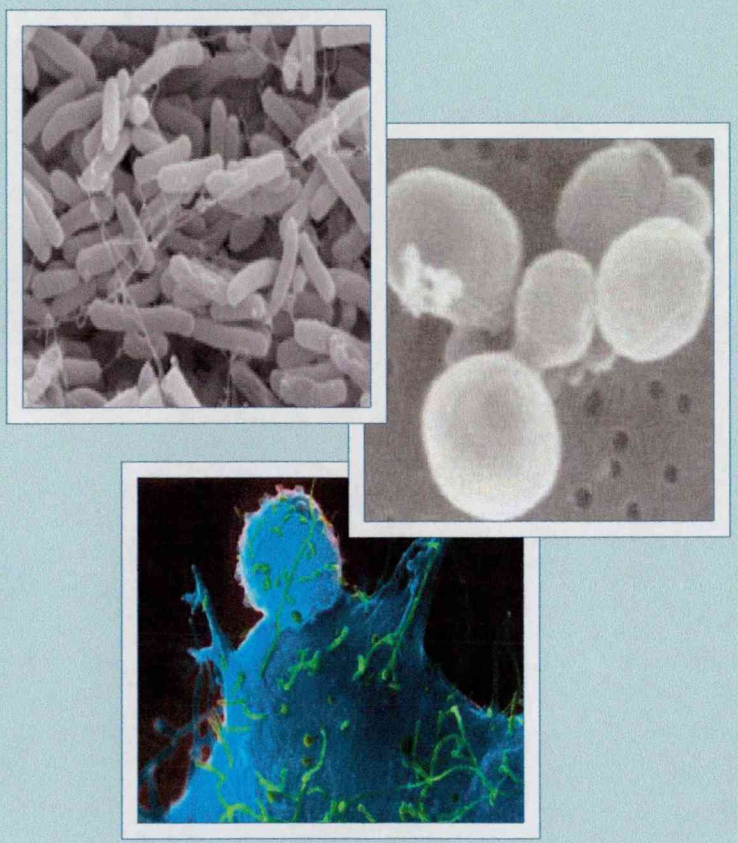
- Resistance to structurally unrelated drugs
- Increased (collateral) sensitivity to different agents
- Altered drug transport (increased efflux, decreased influx)
- Glutatione level and transferase activity
- Topoisomerase activity
- Calcium level
- Intracellular pH
- Membrane potential
- Aminoacid, nucleoside, ion transport
- Plethora of membrane and metabolic changes

P-glycoprotein

Figure 6. The complex phenotype of MDR cells (adapted from Ling[141]).

Chapter 2

AIMS OF THE STUDY



2. AIMS OF THE STUDY

The main aim of the dissertation was to construct models for reverse antibiotic resistance of bacteria and eukaryotic cells.

The goals of the study were the following:

a.). To seek drugs against polyresistant bacteria i.e. drugs against plasmids: to study the antiplasmid effect of some phenothiazines and related compounds. The idea is based on the great finding that promethazine had growth inhibitory effect on mycobacteria (142, 143) and it can eliminate the F' plasmid of *E. coli* (144, 145). The F' lac plasmid of *E. coli* served as a convenient model to study the plasmid curing effects of various tricyclic, because lactose plasmid-carrying colonies can be easily differentiated from plasmid-less pink colonies.

b.) To find out the exact target of phenothiazines in the complex formation resulting in antiplasmid effects. It is suggested that antibacterial, antiplasmid effect of phenothiazines correlate with the binding of phenothiazines to nucleic acids by intercalation. To study this question the antiplasmid effect of promethazine was studied in the presence of complex forming compounds such as caffeine and indole, which can polarize the phenothiazine or decrease its polarization.

c.) To clarify the exact mechanism of action of the drugs on plasmid replication. To investigate interaction between tricyclic compounds and extrachromosomal DNA *pBR322* high copy number plasmid was used as a model plasmid and agarose gel-electrophoresis was performed. The results possibly can explain antiplasmid effect of phenothiazines and structurally related compounds.

d.) In a further study the practical value of the complex formation of the tricyclic compounds with plasmids was investigated on clinical isolates.

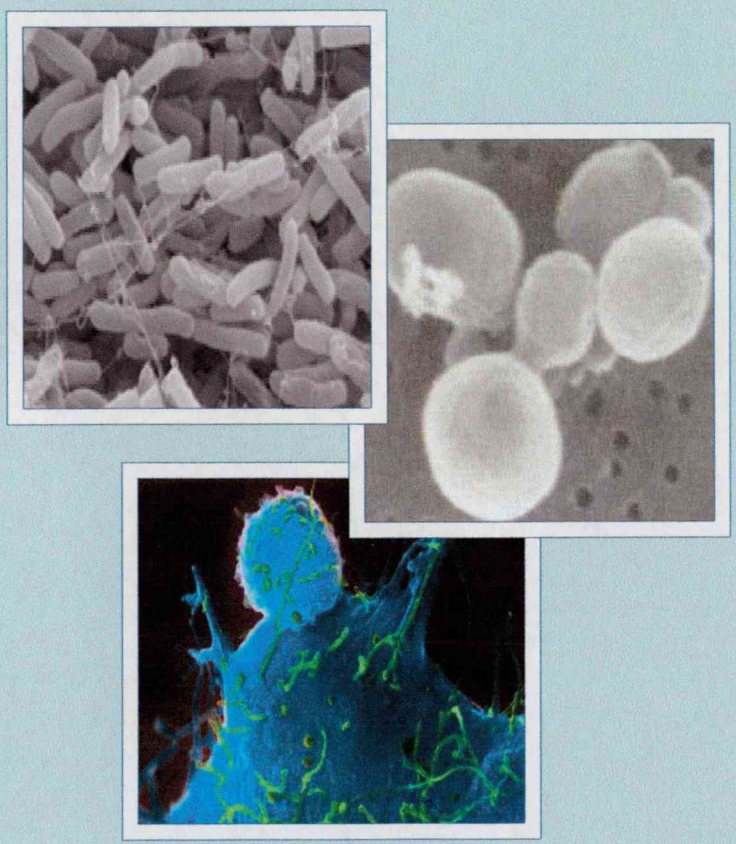
e.) Elimination of yeast plasmids was also studied in the presence of antiplasmid compounds. For this study killer plasmid of *S. cerevisiae* was isolated and used as a model.

f.) To investigate the antifungal effect of some tricyclic antiplasmid drugs in combination with antifungal drugs against clinical isolates (*C. albicans, C. glabrata*).

g.) There are no extrachromosomal nucleic acids responsible for drug resistance in cancer cells (an efflux pump is liable for MDR). Nevertheless, we wanted to test the effect of resistance modifiers on drug accumulation in tumour cells. The inhibitory action of phenothiazines and compounds isolated from plants were tested by flow cytometry.

Chapter 3

MATERIALS AND METHODS



3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Culture media for bacteria and fungi

2xYPD /2x yeast extract-peptone-glucose/ nutrient broth (146) was used for the cultivation and the determination of live cell counts in the cultures of yeast.

2xYPD+P-citrarate buffer: 2 x Yeast extract-peptone-glucose agar buffered with P-citrate was used for the determination of yeasts with killer activity.

MTY /tryptone-yeast extract media/: liquid media was used for the cultivation and the determination of live cell counts in the cultures of *E. coli* bacteria (147).

EMB /eosin-methylene blue/ agar plate was used for the differentiation of lac negative (*lac*$^-$) pink and lac positive (*lac*$^+$) deep purple colonies (148).

LB-broth /Lennox L broth/ was used for the cultivation of *E. coli* ER_{1398} (149, 150). This strain was used for preparation of competent cells.

Luria Agar /Miller's LB agar/ (149) supplemented with *Ampicillin* (100 μg/ml) and *Tetracycline* (12,5 μg/ml) was used for selection of transformed cells.

3.1.2. Media for cell line cultivation

McCoy's 5A medium modified (Gibco BRL) (151, 152, 153):

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

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

3.1.3. Stock solutions 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.

3.1.4. Stock solutions for purification of (DNA/RNA) plasmids by alkaline lysis method

Solution I. (Sol I.): Glucose 50 mM, Tris-HCl 50 mM (pH: 8.0), EDTA 10 mM

Solution II. (Sol II.): NaOH 200 mM, 1% SDS. Prepare on the day of DNA purification.

Potassium acetate solution (for alkaline lysis plasmid preparation):

TE buffer: 10 mM Tris-HCl (pH: 8.0), 1.0 mM EDTA

RNase: 10 mg/ml stock solution

TBE buffer, 20x (for agarose gel electrophoresis):Tris base, boric acid, $Na_2EDTAx2H_2O$

Ethidium bromide: 10 mg/ml stock solution

3.1.5. Stock solutions for preparation of protoplasts

Pre-treating solution: 10 mM Tris-HCl, 0.5 M Na-EDTA and 1 % β-mercaptoethanol in water.

Enzyme solution: In 0.7 M KCl 0.5 % snail enzyme and 0.5 % NovoZym.

**For further details see Appendix I.*

3.1.6. Strains and cell lines

3.1.6.1. Bacterial strains:

1. *Laboratory bacterial strains:*

E. coli K12 LE140 (T_6^R, T_1^S, Sm^R, lac Δ, Su^-, λ^R, Mal^-); *E. coli* ER_{1398} pBR322 *(kindly provided by Erzsébet Magyaródi, SZBK, Szeged)*

2. Clinical isolates:

(kindly provided by Dr. Edit Hajdú, Institute of Clinical Microbiology, Szeged)

E. coli 30906; E. coli 34253; E. coli 34439; E. coli 34561; E. coli 34613; E. coli 42559; E. coli 42883; E. coli 42891; Citrobacter freundii 47051; Enterobacter cloacae 44937

3.1.6.2. Fungal strains

S. cerevisiae T158C; *S. cerevisiae* S6; *S. cerevisiae* 0425; *S. cerevisiae* 0425 P/1 *(kindly provided by Dr. Ilona Pfeiffer, Department of Microbiology, Faculty of Sciences, University of Szeged)*

C. albicans; C. glabrata (kindly provided by Dr. Edit Hajdú, Institute of Clinical Microbiology, Szeged)

3.1.6.3. Tumour cell lines

(provided by Professor Adorján Aszalos, FDA, Washington, USA):

L5178Y mouse T cell lymphoma cell line (PAR)

L5178 mouse T cell lymphoma MDR1/A retrovirus transfected cell line (MDR)

3.1.7. Compounds studied

3.1.7.1. Phenothiazine and its analogues

Promethazine, chlorpromazine, imipramine, amitriptyline, promazine, triflupromazine, trifluoperazine, ethopromazine and 2-dimethylamino ethylchloride were purchased from EGYT Budapest; 9- amino acridine was purchased from Sigma Aldrich Co.

3.1.7.2. Xanthenes and indole

Caffeine was purchased from Fluka; guanosine, GMP (guanosine monophosphate), adenosine, AMP (adenosine monophosphate), cytosine, CMP (cytosine monophosphate) were kindly given by *Professor Harri Lönnberg* from Turku University; indole was purchased from Reanal.

3.1.7.3. Antiarrhytmics

Verapamil (purchased from Sigma). Bepridil, Amiodaron, Quinidine SO_4, Lidocain, Mexiletine, Prajmalinum bitartarat, Disopyramid rythmodan phosphate, Propafenon rythmonorm HCl, Propranolol, Tocaidine HCl, Depasan and Flecaimid acetate were kindly provided by *Professor Gyula Papp*, Department of Pharmacology, University of Szeged.

3.1.7.4. 6,12-dihydro-1-benzopyrano[3,4-b] [1,4]benzothiazin-6-ones and related coumarins

The 6,12-dihydro-1-benzopyrano[3,4-b] [1,4]benzothiazin-6-ones
(6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [7];

2-methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [8];

1,3-dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [9];

1,4-dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [10];

3,4-dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [11];

2-chloro-3-methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [12])
were synthesized according to the method of *K. Tabakovic et al.* (154) by *Professor Noboru Motohashi*, Meiji College of Pharmacy, Tanashi-shi, Tokyo, Japan. The related coumarins (6-methylcoumarin, 7-methylcoumarin and ethyl 3-coumarincarboxylate) were purchased from Aldrich Chemical Co.

3.1.7.5. 2,3,4,5-tetrahydro-1H-3-benzazepinones

7,8-dimethoxy-2-methyl-3-methanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one (KS02), 7,8-dimethoxy-2-methyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one (KM57), 3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one (KN50), 7,8-dimethoxy-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one (KE04), 7,8-

dimethoxy-2-isopropyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one (KI10), 7,8-dimethoxy-2-phenyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one (KP80) were newly synthesized by *Professor Masami Kawase*, Faculty of Pharmaceutical Sciences, Josai University, Japan.

3.1.7.6. 2,3,4,5-tetrahydro-1H-3-benzazepines

7,8-dihydroxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (KF1), 7,8-dihydroxy-3-methyl-2-trifluoromethyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (KF2), 7,8-dihydroxy-2,3,4,5-tetrahydro-1*H*-3-benzazepine (KF3), 7,8-dimethoxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (KF4) were newly synthesized by *Professor Masami Kawase* , Faculty of Pharmaceutical Sciences, Josai University, Japan.

3.1.7.7. Cathecols

Dopamine /DA/ (Sigma Aldrich Co) and Norepinephrine /NE/ (Sigma Aldrich Co) were obtained commercially.

3.1.7.8. Kiwifruit peel extracts

Hexane extracts (KPP-H0-5), acetone extracts (KPP-A0-6), MeOH extracts (KPP-M0-5), 70% MeOH extracts (KPP-70M0-6) were prepared and provided by *Professor Noboru Motohashi,* Meiji College of Pharmacy, Tanashi-shi, Tokyo, Japan.

3.1.7.9. Other studied compound

Diflucan (fluconazole) was obtained commercially from Pfizer.

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* *For the chemical structure of some studied compounds see Appendix II.*

3.2. Methods

3.2.1. Measuring bacterial/fungal density during growth

The overnight culture is small-scale liquid culture growth used to initiate a larger-scale growth. 5.0 ml sterile liquid medium was transferred to a sterile 15 ml tube. The liquid medium was inoculated with an isolated colony from an agar plate using a sterile inoculating loop. The tube was capped and incubated at 37°C with (*E. coli* ER_{1398}) or without shaking (*E. coli* K12 LE140). In the case of fungi the tubes were incubated at 30°C with shaking. The culture has grown until saturation (1 to $2x10^9$ cells/ml), typically over an 8-hour to overnight period. The cell number per ml can be easily estimated by measuring the scattered light at 600 nm using a visible spectrophotometer. To measure bacterial/fungal density the visible spectrophotometer should be blank at 600 nm with a cuvette containing the growth medium (e.g. LB broth). In another cuvette growing bacteria/yeast is added and the optical density

(OD) is measured. An OD reading of over 0.6 is beyond log phase. An OD reading between 0.2 and 0.6 represents cells in late log-phase growth. The OD reading is linear with cell number: 1 OD/ml=$8x10^8$ cells/ml.

3.2.2. Detection of antibacterial/antifungal effect

a.) E. coli

An overnight preculture of *Escherichia coli* K12 LE140 was diluted 10^{-4} and inoculated in 0.05 ml aliquots (approximately $5x10^3$ cells) into 5.0 ml MTY nutrient broth. The various concentrations of tested compounds were added respectively, and the tubes were incubated at 37°C for 24 hours without shaking. The MICs (minimum inhibitory concentrations) were determined with densitometer (Ciba Corning colorimeter 252).

b.) Fungi

An overnight preculture of the fungi was diluted 10^{-4} and inoculated in 0.05 ml aliquots (approximately $5x10^3$) cells into 5.0 ml 2xYPD nutrient broth. The various concentrations of tested compounds were added respectively, and the tubes were incubated at 30°C for 24 hours with shaking. The MIC values were determined with densitometer (Ciba Corning colorimeter 252).

3.2.3. F' lac plasmid elimination

An overnight preculture of *Escherichia coli* K12 LE140 was diluted 10^{-4} and inoculated in 0.05 ml aliquots (approximately $5x10^3$ cells) into 5.0 ml MTY nutrient broth (pH: 7.2-7.3). The various concentrations of tested compounds were added respectively, and the tubes were incubated at 37°C for 24 hours without shaking. Two dilutions with 10^{-4} and 10^{-5} concentrations were prepared from tubes showing growth and plated in 0.1 ml amounts on eosin-methylene blue (EMB) agar (145). The plates were incubated at 37°C for 24 hours. The differential basis of this medium involves two indicator dyes, eosin and methylene blue, which distinguish lactose fermenting and non-lactose fermenting organisms. Acid from lactose fermentation changed the color of the colonies. Large amounts of acid caused a green metallic sheen on the colony surface. Less acid resulted in a pink coloration. The plates were counted for lac^- plasmidless (pink) and lac^+ plasmid containing (deep violet with green metallic sheen) colonies. The percentage (%) of plasmid elimination was counted according to the formula given below:

$$Percentage\ of plasmid\ elimination = \frac{number\ of\ plasmidless\ colonies}{number\ of\ all\ colonies} \times 100$$

3.2.4 Cell transformation

One day before the preparation of competent cells, 5 ml of overnight culture of the strain ER_{1398} was made, beginning from a single colony. Then 100 ml of YTB medium was inoculated with 1 ml of the overnight preculture and was grown at 37°C with shaking to an OD_{550} of 0,25 to 0,3. The bacterial suspension was chilled on ice for 10 minutes. The cells were collected by centrifugation for 10 minutes (4500 rpm, 4°C). The cells were resuspended gently in 50 ml ice-cold 0,1 M $MgCl_2$ by repeated pipetting. The solution was kept on ice during the resuspension. The cells were again collected by centrifugation for 10 minute (4500 rpm, 4°C) and then were resuspended in 3,3 ml ice-cold 0,1 M $CaCl_2$. The suspension was incubated on ice for 1 hour. 200-200 µl aliquots of competent cells were dispensed into individual microcentrifugal tubes and stored on -70°C until needed for transformation (155, 156).

The required number of aliquots of competent cells was thrown. The cells were kept on ice until needed. In sterile tube 1.0 µl pBR322 closed circular plasmid (4363 bp; MW: $2.8x10^6$) was combined with 200 µl competent cells. The tube was incubated on ice for 30 to 60 minutes. The cells were heat shocked by transferring the cells to a 42°C water bath for exactly 2 minutes. Heat shock increases the transformation efficiency but prolonged exposure to heat in $CaCl_2$ buffer kills component cells. 1.0 ml of YTB medium was added to each sample and incubated at 37°C for 60 minutes with gentle orbital shaking. During this period, competent cells had recovered and transformed cells expressed the antibiotic resistance needed to replicate on selective plates. Using sterile glass spreader, 200 µl of transformed cells were spread evenly over the surface of YTB agar plates supplemented with appropriate antibiotics (ampicillin, tetracycline). The plates were incubated with the top of the agar facing up in the 37°C plate incubator for several minutes until inoculum of cells was completely absorbed into the agar. The plates were covered and inverted so that the agar faced down and incubated at 37°C in plate incubator for 12 to 20 hours. After this incubation the colonies were ready for further selection by DNA miniprep (157).

3.2.5. Complex formation between compounds and plasmid DNA

The *E. coli* pBR 322 ER_{1398} strain was grown in 5.0 ml YTB nutrient broth in the presence of tetracycline (100 μg/ml) and ampicillin (25 μg/ml) at 37°C for 16 hours. The overnight culture was distributed in 1.0 ml aliquots into Eppendorf-tubes. The compounds were added to plasmid containing bacterial suspension in various final concentrations (200 μg/ml-5000 μg/ml). The samples were incubated at 37°C for 30 minutes then they were centrifuged for 5 minutes (3000 rpm) and the supernatant was discarded. The pellet was resuspended in 100 μl Sol. I (Tris-HCl, glucose, EDTA) homogenised in vortex and incubated at room temperature for 5 minutes. Then 200 μl of Sol II. (NaOH, SDS) was added to the bacterial suspensions, homogenized and incubated in water bath (0°C) for 5 minutes. After that 150 μl of ice-cold 5 M potassium-acetate was added to the samples. After mixing, samples were incubated further for 5 minutes at 0°C, then centrifuged for 10 minutes (3000 rpm). The supernatant was treated with 400 μl phenol: chlorophorm: isoamyl alcohol (25:24:1). The samples were centrifuged for 1 minute (3000 rpm) and the supernatants were precipitated with 96% ethanol and incubated at room temperature for 5 minutes and then precipitated DNA was centrifuged for 5 minutes (3000 rpm). The supernatant was discarded and the plasmid DNA was washed with 70% ethanol. The ethanol was discarded and the samples were dried. The extracted plasmid DNA was dissolved in 20 μl Tris-EDTA (TE) buffer, containing RNase (20 μg/ml). 5 μl of RNase treated plasmid DNA solutions were applied to 0.7% agarose gel (with 0.5 μg/ml ethidium bromide). Agarose gel electrophoresis was performed for 20 minutes (200 V). The various forms of plasmid DNA were detected under UV (ultra-violet) lamp (158).

3.2.6. Detection of killer activity in *S. cerevisiae* T-158C

The *S. cerevisiae* T158C strain with killer activity was cultivated in 2xYPD for 3 days at 30°C with shaking. The sensitive *S. cerevisiae* S/6 was grown in the same way for 24 hours. After one-day cultivation the sensitive strain was plated massively on phosphate-citrate buffered 2xYPD plate (pH: 4.2-4.4). From the *S. cerevisiae* T158C cell suspension 20 μl was taken out and dropped on this plate. The plates were incubated at 30°C for three days. An inhibition zone around *S. cerevisiae* T158C dropped on the plate indicated the killer activity.

3.2.7. Inhibition of killer activity in *S. cerevisiae* T158C

10^{-4} dilution was made from killer toxin producing *S. cerevisiae* T-158C strain. The tested compounds were added in different concentrations into the tubes and the samples were

incubated for 3 days at 30°C with shaking. After incubation 10^{-4} dilutions were made from the samples and 100 µl from each treated cell suspension were plated on 2xYPD plates. The plates were incubated at 30°C till the colonies had grown out. From each plate 20-20 colonies were taken and suspended in 100 µl 0.8 KCl and as it was described above dropped on the plates containing the sensitive strain. After three days of incubation at 30°C the presence or absence of the inhibition zone was checked.

3.2.8. Protoplast preparation from *S. cerevisiae* T-158C/S6

The *S. cerevisiae* T158C and *S. cerevisiae* S6 strains were grown in 2xYPD medium for 24 hours at 30°C. Then the cells were centrifuged for 10 minutes (3000 rpm) and the supernatant was discarded. The cells (10^8-$5x10^8$ cell/ml) were resuspended in pre-treating solution (10 mM Tris-HCl; 0.5 M Na-EDTA; 1% ß-mercaptoethanol; pH: 9.0) and incubated at room temperature for 20 minutes. The samples were centrifuged for 10 minutes (3000 rpm) and the supernatant was discarded. The cells (10^8-$5x10^8$ cell/ml) were resuspended in enzyme solution (0.5% snail-enzyme; 0.5% NovoZym) and incubated at room temperature for 3 hours. The samples were centrifuged for 10 minutes (3000 rpm). The achieved protoplasts were washed with 0.7 M KCl.

3.2.9. Complex formation studies between compounds and extrachromosomal RNA of *S. cerevisiae*

As it was previously described protoplasts were made. The protoplast suspension was distributed into 1.0 ml aliquots in Eppendorf centrifuge tubes. The samples were centrifuged for 10 minutes (3000 rpm). The supernatant was resuspended in 1ml 0.8 M KCl. The tested compounds were added and the samples were incubated at 30°C for 20 minutes then as it was described in the case of plasmid DNA extra chromosomal plasmid RNA was isolated and detected under UV light after agarose gel electrophoresis was performed.

3.2.10. Evaluation of combined effect of fluconazole (Diflucan) with promethazine by Checkerboard method

The checkerboard method is the technique used most frequently to assess antimicrobial combinations *in vitro*. The concentrations tested for each antimicrobial or antiplasmid compounds typically ranged from 4 to 5 dilutions below the MIC, using 2-fold dilutions of tested compounds.

Thus, the checkerboard consists of columns in which each well contains the same amount of the compounds being diluted and the rows in which each well contains the same amount of the other tested compound being diluted. The result is that each square in checkerboard (which represents on well) contains unique combination of the two drugs tested. The dilutions of the antimicrobial or antiplasmid agents were prepared in 2xYPD broth for the interactions in the antifungal studies. The drug containing solutions designated in the microtiter trays. Although we have expressed the antimicrobial concentrations as multiples or fractions of the MIC, they are usually expressed in μg/ml. *Eliopoulos and Moellering* (159) as synergism, additivity indifference and antagonism evaluated the results of the combined use of compounds. Fungal strains were tested by the microdilution checkerboard technique described by *Eliopoulos and Moellering* (159). Fungal dilutions from the logarithmic-growth phase were prepared and subsequently distributed into microtiter trays containing various drug regimen concentrations. The final inoculum size in the microtiter trays was approximately 10^5 colony forming unit (CFU)/ml. Concentrations of each compound ranged from four to five times below the MIC to two times of the MIC for the yeast being tested. Inoculated microtiter trays were incubated at 30°C for a period of 24 h and then the inhibition of fungal growth was established. In order to evaluate the outcome of the drug combination, fractional inhibitory concentration (FIC) indices were calculated as FC_A+FIC_B, when FIC_A and FIC_B represent the minimum concentrations that inhibited organism growth for drugs A and B, respectively. Individual checkerboard runs were replicates, a mean FIC index was calculated and applied to commonly utilized definition of synergy, and classified as either synergistic (≤0,5), additive (0,51-1,0) or indifferent (1,01 but ≤4,0).

3.2.11. Cell fluorescence uptake and mdr reversal effect

The L5178 mouse T-cell lymphoma cell line was infected with the pHa MDR1/A retrovirus (160). The MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/ml colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, glutamine and antibiotics. The cells were adjusted to a concentration of $2x10^6$ /ml and resuspended in serum free McCoy's 5A medium and were distributed into 0.5 ml aliquots in Eppendorf centrifuge tubes. Test compounds were added from 0.5 to 5 μl of the 1.0 mg/ml stock solutions and the samples were incubated for 10 minutes at room temperature. Then 10 μl of the indicator Rhodamine-123 (5.2 μM final concentration) was

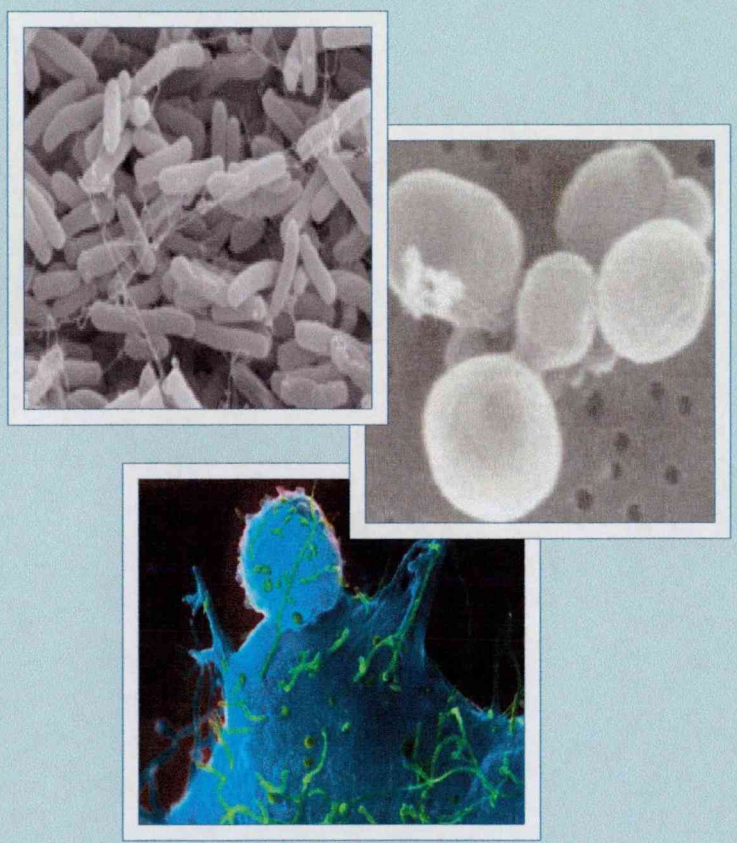
added to the samples and the cells were incubated for further 20 minutes at 37°C, washed twice and resuspended in 0.5 ml PBS for FACS (Fluorescence Activated Cell Sorter) analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instruments.

The fluorescence activity ratio was calculated by the following equation:

$$Fluorescence\ Activity\ Ratio = \frac{(mdr\ treated / mdr\ control)}{(parental\ treated / parental\ control)}$$

Chapter 4

RESULTS AND DISCUSSION



4. RESULTS AND DISCUSSION

4.1. Model experiments for F' lac plasmid elimination

4.1.1. Promethazine and structurally related compounds

Laborit described the action of phenothiazines on the central nervous system as painkillers in 1949 (161). Soon it was pointed out that promethazine had a growth inhibitory effect on mycobacteria *in vitro* (143). Later the antibacterial effect of phenothiazine compounds was tested on wide variety of Gram-positive and Gram-negative bacteria (162).

F' lac metabolic plasmid served as convenient model to study the plasmid curing effects of various tricyclics, because lactose plasmid-carrying colonies (deep violet with green metallic sheen) can be easily differentiated from plasmid-less pink colonies (Figure 7.).

Many tricyclic psychopharma with an antibacterial effect eliminate the F' plasmid (163).

Promethazine as control was effective in the elimination of plasmid in this study (Table 2.). The effect of other structurally related compounds was also concentration dependent. The only exception was 2-dimethylamino ethylchloride HCl-this compound had neither antibacterial nor antiplasmid effect on *E. coli* K12 LE120. This action was rather specific and it was depending on chemical structure. We suggested that the basis of antibacterial effect of phenothiazines and related compounds was an increased permeability of the membrane (163) while the plasmid curing effect was a result of a complex formation of the studied compounds with the plasmid DNA (163).

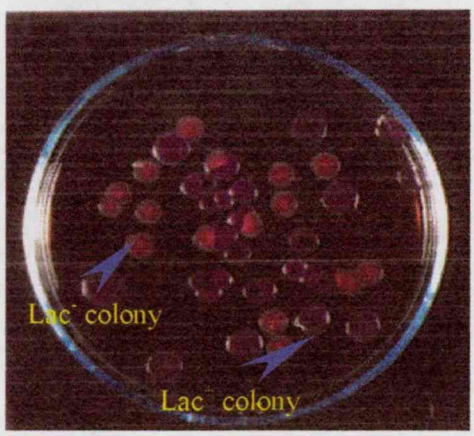


Figure 7. *E. coli* lac^+ and lac^- colonies on EMB agar plate.

Table 2. Plasmid elimination effect of promethazine and related compounds.

Compounds	Concentration (µg/ml)	Plasmid elimination % (mean)
Promethazine	20	0,00
	40	0,09
	60	11,35
	80	78,2
Imipramine	20	0,00
	40	0,11
	60	0,34
	80	10,57
	100	85,87
Triflupromazine HCl	10	0,00
	20	0,00
	30	7,66
	40	2,77
	50	-
Promazine HCl	10	0,00
	20	0,46
	30	2,31
	40	57,45
	50	43,5
Trifluoperazine 2 HCl	20	0,10
	40	37,92
	60	70,91
	80	0,00
	100	0,00
Amitriptyline HCl	20	0,20
	40	0,27
	60	4,89
	80	22,63
	100	-
Ethopromazine HCl	20	0,34
	40	2,83
	60	17,84
	80	39,5
	100	0,00
2-Dimethylamino ethylchloride HCl	20	0,00
	40	0,00
	60	0,00
	80	0,00
	100	0,00

4.1.2. Reduction of plasmid elimination of promethazine in the presence of caffeine GMP and indole

It was suggested that antibacterial, antiplasmid and antimutagenic effect of phenothiazines (144,164) can be correlated with the binding of phenothiazines to nucleic acids by intercalation (165, 166) or other type of interaction (charge transfer (CT) complex formation (167)). The determination of the exact binding points is difficult because the macromolecules such as nucleic acids have different groups which can take part in their binding. An interaction between nucleic acid and phenothiazine may be formed by the binding between base moiety, external sugar and phosphate moiety or by the multiple bindings among linker

and groups simultaneously. One example for the interaction of phenothiazines with guanosine mononucleotide has been described (Figure 8.) (168). An another type of interaction between the aromatic part of DNA and phenothiazines could be the stacking interaction.

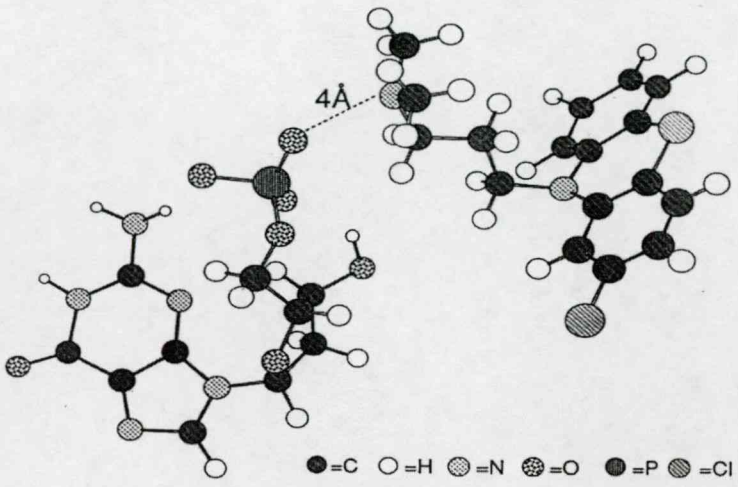


Figure 8.: Interaction model of CPZ with dGMP of Z-DNA.

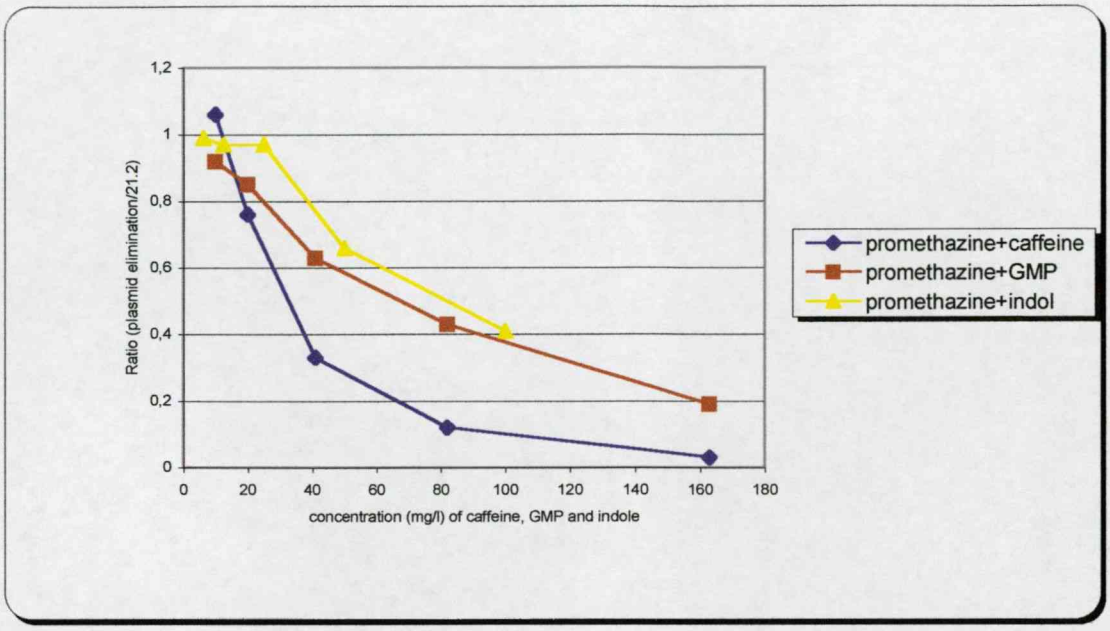
The *stacking interaction** could be responsible for the biological activities of phenothiazine therefore the antiplasmid effect of promethazine was studied in the presence of guanosine monophosphate (GMP), caffeine and indole. Promethazine alone was effective in the elimination of plasmid and served as a control in the study (Table 3.).

The caffeine having large polarizing power significantly reduced the antiplasmid effect of promethazine and the extent of reduction was concentration-dependent (Fig. 9.). Using caffeine alone, the F' lac plasmid was not eliminated from the bacterial cells.

On the contrary, the indole reduced the effect of promethazine slightly, depending on the concentration (Fig. 9.).

A combination of promethazine and GMP did not show significant reduction of plasmid elimination by promethazine compared to the combination effect of caffeine. The extent of reduction of plasmid elimination was similar to the effect of indole (Fig. 9.).

Figure 9. Ratio of reduction for plasmid elimination by promethazine (60 mg/l) for caffeine, GMP and indole.



The observed reduction of the plasmid elimination activity of promethazine could be interpretated by a competition between caffeine, indole and GMP and some physiological substrates or phenothiazine, which have a structural similarity or common target, could be suggested.

***Description of the stacking interaction:** In the course of the stacking interaction, two aromatic molecules (with a closely planar aromatic part) are located to each other parallely at approximately 3.4- 3.6 Å. There are some idea concerning the theory of the stacking interaction. The most accepted one is the theory of *Lawacek and Wagner* (169). According to this idea, the aromatic molecules could be characterised with polarizing power and with polarizability. The molecules containing many chemical bonds with large dipole momentum (C-N, C-O bonds) have large polarizing power. Molecules containing less dipole character and more aromatic character have large polarizability. According to this idea the aromatic molecules could be arranged in an order. The order of the polarizing power for stacking interaction is *caffeine>guanine>adenine>purine>indole*. Simple aromatic compounds with mostly C-C and C=C bonds, such as indole also have high polarizing ability with the following order: *indole>adenine>purine>guanine>caffeine*.

Table 3. Possible complex micelle formation between promethazine and nucleotides inside the bacterial cells (the effect of micelle formation on the plasmid elimination of promethazine).

Compounds	Concentration (µg/ml)	Molar ratio	Plasmid elimination % (mean)
Control	0,00	-	0,00
Promethazine	20	-	0,32
	40	-	3,93
	60	-	59,39
	80	-	MIC
Promethazine+GMP	60+18,3	1:2	0,17
	60+36,6	1:4	0,26
	60+73,2	1:1	0,45
	60+146	2:1	12,5
	60+292,8	4:1	0,11
Promethazine+Guanosine	60+15	1:2	0,00
	60+30	1:4	0,00
	60+60	1:1	0,00
	60+120	2:1	0,00
	60+240	4:1	0,30
Promethazine+AMP	60+18,75	1:2	2,76
	60+37,5	1:4	3,13
	60+75	1:1	0,70
	60+150	2:1	2,63
	60+300	4:1	2,80
Promethazine+Adenosine	60+13,25	1:2	0,04
	60+26,5	1:4	0,046
	60+53	1:1	0,26
	60+106	2:1	0,30
	60+212	4:1	0,33
Promethazine+CMP	60+18,25	1:2	0,13
	60+37,03	1:4	0,71
	60+74,07	1:1	3,65
	60+148,14	2:1	2,70
	60+296,28	4:1	1,84
Promethazine+Cytosine	60+10	1:4	0,00
	60+20	1:2	1,91
	60+40	1:1	8,53
	60+80	2:1	0,00
	60+160	4:1	0,00

A third molecule (caffeine, GMP and indole) could influence the complex formation between promethazine and plasmid DNA in the following ways:

- The third molecule could form a stronger complex with plasmid DNA than the promethazine. This molecule would be the competing agent against promethazine in the interaction with the plasmid DNA. In this case the third molecule has the characteristics of promethazine.

- The third molecule could also form a stronger complex with promethazine than the plasmid DNA. Consequently, the concentration of free promethazine would decrease in the presence of third compound. In this case, the plasmid DNA-like compound reduces the antiplasmid effect of promethazine.

The difference between Raman spectra of chlorpromazine-GMP system predicts an interaction between the phosphate group of GMP (170). Because both, the caffeine and indole (two compounds, forming stacking interaction) have an effect on plasmid elimination of phenothiazines, it can be suggested that the stacking interaction is present in a complex formation between promethazine and DNA. The effect of nucleotides was similar to the effect of indole, the interaction between phenothiazine and caffeine, (indole, GMP, AMP, CMP) could be suggested as a main driving force in the interpretation of the above observation. The interaction of a complex formation between promethazine and caffeine is stronger than that of complex formation between promethazine and indole. If two interaction types including stacking exist, it means that at least two types of interactions are present for the complex formation between phenothiazines and plasmid DNA. One is an ionic interaction between the linker of phenothiazine and phosphate group of DNA. The second is a stacking interaction between aromatic sites of phenothiazines and nucleic acid bases such as guanosine, with a higher affinity than that of adenosine. On the basis of the weaker stacking interaction, the weaker antiplasmid competition effect of adenosine monophosphate (AMP) can be understood.

We concluded that promethazine might form a complex with guanine-cytosine (G-C) rich regions of DNA. The native uncomplexed sites of the G-C rich regions are necessary for the normal plasmid replication. The phenothiazine complexes, however, should not maintain the physiological functions of the G-C rich regions in the ccc form of plasmid DNA.

4.1.3. Effect of antiarrhytmics on the F'lac plasmid elimination

Based on the experiments with phenothiazines some antiarrhytmics with tricyclic chemical structure (see *Appendix II*) were tested systematically.

It was supposed that membrane effects could be involved in the plasmid elimination (163).

Only one compound propranolol had remarkable effect on F'lac plasmid elimination, while other antiarrhytmics had weak effects as concerns the loss of the bacterial plasmids responsible for the lactose metabolism or antibiotic resistance (Table 4.). Apparently some relationship exists between the chemical structures (hydrophobic and cationic properties) and

the antiplasmid effects of the tested compounds and this can be exploited theoretically to overcome the antibiotic resistance of bacteria.

Table 4. F' lac plasmid elimination effect of antiarrhytmics.

Compounds	Concentration (μg/ml)	Plasmid elimination % (mean)
Control	0,0	0,0
Promethazine	20	0,0
	60	20,2
	80	26,7
	100	17,2
Bepridil	160	1,50
	200	3,60
Amiodaron	160	0,40
	200	0,50
Quinidine SO_4	160	1,00
	200	0,51
Lidocaine	120	0,59
	160	0,70
Mexiletine	160	0,00
	200	0,18
Prajmalinum bitartarat	160	0,80
	200	0,75
Disopyramid rythmodan phosphate	160	0,15
	200	0,00
Propafenon rythmonorm HCL	70	0,00
	140	0,00
Propranolol	160	17,50
	200	52,00
Tocaidine HCl	160	0,49
	200	1,13
Verapamil	200	0,00
	250	0,06
Depasan	400	0,00
	500	0,00
Flecaimid acetate	400	0,00
	500	0,00

4.1.4. Antiplasmid activity of 6,12-dihydro-1-benzopyrano[3,4-b] [1,4] benzothiazin-6-ones

Some 6,12-dihydro-1-benzopyrano[3,4-b] [1,4] benzothiazin-6-ones showed an effect on the multidrug resistance efflux pump of mouse lymphoma cells *in vitro* (171). In a further study the antiplasmid activity by elimination of F' lac was tested. Only one compound the 7-methylcoumarin (No. 14.) had a remarkable effect, while three other derivatives (No. 8; No. 11.; No. 12.) had slight effect. The other coumarins were ineffective (Table 5.).

The activity of 7-methylcoumarin is probably related to the electrophilic-superdelocalization induced by methyl substitution at 7 position. If this correlation were true then we would expect similar effects in case of compounds (No.9.; No. 11.; No. 12.), however, these

compounds were weak. The effect of compound No. 9. was reduced by methylation at position 1 due to a compensating electronic distribution.

Table 5.: Antiplasmid activity of 6,12-dihydro-1- benzopyrano[3,4-b] [1,4] benzothiazin-6-ones and related coumarins.

Compound's No.	R_1	R_2	R_3	R_4	Elimination of F' lac (μg/ml)	Antiplasmid effect %
7	H	H	H	H	200	0.0
8	H	CH_3	H	H	>200	0.05/at 160 μg
9	CH_3	H	CH3	H	200	0.0
10	CH_3	H	H	CH_3	200	0.0
11	H	H	CH_3	CH_3	>200	0.1/at 160 μg
12	H	Cl	CH_3	H	200	0.0
13 (6-methylcoumarin)					200	0.0
14 (7-methylcoumarin)					95	32.0/at 70 μg
15 (ethyl 3-coumarin-hyphen carboxilate)					200	0.0

*For chemical structure see *Appendix II.*

4.1.5. Plasmid elimination by 3-benzazepines

The search for novel agents has led to the synthesis of 3-benzazepines (172, 173). It was suggested in previous experiments, some change in the molecular structure could modify the antiplasmid effect. Therefore we investigated the interaction on plasmid-eliminating activity of 3-benzazepines in competition with known resistance modifiers such as promethazine or verapamil.

The KN50 itself was able to eliminate the F' lac plasmid with relatively high frequency (27%). In general the antimicrobial effect of 3-benzazepines was very low. The majority of compounds had no MIC values below 200 μg/ml on *E. coli* (Table 7.). Interestingly the compound KF4 could inhibit the antiplasmid effect of promethazine. In the presence of 200 μg/ml of verapamil this benzazepine was still ineffective. The antiplasmid effect is structure

dependent (Table 5.). This suggests that interaction between promethazine and KF4 exists in plasmid elimination effects.

As new biological effects of 3-benzazepines are confirmed, additional targets for plasmid can be supposed which is independent from the binding sites of phenothiazines (calmodulin) and verapamil (Ca-channel blocker).

Table 5. F' lac plasmid elimination by a combination of promethazine with KF4 and verapamil with KF4.

Compounds	Concentration (µg/ml)	Plasmid elimination % (mean)
Control	0,0	0,0
Promethazine	20	0,0
	40	0,1
	60	3,52
	80	70,6
	100	MIC
Promethazine+KF4	20+100	0,0
	40+100	0,0
	60+100	0,0
	80+100	0,0
	100+100	0,0
Verapamil+KF4	200+10	0,0
	200+20	0,0
	200+50	0,0
	200+100	0,0

Because of their structural similarity to 3-benzazepines (see *Appendix II.*) dopamines (dopamine (DA) and norepinephrine (NE)) were used as controls in this study. These compounds had no MIC values below 200 µg/ml and they could not eliminate the metabolic plasmid of *E. coli* (Table 6.).

Table 6. Plasmid curing effect of dopamines.

Compounds	Concentration (µg/ml)	Plasmid elimination % (mean)
Control	0,0	0,0
Promethazine	20	0,0
	40	0,0
	60	20,95
	80	84
	100	-(MIC)
NE	20	0,0
	40	0,0
	100	0,0
	200	0,14
DA	20	0,0
	40	0,0
	100	0,38
	200	0,0

Table 7. F' lac plasmid elimination of 3-benzazepines and related compounds.

Compounds	Concentration (µg/ml)	Plasmid elimination % (mean)
Control	0,0	0,0
Promethazine	20	0,0
	40	0,0
	60	20,95
	80	84
	100	-(MIC)
KSO2	20	0,45
	40	0,0
	100	0,0
	200	18,75
KM57	20	0,59
	40	0,15
	100	0,0
	200	13,76
KN50	20	0,0
	40	0,0
	100	0,37
	200	27,88
KF1	20	0,77
	40	0,13
	100	2,44
	200	-(MIC)
KF2	20	0,0
	40	0,28
	100	3,2
	200	-(MIC)
KF3	20	0,0
	40	0,045
	100	1,2
	200	-(MIC)
KF4	20	0,0
	40	0,0
	100	0,0
	200	0,0
KI10	20	13,33
	40	0,2
	100	0,0
	200	0,0
KP80	20	0,0
	40	0,0
	100	0,15
	200	-(MIC)
KE04	20	0,01
	40	0,0
	100	0,0
	200	0,0

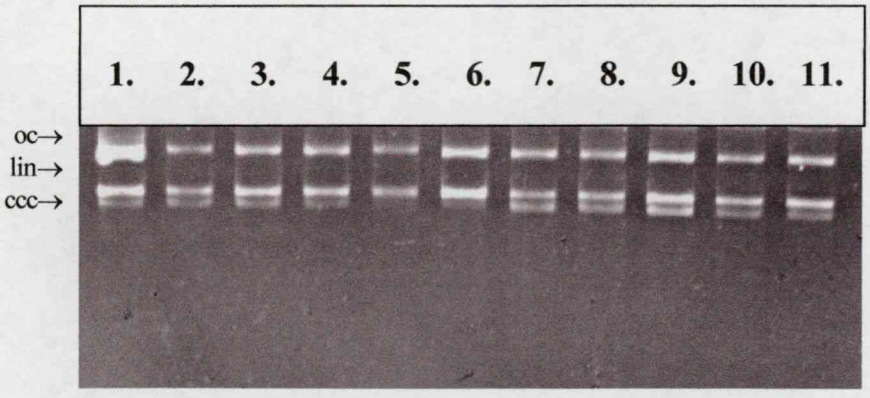
4.1.6. The effect of tetrahydrobenzazepines on *pBR322* plasmid DNA

The tetrahydro-3-benzazepines were subjected to interact with *E. coli* plasmid DNA. In the control sample the covalently closed circular (ccc) form of plasmid DNA ran faster than the linear (lin) form and the two conformations can be seen (Fig. 9.) The open circular (oc) form ran slower, relatively far from linear form. In case of 2,3,4,5-tetrahydro-1H-3-benzazepine treated plasmid-containing cells the oc form can be found in each sample. Similar situation exists with linear form. However compounds 5. and 6. (KI10 and KF4) were able to form complexes with the ccc form of plasmid DNA, just like phenothiazines, which also have some specificity to ccc form of plasmid DNA (163). The results showed an evidence for the selective complex formation of two active benzazepines with the superhelical form of plasmid DNA, meaning that this biological effect is dependent on the substituents of the benzazepine molecule.

The ccc form of plasmid DNA is maintained by bacterial gyrase (topoisomerase) which can be inhibited by benzazepines. Theoretically it is also possible that a simple interaction of two benzazepines (5. and 6.) leads to the unwinding of ccc form into the oc form, which is due to a single nick introduced into one strand of plasmid DNA.

Two compounds (5. and 6.) selectively inactivate the replicative form of plasmid DNA that encodes the antibiotic resistance (tetracycline (Tc) and ampicillin (Ap)) in bacteria.

Figure 10. The effect of tetrahydrobenzazepines on pBR322 plasmid DNA isolated from *E. coli*.



Compounds:

1. control; **2.** KF1; **3.** KF2;
4. KF3; **5.** KF4; **6.** KI10
7. KN50; **8.** KE04;
9. KS02; **10.** KP80;
11. KM57

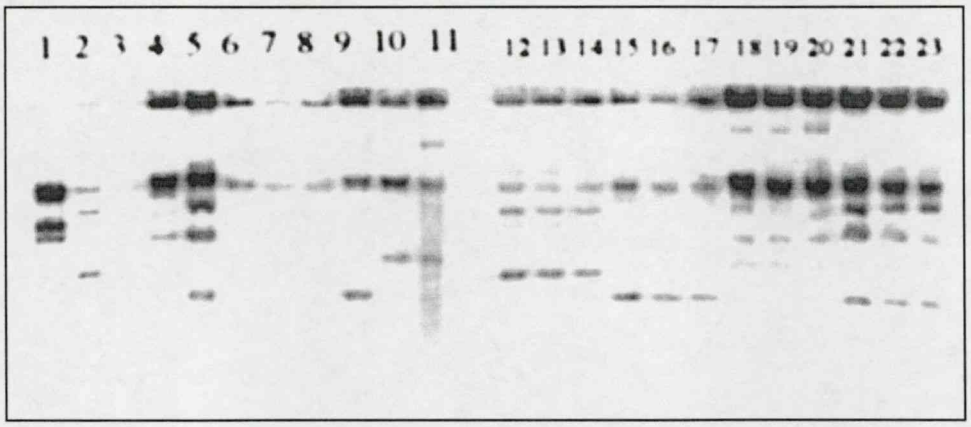
4.2.Complex formation of the tricyclic compounds with plasmids purified from clinical isolates.

In this model study we wanted to investigate whether the plasmid curing effect of tricyclic compounds is valid for clinical isolates. We chose promethazine and 9-amino acridine (9-AA) for this study because the plasmid curing activity of these two compounds on *E. coli,* carrying metabolic F' lac plasmid had been proven previously (163).

The cells were treated with high concentrations of these two compounds and electrophoresis was performed (Fig. 11.).

As it can be seen (Fig. 11.) 9-amino acridine was more effective than promethazine in complex formation with plasmid DNA.

Figure 11. Plasmid profile of clinical isolates.



1. λ ssp marker; **2**. *E. coli* 30906; **3**. *E. coli* 42883; **4**. *E. coli* 34613; **5**. *E. coli* 34561; **6**. *E. coli* 34439; **7**. *E. coli* 42559; **8.** *E. coli* 34253; **9**. *E. coli* 42891; **10**. *Citrobacter freundii* 47051; **11**. *Enterobacter cloacae* 44937; **12** *E. coli* 30906 *control*; **13**. *E. coli* 30906 *promethazine treated*; **14**. *E. coli* 30906 *9-AA treated*; **15**. *E. coli* 42883 *control;* **16**. *E. coli* 42883 *promethazine treated*; **17.** *E. coli* 42883 *9-AA treated*; **18.** *E. coli* 34613 *control*; **19**. *E. coli* 34613 *promethazine treated*; **20**. *E. coli* 34613 *9-AA treated*; **21**. *E. coli* 34561 *control*; **22**. *E. coli* 34561 *promethazine treated*; **23.** *E. coli* 34561 *9-AA treated.*

4.3. Effect of tricyclic compounds on yeasts

4.3.1. Effect of tricyclic compounds on the extrachromosomal RNA plasmids of yeasts

S. cerevisiae killer toxins are protein molecules secreted by killer strains carrying the specific satellite dsRNA (killer virus) (174, 175, 176); the replication complement L-A dsRNA genomic segment of the virus is a 4.6 kbp dsRNA encoding the major capsid protein and a capsid-RNA polymerase fusion protein generated by a translational frame shift mechanism (175, 177). The plasmid encoding killer activity is 1,9 kbp dsRNS (M28 toxin) (178). Killer strains are immune to their own toxin but remain susceptible to other killer toxins.

Killer toxin producing strain of *Saccharomyces cerevisiae* (*S. cerevisiae* T-158C) served to identify the plasmid elimination effect of tricyclic compounds on linear extrachromosomal yeast plasmids.

Promethazine and related compounds did not eliminate the killer plasmid of *S. cerevisiae* probably they could not penetrate into the yeast cell (Table 8.).

Table 8.: Effect of tricyclic promethazine and 9-AA on killer plasmid elimination.

Compounds	Concentration (μg/ml)	Killer plasmid elimination %	Number of colonies
Promethazine	40	0.0	642
	60	0.0	532
	80	0.0	321
	100	*MIC*	-
Trifluoperazine	4	0.0	625
	6	0.0	458
	8	0.0	321
	10	*MIC*	-
Amitriptyline	40	0.0	630
	60	0.0	436
	80	0.0	221
	100	*MIC*	-
9-amino acridine (9-AA)	10	10	679
	20	30	456
	40	40	331
	60	50	213
	80	*MIC*	-

As it is known acridine orange, an intercalating tricyclic dye has showed a high curing percentage of the killer character (179). Therefore we tested 9-amino-acridine for killer plasmid curing. 9-amino-acridine was effective as killer character eliminating agent (Table 8.). The 9-amino acridine treated strains were dropped on the plates containing the sensitive strain (*S. cerevisiae S6*). After three days of incubation at 30°C the presence or absence of the inhibition zone was checked (Fig. 12.).

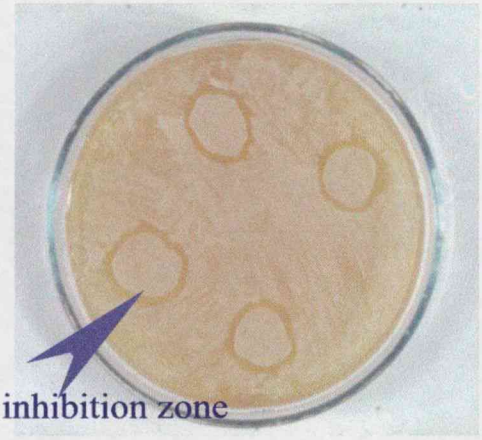


Figure 12. Killer character of *S. cerevisiae T-158C.*

The elimination was proven by gel-electrophoresis. The figure 13. shows that the 1,9 kbp dsRNS band is missing from the 9-amino acridine treated *S. cerevisiae*.

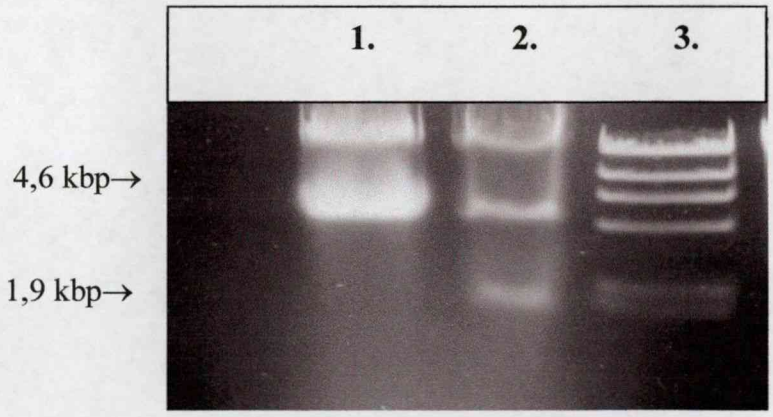


Figure 13. Killer plasmid elimination of *S. cerevisiae* by 9-amino acridine.

1. 9 AA treated *S. cerevisiae T-158C*
2. untreated *S. cerevisiae T-158C* (control)
3. marker

4.3.2. Evaluation of combined effect of fluconazole (Diflucan) with promethazine

A great deal of experimental and clinical effort with the aim of increasing antifungal activities while reducing adverse effects has been made in the last two decades. These efforts include the combined use of two distinct antimicrobial agents: for example amphotericin B plus rifampin (180) etc. It has been verified that promethazine has antibacterial and antiplasmid activity. It is supposed that the bactericidal and antiplasmid effect of promethazine is based on its inhibitory effect on bacterial DNA gyrase (163).

In the present study we demonstrated by checkerboard microdilution method that promethazine enhances the *in vitro* activities of fluconazole against two laboratory strains (*S. cerevisiae* 0425 S288 and *S. cerevisiae* 0425 S288 P/1) and against two clinical isolates (*C. albicans* and *C. glabrata).*

Promethazine alone also showed antifungal activity (MIC value at 125 μg/ml in case of laboratory strains, 250 μg/ml in case of clinical isolates).

Table 9.: Effect of fluconazole in combination with promethazine on *S. cerevisiae* 0425 S288 and *S. cerevisiae* 0425 S288 P/1 laboratory strains.

S. cerevisiae 0425 S288			***S. cerevisiae 0425 S288 P/1***		
Compounds	**FIX**	**Interaction**	**Compounds**	**FIX**	**Interaction**
fluconazole+promethazine	0.5	***synergistic***	fluconazole+promethazine	0.37	***synergistic***

Table 10.: Effect of fluconazole in combination with promethazine *C. albicans* and *C. glabrata* clinical isolates.

C albicans			***C. glabrata***		
Compounds	**FIX**	**Interaction**	**Compounds**	**FIX**	**Interaction**
fluconazole+promethazine	0.47	***synergistic***	fluconazole+promethazine	0.5	***synergistic***

Further studies are required to elucidate the possible mode of action by which promethazine acts synergistically with fluconazole. One presumed explanation for the observed synergism is that the intracellular entry of promethazine through fungal plasma membranes, whose integrities were interrupted by the action of antifungal agent with which it is used in combination could be greatly enhanced. Inhibition by the promethazine on fungal topoisomerase II, a homologous of bacterial gyrase might occur.

4.4. Reversal of MDR in cancer cells

4.4.1. MDR reversal activity of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones

As it was shown earlier, benzo[a]phenothiazines have antitumour activity against some tumour cells (181). 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones are structurally very similar to the structure of benzo[a] phenothiazines (see *Appendix II.*). Based on our estimated structure-activity relationship, it was expected that 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones may have some antitumour activity and may cause reversal on resistant tumour cells. The coumarins, which are included in these compounds, have also shown antitumour activity by the inhibition of HIV integrase (182).

Our purpose was to show the mdr reversal activity of seven 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones. Verapamil was used as a control for mdr reversal. The coumarins used in this study were slightly effective (Table 11.). A moderate mdr reversal activity was shown by three of 6,12-dihydro-1-benzopyrano [3,4-b] [1,4] benzothiazin-6-ones (No. 8., 9., 10.) at 20 μg concentration. Three 6,12-dihydro-1-benzopyrano [3,4-b] [1,4] benzothiazin-6-ones (No. 7., 11., 12.) without methyl or benzo group at positions 1 or 2 together reduced the rhodamine accumulation in tumour cells, probably by inducing the efflux pump system or by causing a direct membrane injury (Table 11.). Position 4 must be free or low in electron density for the mdr reversal effect. The one exeption is compound No. 10. (Table), in which the substitutions at positions 1 and 4 neutralized each other. The ineffectiveness of the compounds was probably correlated with the lower or reduced cell size, however granulation did not change remarkably in the cells. There was no toxic effect in the applied concentration, and the cell size did not change in flow cytometry.

Table 11.: The effect of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones (7-12) and related coumarines (13-15) on the mdr reversal on L-5178 tumour cells with multidrug resistance.

Compound's number	Concentration (μg)	Cell size ratio (FSC)	Cell Granulation ratio (SSC)	Fluorescence one height (FL-1)	Fluorescence activity ratio
par	*Control*	501.31	335,65	1133.80	-
mdr+R123	*Control*	552.47	385,44	18.78	1.00
verapamil	8	540.50	381,20	159.37	8.49
7	2	551.31	375,07	9.22	0.49
	20	533.43	400,03	10.70	0.57
8	2	557.39	375,09	10.71	0.57
	20	541.54	388,52	29.27	1.56
9	2	553.48	379,05	14.64	0.78
	20	544.72	375,61	26.90	1.43
10	2	551.91	385,77	13.82	0.74
	20	543.69	380,65	20.86	1.11
11	2	540.58	368,60	8.33	0.44
	20	553.07	374,34	11.25	0.60
12	2	550.50	386,64	9.61	0.51
	20	544.76	382,16	11.91	0.63
13	2	558.61	396,64	9.90	0.53
	20	559.92	389,56	9.72	0.52
14	2	559.13	393,86	10.94	0.58
15	2	522.56	397,48	10.76	0.57
	20	513.96	371,64	11.95	0.64

4.4.2. MDR reversal activity of 3-benzazepines

The mdr reversing effects of benzazepines by their chemical structures were compared to that of verapamil, using a mouse leukaemia cell line (L-5178 cells). The effects were measured by fluorescence ratio between treated and untreated cell groups. Compound No.8. (KF2) has the highest activity on mdr reversal (Fluorescence activity ratio: *8.38*) (Table 12.) among 12 compounds used in this research and the compound No. 8. was 2-fold more potent than verapamil (Fluorescence activity ratio: *4.18*). This compound might be an anti-mdr inducing agent with a great interest, because the affinity of compound No.8. to two dopamine D_1 and D_2 receptors was reduced by introduction of trifluoromethyl (CF_3) group at 2^{nd} position of

benzazepine ring (173): Compound No.6. (KP80) seems to have reasonable activities against all 60 cancer cell lines (183).

Table 12.: The effect of 3-benzazepines (1-10) and two analogues (DA, NE) on lymphoma 5178 cells with multidrug resistance.

Compound's number	Concentration (μg)	Cell size ratio (FSC)	Cell Granulation ratio (SSC)	Fluorescence one height (FL-1)	Fluorescence activity ratio
par	Control	504.86	184.75	1060.98	49.98
mdr+R123	Control	552.95	211.52	21.23	1.00
verapamil	5	545.74	216.62	88.72	4.18
1. (KS02)	5	473.30	188.91	9.85	0.46
2. (KM57)	5	425.16	205.05	18.69	0.88
3. (KN50)	5	494.57	193.54	12.97	0.61
4. (KE04)	5	467.29	188.79	16.39	0.77
5. (KI10)	5	491.72	190.79	44.77	2.11
6. (KP80)	5	507.50	197.30	13.89	0.65
7. (KF1)	5	469.78	187.09	17.01	0.80
8. (KF2)	5	447.77	191.53	177.91	8.38
9. (KF3)	5	477.28	189.43	16.12	0.76
10. (KF4)	5	507.71	198.41	28.68	1.35
DA	2	579.02	251.38	21.77	0.84
	20	571.20	251.12	23.47	0.93
NE	2	569.10	256.09	21.32	0.82
	20	564.14	254.11	19.53	0.80

4.4.3. MDR reversal activity of kiwifruit peel extracts

Kiwifruit, *Actinidia deliciosa* (*Actinidaceae*) has been mainly cultured in tropics and subtropics. To assess the *in vitro* antioxidant potential of kiwifruit extracts, nucleotides were treated in the gel with H_2O_2 (184). The extracts reduced the H_2O_2-induced DNA breaks, suggesting its antioxidant activity (184). Kiwifruit juice efficiently inhibits the nitrosation both *in vitro* and *in vivo*, possibly by various components such as ascorbic acid and other unidentified nitrile scavengers (185). We have investigated the mdr reversal activity of kiwifruit peel extracts. Mdr reversal activity and cytotoxic activity were found in the same fractions of hexane (H4, H5), acetone (A4) and methanol extract (70M4) (Table 13.).

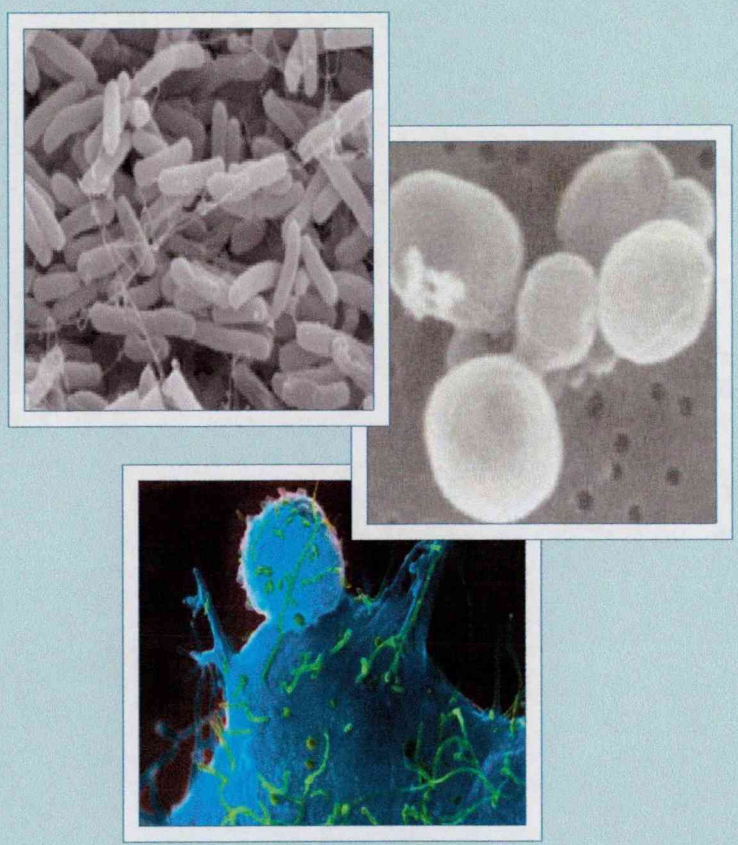
Reversal effects of kiwifruit extracts can be based on the modification of P-gp transmembrane efflux pump. However further purification of these substances is required.

Table 13.: Multidrug resistance reversion by kiwifruit peel extracts in lymphoma 5178 cells.

Compound's number	Concentration (μg/ml in DMSO)	Cell size ratio (FSC)	Cell granulation ratio (SSC)	Fluorescence on height (FL-1)	Fluorescence activity ratio
par	Control	394	131	4104.0	78.77
mdr+R123	Control	413	142	52.1	1.00
verapamil	10	415	110	842.0	16.16
H0	200	532	174	128	2.09
H1	200	487	160	92	1.51
H2	200	584	177	199	3.21
H3	200	482	230	420	6.89
H4	200	435	304	977	16.01
	20	452	266	767	5.52
H5	200	430	295	1165	19.09
	20	398	217	473	3.40
A0	200	332	116	112	1.31
A1	200	334	106	68	0.79
A2	200	322	132	119	1.39
A3	200	380	84	536	6.24
A4	200	380	147	1193	13.90
	20	461	188	406	2.92
A5	200	269	166	75	0.89
A6	200	363	143	101	1.17
M0	200	458	156	95	1.56
M1	200	482	149	103	1.69
M2	200	503	204	1040	17.05
	20	511	205	427	3.07
M3	200	460	198	264	4.32
M4	200	498	142	77	1.26
M5	200	465	155	55	0.89
70MO	20	416	120	82	0,79
70M1	20	417	123	72	0.69
70M2	20	468	103	72	0.69
70M3	20	422	104	70	0.67
70M4	20	486	111	56	0.54
70M5	20	416	125	106	1.02
70M6	20	405	111	107	1.03

Chapter 5

SUMMARY

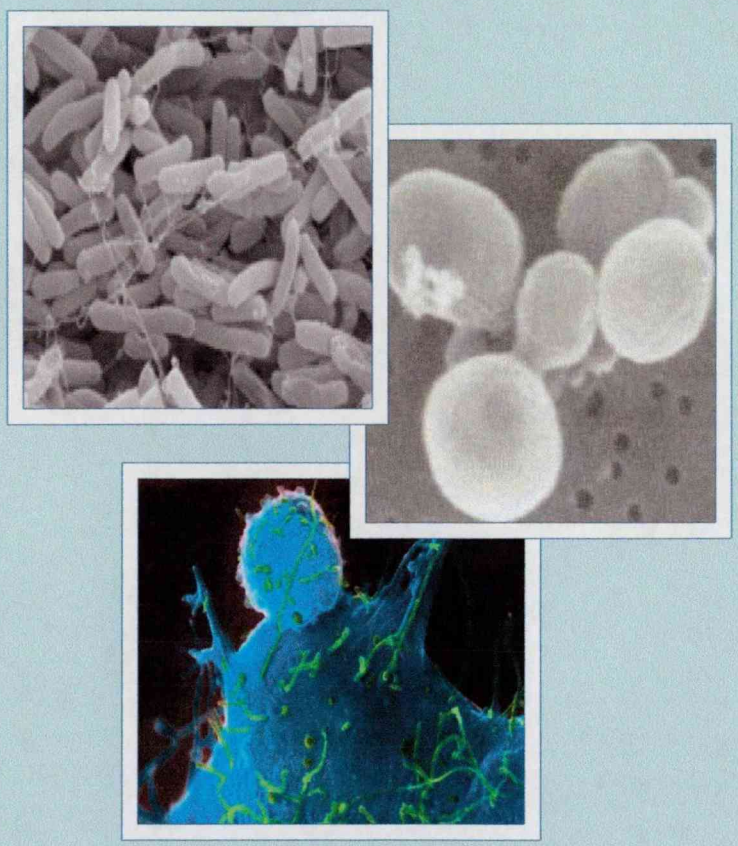


5. SUMMARY

Since their discovery during the 20th century, antimicrobial agents have substantially reduced the threat posed by infectious diseases. By helping to bring many serious infectious diseases under control, these drugs have also contributed to the major gains in life expectancy experienced during the later part of the last century. These gains are now seriously jeopardised by the emergence and spread of antimicrobial resistance, which is a complex problem driven by numerous interconnected factors, many of which are linked to the misuse of antimicrobials and thus amenable to change. On the other hand intrinsic or chemotherapy-induced multidrug resistance, the principal mechanisms by which many cancers develop resistance to chemotherapy is a major factor in the failure of cancer chemotherapy and indicates a great problem in clinical oncology. The resistance against antimicrobial agents and cancer cells appeared in the last decades, but only limited number of new drugs are being discovered. Therefore the research and development of structurally new, effective medicaments are necessary. Starting from the fact that promethazine, a tricyclic non-antibiotic has antibacterial, antiplasmid and antimutagenic (also anticancer) effect, I aimed to study the multidrug resistance reversal activity of some hetrocyclic compounds, initiate bacteria, yeasts and tumour cells in these model experiments, to find more effective compounds than promethazine. It was suggested that complex formation of tricyclic compound with plasmid DNA was involved in antiplasmid activity. The possible mechanism of interaction between nucleic acid and phenothiazines was clarified. It was concluded that promethazine might form a complex with guanosine-cytosine rich regions of DNA. The G-C rich regions are essential for plasmid replication. Some 3-benzazepines in high concentration eliminate the covalently closed circular (ccc) form of plasmid DNA by complex formation. The same holds for clinical isolates of bacteria: promethazine and 9-amino acridine could form a complex with DNA. Promethazine and related compounds did not eliminate the killer plasmid *of S. cerevisiae* but 9-amino acridine was effective as killer character eliminating agent. In evaluation of combined effect of promethazine with fluconazole the achieved results show that promethazine could enhance the *in vitro* activity of fluconazole. This finding can open up new perspectives for treating multidrug resistance (mdr) in yeasts using combinations of antifungal agents with non-antibiotic drugs. Some newly synthesized compounds (e.g. 3-benzazepines) proved to be very promising agents for mdr reversing in cancer cells. The results of this study can open up new prospects to recognize some new roles in the interaction of drugs in elimination of mdr. We assume that these data may lead to further ideas for the development of more effective chemotherapeutic agents in the future.

Chapter 6

ÖSSZEFOGLALÓ

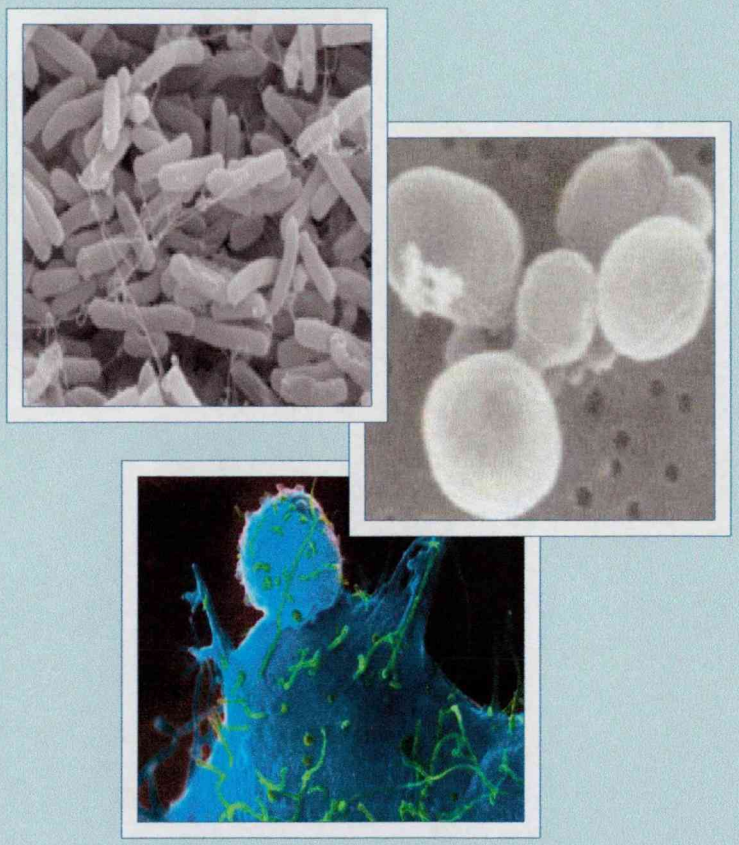


6. ÖSSZEFOGLALÓ

Az antimikrobiális szerek a XX. században bekövetkezett felfedezésük óta jelentősen visszaszorították a fenyegető fertőző betegségeket. Segítségükkel nőtt az emberek várható élettartama, mivel a komoly fertőző betegségek kontrollálhatóvá váltak. Mindezek az eredmények veszélybe kerültek az antimikrobiális rezisztencia megjelenésével és rohamos terjedésével. Ez a jelenség egy összetett probléma, több, szorosan összefüggő tényező befolyásolja, melyek közül számos kapcsolatban áll az antibiotikumok helytelen alkalmazásával. Másrészről a tumor sejtekben kemoterápia indukálta multidrog rezisztencia mechanizmusok teszik hatástalanná a kezelést és okoznak nagy gondot a klinikai onkológiában. Az antimikrobiális és antitumor rezisztencia az elmúlt évtizedekben jelent meg, ugyanakkor kevés új gyógyszer került bevezetésre a hatástalanok pótlására. Ezért elengedhetetlenné vált új támadáspontokra ható gyógyszerek kutatása és fejlesztése. Abból a tényből kiindulva, hogy a promethazin, egy triciklikus nem antibiotikum jellegű gyógyszer antibakteriális, antiplazmid, valamint antimutagén (és antitumor) hatással rendelkezik, célul tűztem ki heterociklusos vegyületek rezisztencia csökkentő hatásának vizsgálatát, bevonva baktériumokat, élesztőgombákat és tumor sejteket a modell kísérletekbe, abban a reményben, hogy a promethazinnál hatékonyabb vegyületet találok. Feltételeztük, hogy a triciklikus vegyületek antiplazmid aktivitásáért a plazmid DNS-el történő komplex képzésük felelős. Munkám során tisztáztam a plazmid nukleinsav és a phenothiazinok közötti interakció egyik lehetséges formáját: megállapítottam, hogy a promethazin a DNS guanozin-citozin gazdag régiójával alkothat komplexet. Ez régió elengedhetetlen a plazmid replikációhoz. Egyes 3-benzazepin származékok magas koncentrációban alkalmazva károsítják, illetve destabilizálják a plazmid DNS kovalensen zárt, cirkuláris (ccc) formáját komplex képzés révén. Ugyanez érvényes volt a klinikai vizsgálati anyagokból izolált baktérium speciesekre is. A kontrollként alkalmazott planáris szerkezetű 9-amino akridin komplexet képzett a DNS-el. A promethazin és szerkezetileg hasonló vegyületek nem törölték a *S. cerevisiae* killer plazmidját, ugyanakkor a 9-amino akridin hatékony plazmid eliminálónak bizonyult. A promethazin flukonazollal kombinálva *in vitro* felerősítette a flukonazol aktivitását rezisztens *Candida* törzsekben. Ez a felismerés lehetőséget nyújthat az antibiotikum rezisztens gombák elpusztítására, gombaellenes szerek nem antibiotikum jellegű gyógyszerekkel történő kombinációjával. Egyes újonnan szintetizált vegyületek (pl. 3-benzazepin származékok) igen ígéretesnek bizonyultak a multidrog rezisztencia eliminálásában, tumor sejtekben. Jelen tanulmány eredményei új lehetőségeket nyithatnak a multidrog rezisztencia csökkentésében és visszafordításában szerepet játszó tényezők megismerésében. Úgy véljük, ezek az adatok a jövőben új perspektívákat adhatnak kemoterápiás szerek kifejlesztéséhez.

Chapter 7

ACKNOWLEDGEMENTS



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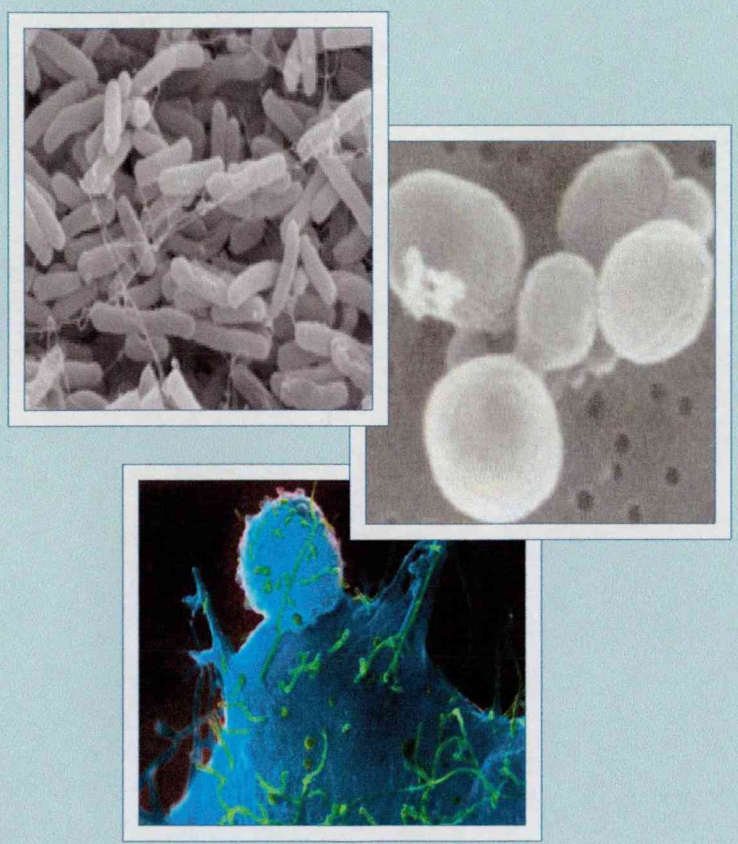
Chapter 8

REFERENCES



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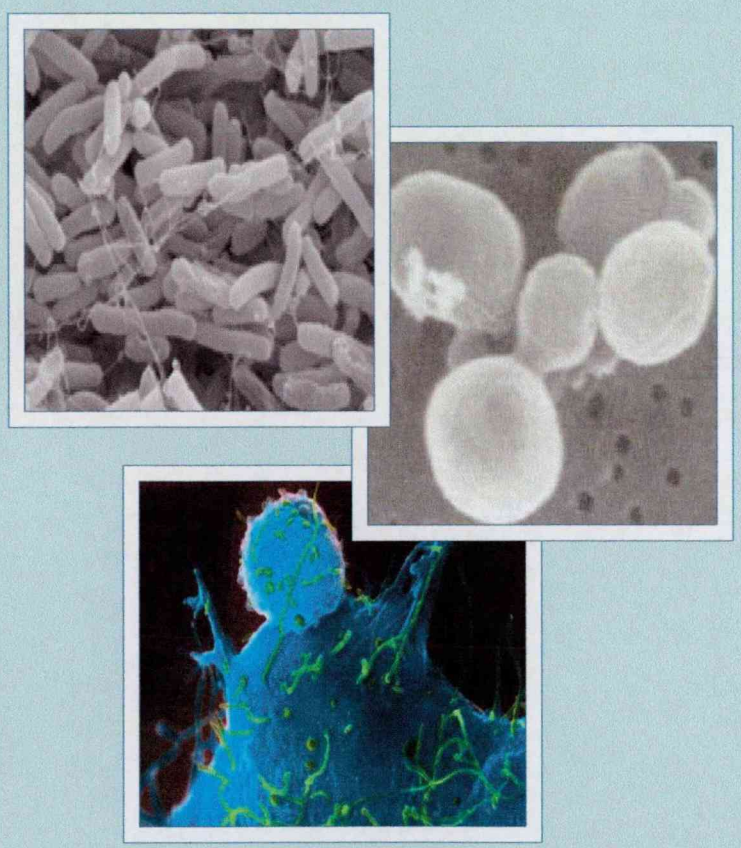
APPENDIX



Appendix I.

1. Preparation of nutrient broth for bacteria and fungi

MTY /Tryptone-Yeast extract media/

NH_4Cl	1.0 g (Reanal; Cat. No. 04110-1-22)
K_2HPO_4	7.0 g (Reanal; Cat. No. 08790-1-08)
$NaH_2PO_4x2H_2O$	3.0 g (Reanal; Cat. No. 24430-1-08)
NaCl	2.0 g (Reanal; Cat. No. 24640-1-01)
TRYPTONE	10.0 g (Reanal; Cat. No. 20571-0-33)
YEAST EXTRACT	1.0 g (Difco; Cat. No. 0127-01)
(1.5%AGAR)	15.0 g (Biolabs)
DISTILLED WATER	1000 ml

Adjust pH to 7.2-7.3 with 10M NaOH (Reanal; Cat. No.24576-1-08)

Autoclave on liquid cycle (121°C; 0,5 MPa) for 20 minutes

YTB /Yeast extract-Tryptone Broth/

TRYPTONE	10.0 g (Reanal; Cat. No. 20571-0-33)
NaCl	5.0 g (Reanal; Cat. No. 24640-1-01)
YEAST EXTRACT	5.0 g (Difco; Cat. No. 0127-01)
(1.5% AGAR)	15.0 g (Biolabs)
AMPICILLIN	100 μg/ml (Beecham Research Laboratories, England)
TETRACYCLINE	12.5 μg/ml (Chinoin)

Autoclave on liquid cycle (121°C; 0,5 MPa) for 20 minutes

2xYPD /2 x Yeast extract-peptone-glucose media/:

YEAST EXTRACT	10.0 g (Difco; Cat. No. 0127-01)
PEPTONE	20.0 g (Difco; Cat. No. 0118-01)
GLUCOSE	20.0 g (Reanal; Cat. No. 07072-1-08)
(AGAR)	20.0 g (Biolabs)
DISTILLED WATER	1000 ml

Autoclave on liquid cycle (121°C; 0,5 MPa) for 20 minutes

2xYPD+P-citrarate buffer:

YEAST EXTRACT	10.0 g (Difco; Cat. No. 0127-01)
PEPTONE	20.0 g (Difco; Cat. No. 0118-01)
GLUCOSE	20.0 g (Reanal Cat. No. 07072-1-08)
(2% AGAR)	20.0 g (Biolabs)
Na_2HPO_4	13.78 g (Reanal; Cat. No. 08972-1-01)
DISTILLED WATER	1000 ml
CITRIC ACID	12.92 g (Reanal) for adjust the pH to 4.2-4.4

Autoclave on liquid cycle (121°C; 0,5 MPa) for 20 minutes.

Media for cell line cultivation:

*McCoy's 5A medium modified (*Gibco BRL; Cat. No. 21500-046*):*

PAR:	in 100 ml
Streptomycin	1.0 ml (Sigma; Cat. No. S6501)
Nystatin	0.1 ml (Gibco BRL; Cat. No. 15345-010)
200mM L-glutamine	1.0 ml (Gibco BRL; Cat. No.15032-014)
10% heat inactivated horse serum	10.0 ml (Gibco BRL; Cat. No.26050-039)

MDR:	in 100 ml
Streptomycin	1.0 ml (Sigma; Cat. No. S6501)
Nystatin	0.1 ml (Gibco BRL; Cat. No. 15345-010)
200mM L-glutamine	1.0 ml (Gibco BRL; Cat. No.15032-014)
10% heat inactivated horse serum	0.1 ml (Gibco BRL; Cat. No.26050-039)
Colchicine	60 µg/ml (Sigma; Cat. No. C3915)

2. Preparation of stock solutions

Note: *All reagents are prepared and diluted using deionized water.*

Antibiotics

Ampicillin 100 mg/ml, sterile filtered water (1000x stock)

Tetracycline 12,5 mg/ml in 50% (v/v) ethanol, sterile filtered (1000x stock). Store in dark!

Store antibiotic stock solutions at -20°C until needed.

Add 1.0 ml of 1000x stock per L to broth for liquid culture, or agar solution (after cooling to 50°C)

Preparation of competent cells

Calcium chloride ($CaCl_2$) 1.0 M:

Dissolve 14.7 g $CaCl_2x2H_2O$ (Reanal; Cat. No. 16381-1-01) in 100 ml water. Autoclave, and store as sterile solution at room temperature.

Magnesium chloride ($MgCl_2$) 1.0 M:

Add 20.3 g $MgCl_2x6H_2O$ (Reanal; Cat. No. 20281-1-01) to a final volume of 100 ml water. Autoclave to sterilize and store as a sterile solution at room temperature.

Potassium chloride (KCl), 1.0 M:

Combine 7.45 g KCl (Reanal; Cat. No. 18050-1-08) with 100 ml water. Autoclave and store at room temperature.

Plasmid purification by alkaline lysis method

Stock solutions for Sol I.:

***0.5 M glucose*:**

Add 0.99 g to a final volume of 10 ml water. Autoclave to sterilize and store as sterile solution at room temperature

Tris-HCl 2.0 M, pH: 8.0:

Dissolve 24.22 g Tris base (Reanal; Cat. No. 30750-2-99) in 70 ml water. Adjust the pH by adding about 8.4 ml concentrated HCl. Add water to a final volume of 100 ml.

Note: The pH of Tris is pH dependent, decreasing about 0.03 pH units per °C increase in temperature.

Ethylenediamine tetraacetic acid (EDTA) 0.25 M:

Dissolve 4.65 g EDTA (Reanal; Cat. No. 09130-1-01) in 50 ml water. Autoclave and store at room temperature.

Stock solutions for Sol II.:

NaOH 10 N:

Carefully dissolve 40 g NaOH pellets in water, to final volume of 100 ml. Store in plastic container at room temperature. Do not autoclave!

Sodium dodecyl sulfate (SDS), 10% (w/v):

Dissolve 10 g SDS (Sigma; Cat. No. L4390) in 80 ml water. Warming the solution to 50°C often accelerates dissolution. Add the water to a final volume of 100 ml. Do not autoclave this solution, it is already sterile.

Potassium acetate solution (for alkaline lysis plasmid preparation):

Combine 29.4 g potassium acetate (Reanal; Cat. No. 17830-1-08), 11.5 ml glacial acetic acid (Sigma; Cat. No. A6283) and water to a final volume of 100 ml. Store at room temperature.

Preparation of yeast protoplast

Pre-treating solution (for protoplast preparation):

Combine 10 mM Tris-HCl, 0.5 M Na-EDTA and 1 % β-mercaptoethanol (Sigma; Cat. No. M6250) in water. Store at room temperature.

Enzyme solution (for protoplast preparation):

Combine in 0.7 M KCl (Reanal; Cat. No.18050-1-22) 0.5 % snail enzyme and 0.5 % NovoZyme (Sigma; Cat. No. L1412). Store at 4°C.

Gel-electrophoresis

TE buffer:

10 mM Tris-HCl, pH: 8.0

1.0 mM EDTA

RNase 10 mg/ml stock solution:

Dissolve 10 mg of RNase in 1.0 ml water and put the solution into a hot water-bath for 10 minutes to inactivate the rest of the DNase. Store at -20°C.

TBE buffer, 20x (for agarose gel electrophoresis):

Combine 121 g Tris base, 61.7 g boric acid (Reanal; Cat. No. 06000-1-01), 7.44 g $Na_2EDTAx2H_2O$, and ad water to final volume of 1.0 l.

Note: A precipitate may form in this solution with storage at room temperature. The buffer works well despite of the precipitate

Ethidium bromide, 10 mg/ml

Add 0.2 g ethidium bromide (Sigma; Cat. No. E8751) to 19.8 ml water to dissolve. Store the solution at 4°C in a foil-wrapped bottle to prevent exposure to light. Handle with care!

3. Preparation of agar plates for bacterial/fungal growth

After autoclaving the cultivation broth to pour plates, the agar was allowed to cool to 50°C. Antibiotics (or other additives) were added if needed, and mixed. Liquid agar was poured (about 30 to 40 ml) into 100 mm sterile petri dishes. The plates were allowed to cool with consequent hardening of the agar. It was generally advisable to pour plates at least one day in advance to allow time for the plates to dry at room temperature before use.
After drying the plates were stored at 4°C until needed
The agar may be stored at room temperature in bottles of convenient volume, and melted as needed by briefly reautoclaving or heating in microwave oven.

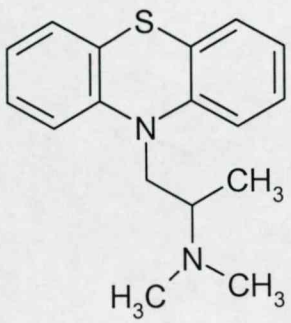
4. Long-term storage

a. Long-term storage of bacterial/fungal strains
After streaking bacterial/fungal strains can be stored at 4°C for several months. Valuable strains of bacteria/fungi can be stored at -70°C as glycerol stocks. To freeze cells as a glycerol stock, a sterile glycerol/4:1: v/v water solution was prepared by autoclaving. 1.0 ml of the sterile 80% glycerol solution (Reanal; Cat. No. 11290-0-01) was added to 2.5 ml of logarithmically growing (OD 0.1 to 0.6) culture of the strain. The two solutions were mixed and the mixture was transferred to a Nunc tube, which was frozen at -70°C.
b. Long-term storage of cell lines:
Cell lines (PAR and MDR) can be stored in liquid nitrogen. To freeze cells in liquid nitrogen, 7.0 million/ml cells were added to 10% dimethyl sulfoxide (Reanal; Cat. No. 08860-1-08) containing McCoy's medium and transferred to a Nunc tube, which was frozen at -70° C. After 24 h, the tubes were transferred into liquid nitrogen.

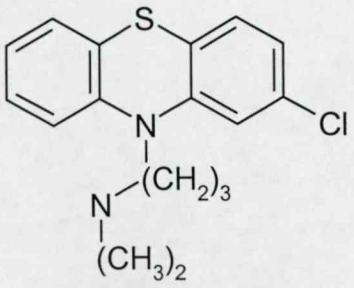
Appendix II.

Chemical structure of some studied compounds

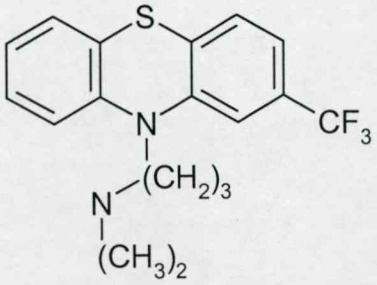
1. Promethazine and related compounds



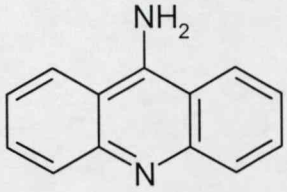
promethazine



chlorpromazine

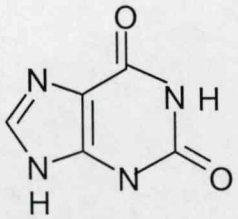


triflupromazine

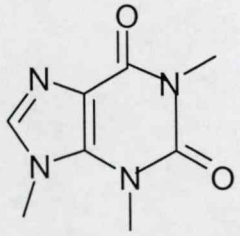


9-amino acridine

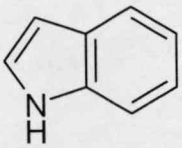
2. Xanthenes and indole



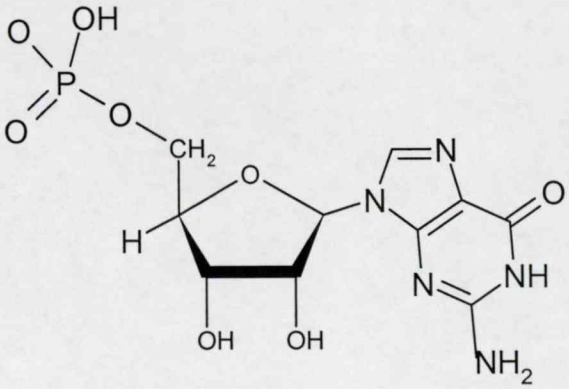
xanthine



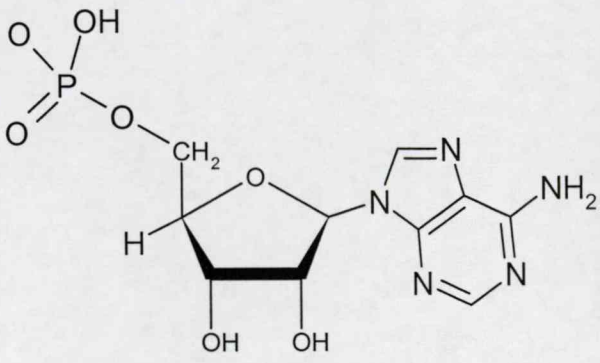
caffeine



indole

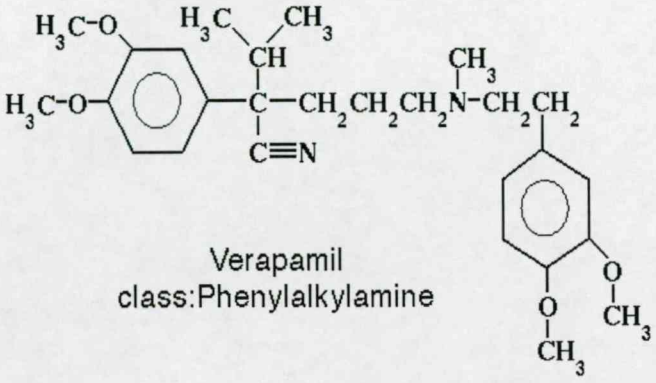


2'-deoxyguanosine
5'monophosphate

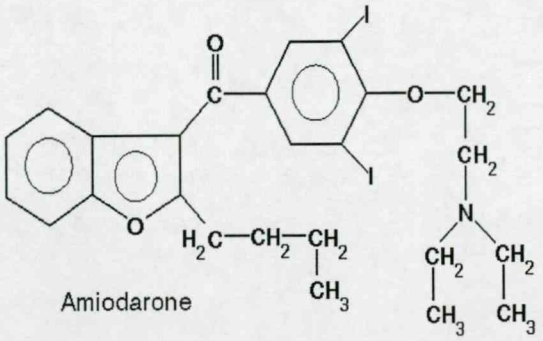


2'-deoxyadenosine
5'-monophosphate

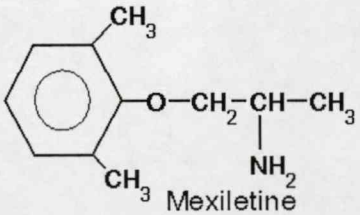
3. *Structure of some studied antiarrhytmics*



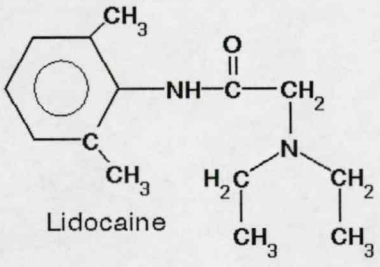
Verapamil
class:Phenylalkylamine



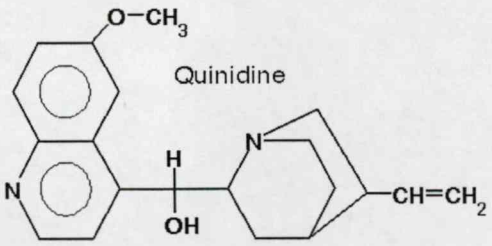
Amiodarone



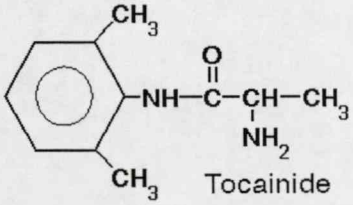
Mexiletine



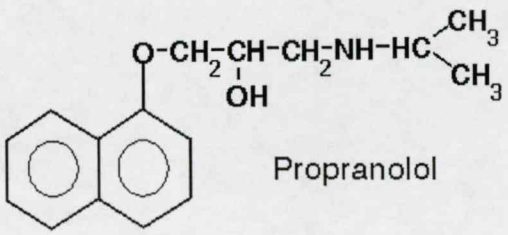
Lidocaine



Quinidine



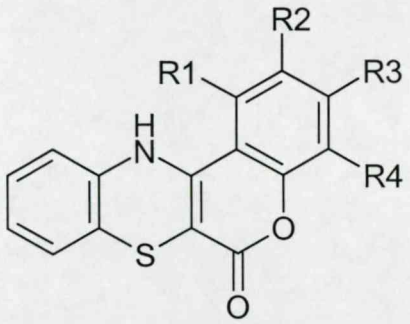
Tocainide



Propranolol

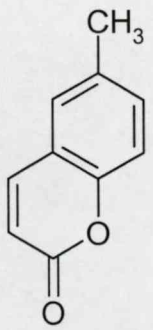
4. 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones and related coumarins

Benzo[a]phenothiazine

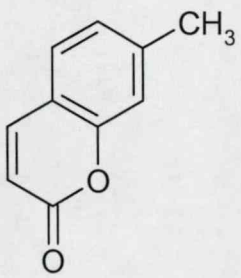


6,12-dihydro-1-benzopyrano[3,4b][1,4]benzothiazin-6-ones (*7-12*)

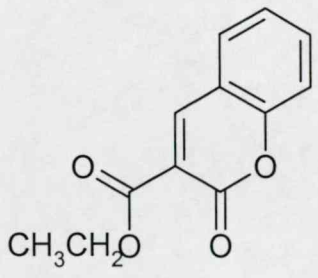
Compound's No.	***R1***	***R2***	***R3***	***R4***
7	H	H	H	H
8	H	CH_3	H	H
9	CH_3	H	CH_3	H
10	CH_3	H	H	CH_3
11	H	H	CH_3	CH_3
12	H	Cl	CH_3	H



6-methylcoumarin

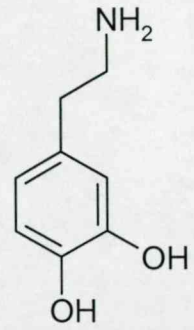


7-methylcoumarin

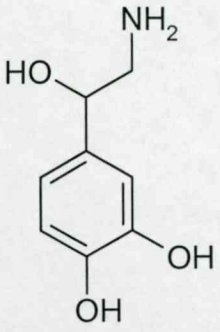


Ethyl 3-coumarin-hypen carboxylate

5. Cathecols

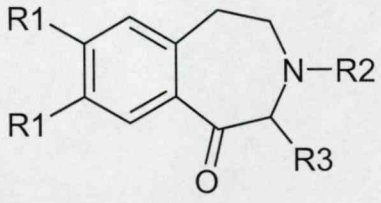


dopamine

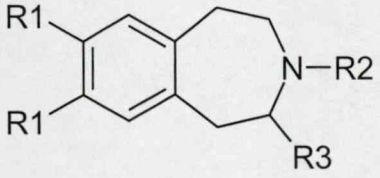


norepinephrine

6. 3-benzazepines



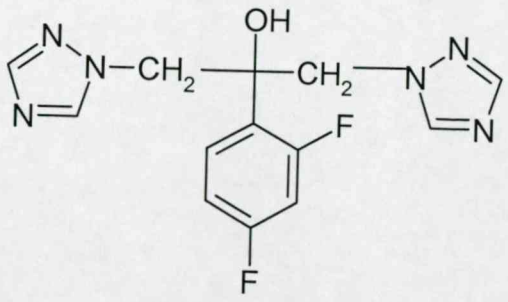
Benzazepinones



Benzazepines

Compound	***R1***	***R2***	***R3***
Benzazepinones			
KS02	MeO	Ms	Me
KM57	MeO	Tf	Me
KN50	H	Tf	H
KE04	MeO	Tf	H
KI10	MeO	Tf	Me_2CH
KP80	MeO	Tf	Ph
Benzazepines			
KF1	HO	H	CF_3
KF2	HO	Me	CF_3
KF3	HO	H	H
KF4	MeO	H	CF_3

7. ***Other studied compounds***



Diflucan (fluconazole)

Full papers

FULL PAPERS