PLASMID CURING IN ECOSYSTEMS OF BACTERIA, EFFLUX PUMP INHIBITORS IN BACTERIA AND CANCER CELLS

Ph.D. Thesis

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CONTENTS

LIST OF PUBLICATIONS

Full papers published on the subject of the thesis:

I. **Molnár A**, Amaral L, Molnár J. Antiplasmid effect of promethazine in mixed bacterial cultures. *International Journal of Antimicrobial Agents* 2003; 22: 217-222. ***IF: 1.950***

II. **Molnár A**, Wolfart K, Kawase M, Motohashi N, Molnár J. Effect of a trifluoromethyl ketone on the motility of proton pump deleted mutant of *Escherichia coli* strain and its wild type. *In Vivo* 2004; 18(4): 505-509. ***IF: 0.753***

III. Spengler G, **Molnár A**, Klausz G, Mándi Y, Kawase M, Motohashi N, Molnár J. Inhibitory action of a new proton pump inhibitor, trifluoromethyl ketone derivative, against the motility of clarithromycin-susceptible and-resistant *Helicobacter pylori*. *International Journal of Antimicrobial Agents* 2004; 23: 631-633. ***IF: 1.950***

IV. Motohashi N, Wakabayashi H, Kurihara T, Fukushima H, Yamada T, Kawase M, Sohara Y, Tani S, Shirataki Y, Sakagami H, Satoh K, Nakashima H, **Molnár A**, Spengler G, Gyémánt N, Ugocsai K, Molnár J. Biological activity of barbados cherry (acerola fruits, fruit of *Malpighia emarginata* DC) extracts and fractions. *Phytotheraphy Research* 2004; 18(3): 212-223. ***IF: 0.803***

V. Madureira AM, Spengler G**, Molnár A**, Varga A, Molnár J, Abreu PM, Ferreira MJ. Effect of cyloartranes on reversal of multidrug resistance and apoptosis induction on mouse lymphoma cells. *Anticancer Research* 2004; 24(2B): 859-864. ***IF: 1.347***

Full papers related to the subject of the thesis:

VI. Hohmann J, Forgo P, Molnár J, Wolfard K, **Molnár A**, Thalhammer T. Antiproliferative Amaryllidaceae alkaloids isolated from the bulbs of *Sprekeleia formosissima* and *Hymenocalis x festalis*. *Planta Medica* 2001; 68: 454-57. ***IF: 1.879***

VII. Misbahi H, Brouant P, Hevér A, **Molnár A,** Wolfard K, Spengler G, Mefetah H, Molnár J, Barbe J. Benzo[b]-1,8-naphthyridine derivatives: synthesis and reversal activity on multidrug resistance. *Anticancer Research* 2002; 22: 2097-2102. ***IF: 1.347***

VIII. Molnár J, **Molnár A**, Mucsi I, Pinter O,. Nagy B, Varga A. and. Motohashi N. Reversal of multidrug resistance in mouse lymphoma cells by phenothiazines. *In Vivo* 2003; 17: 145-150. ***IF: 0.753***

IX. Wolfart K, **Molnár A**, Kawase M, Motohashi N. and Molnár J. Effects of trifluoromethyl ketones on the motility of *Proteus vulgaris*. *Biological and Pharmaceutical Buletin* 2004; 27(9): 1462-1464. ***IF: 1.124***

X. Molnár J, Gyémánt N, Mucsi I, **Molnár A**, Szabó M, Körtvélyesi T, Varga A, Molnár P, Tóth G. Modulation of multidrug resistance and apoptosis of cancer cells by selected carotenoids. *In Vivo* 2004; 18(2): 237-244. ***IF: 0.753***

XI. Gyémánt N, **Molnár A**, Spengler G, Mándi Y, Szabó M. and Molnár J. Bacterial models for tumor development. *Acta Microbiologica et Immunologica Hungarica* 2004; 51(3): 321-332. ***IF: 0.0***

XII. Molnár J, **Molnár A**, Spengler G. and Mándi Y. Infectious plasmid resistance and efflux pump mediated resistance. *Acta Microbiologica et Immunologica Hungarica* 2004; 51(3): 333-349. ***IF: 0.0***

XIII. Spengler G, **Molnár A**, Klausz G, Mándi Y, Kawase M, Motohashi N. and Molnár J. The antimotility action of a trifluoromethyl ketone on some Gram-negative bacteria. *Acta Microbiologica et Immunologica Hungarica* 2004; 51(3): 351-358. ***IF: 0.0***

XIV. Madureira AM, **Molnár A**, Abreu PM, Molnár J, Ferreira MJ. A new sesquiterpene – coumarine ether and new abietane diterpene and their effects as inhibitors of P-glycoprotein. *Planta Medica* 2004; 70(9): 828-833. ***IF: 1.879***

XV. Kawase M, Sakagami H, Motohashi N, Hauer H, Chatterjee SS, Spengler G, Vigyikanne AV, **Molnar A**, Molnar J. Coumarine derivatives with tumor specific cytotoxicity on multidrug resistance reversal activity. *In Vivo* 2005; 19(4): 705-711. ***IF: 0.753***

XVI. Viveiros M, Jesus A, Brito M, Leandro C, Martins M, Ordway D, **Molnar AM**, Molnar J, Amaral L. Inducement and reversal of tetracycline resistance in *Escerichia coli* K12 and expression of proton gradient dependent multidrug efflux genes. *Antimicrobial Agents and Chemotherapy* 2005; 49(8): 3578-3582. ***IF: 4.246***

XVII. Hendricks O, **Molnár A**, Butterworth T, Butaye P, Kolmos HJ, Christiansen JB. and Kristiansen JE. *In vitro* activity of phenothiazine derivatives on *Enterococcus faecalis* and *Enterococcus faecium. Basic & Clinical Pharmacology & Toxicology* 2005; 96(1): 33-36. ***IF: 1.342***

XVIII. Sharples D, Spengler G, Molnar J, Antal Z, **Molnar A**, Kiss JT, Szabo JA, Hilgeroth A, Gallo S, Mahamoud A, Barbe J. The interaction between resistance modifiers such as pyrido (3,2-g) quinolone, aza-oxafluorene and pregnane derivatives with DNA, plasmid DNA and tRNA. *European Journal of Medicinal Chemistry* 2005; 40(2): 195-202. ***IF: 1.168***

Presentations related to the subject of the thesis:

I. Hohmann J, Rédei D, Máthé I, Forgo P, Blazsó G, Falkay Gy, Molnár J, Wolfard K, **Molnár A.** and Thalhammer T. Chemical and pharmacological investigation of macrocyclic diterpenoids isolated from *Euphorbia* species. 6th International symposium of poisonous plants, Glasgow, 6-10 August, 2001.

II. **Molnár A**, Molnár J. Baktériumfajok kokultivációja, hatása a plazmideliminációra. Magyar Mikrobiológiai Társaság Jubileumi Nagygyűlése, Balatonfüred, 2001. október 10-12.

III. **Molnár A**, Molnár J. Plasmid curing action of promethazine in mixed bacterial populations. COST ACTION B16 "Reversal of Antibiotic Resistance", Marseille, 30. November, 2001.

IV. **Molnár A**, Molnár J. Antiplasmid effect in mixed bacterial communities. COST ACTION B16 "Reversal of Antibiotic Resistance (by inhibition of membrane transport)", Paris, 3-4. May, 2002.

V. **Molnár A,** Spengler G, Schelz Zs, Molnár J. Multidrog-rezisztencia visszafordítása fenothiazin és perfenazin származékokkal, valamint növényi kivonatokkal. III. Sejtanalitikai Konferencia, Budapest, 2002. május 16-18.

VI. **Molnár A**, Molnár J. Plazmideliminációó vegyes baktérium kultúrákban. Magyar Kemoterápiai Társaság XVII. Kongresszusa, Szeged, 2002. június 7-8.

VII. **Molnár A**, Molnár J. Promethazin plazmideliminálóó hatása vegyes baktérium kultúrákban. Magyar Mikrobiológiai Társaság Kongresszusa, Balatonfüred, 2002. október 8-10.

VIII. Molnár J, **Molnár A**, Gyémánt N, Molnár P, Tóth G. Modulation of multidrug resistance on cancer cells by selected carotenoids. 14th International Congress on Anti-Cancer Treatment, Paris, 1-4. February, 2003.

IX. Molnár J, **Molnár A**, Eckert G, Keyzer H, Gaal D, Motohashi N, Thornton B. Possible role of negative entropy in tumor growth. 14th International Congress on Anti-Cancer Treatment, Paris, 1-4. February, 2003.

X. **Molnár A**, Amaral L, Molnár J. Plasmid curing effect of promethazine in mixed bacterial cultures under different environmental conditions. 14th Intarnational Congress of the Hungarian Society for Microbiology, Balatonfüred, 9-11. October, 2003.

XI. **Molnár A**, Amaral L, Molnár J. Plasmid curing effect in mixed bacterial communities. 5th European Congress of Chemotherapy and Infection, Rhodes, 17-20. October, 2003.

XII. **Molnár A**, Molnár J. Antiplazmid hatás vizsgálata vegyes baktérium tenyészetekben. Magyar Kemoterápiai Társaság XVIII. Kongresszusa Budapest, 2004. Január 22-24.

XIII. Molnár J, **Molnár A**, Gyémánt N, Ugocsai K, Gaál D, Hohmann J, Körtvélyesi T, Molnár P, Ocsovszki I, Varga A. Multidrog-rezisztencia gátlása különböző tumorsejtekben *in vitro*. Magyar Kemoterápiai Társaság XVIII. Kongresszusa, Budapest, 2004. Január 22-24.

LIST OF ABBREVIATIONS

ABC: ATP Binding Cassette
AMP: Ampicillin
BHI: Brain Heart Infusion
CFU: Colony Forming Unit
CRHP: Clarithromycin Resistant *Helicobacter pylori*
CSHP: Clarithromycin Sensitive *Helicobacter pylori*
CT: Charge Transfer
DMSO: Dimethyl sulfoxide
EMB: Eosine Methylene Blue
HGT: Horizontal Gene Transfer
HOMO: Highest Occupied Molecular Orbital
LUMO: Lowest Unoccupied Molecular Orbital
MATE: Multidrug and Toxic Compound Extrusion
MFS: Major Facilitator Supefamily
MDR: Multidrug Resistance
MRP1: Multidrug Resistance Associated Protein 1
MRSA: Multidrug Resistant *Staphylococcus aureus*
MTY: Minimal Tryptone Yeast
PAR: Parental
PBP: Penicillin Binding Protein
PBS: Phosphate Buffered Saline
PCP: Prochloperazine
PMF: Proton Motive Force
RND: Resistance Nodulation Division
SAR: Structure Activity Relationship
SMR: Small Multidrug Resistance
SDS: Sodium Dodecyl Sulfate
THIO: Thioridazine
VAN: Vancomycin
VISA: Vancomycin Intermediate Resistant *Staphylococcus aureus*
VRE: Vancomycin Resistant *Enterococcus faecalis*

I. INTRODUCTION

1.1. Factors contributing to antimicrobial resistance

In the past 50 years the development and subsequent clinical use of antibiotics led to successful treatment of many infectious diseases. The introduction of penicillin into general medical practice in 1944 made the formerly deadly illnesses like wound infections or pneumonia curable, leading to a significant increase in life expectancy [1].

However, the introduction of natural, semi-synthetic and synthetic antimicrobials to clinics and agriculture contributed to the emergence of resistant microorganisms [2]. There is a growing body of evidence that the medical and non-medical misuse and overuse of antimicrobials facilitate the rapid dissemination of antibiotic resistance among microorganisms, especially human pathogens due to the strong selective pressure [3, 4]. Today, resistance to antibiotics has become a major public health concern [5, 6, 7]. Rates of antibiotic resistance are reaching alarming levels in Southern and Central Europe, standing in correlation with the amount of antibiotics used [8]. Recently it was reported that the outpatient antibiotic use was lower in the northern regions, moderate in middle regions, and higher in southern regions in Europe (Figure1).

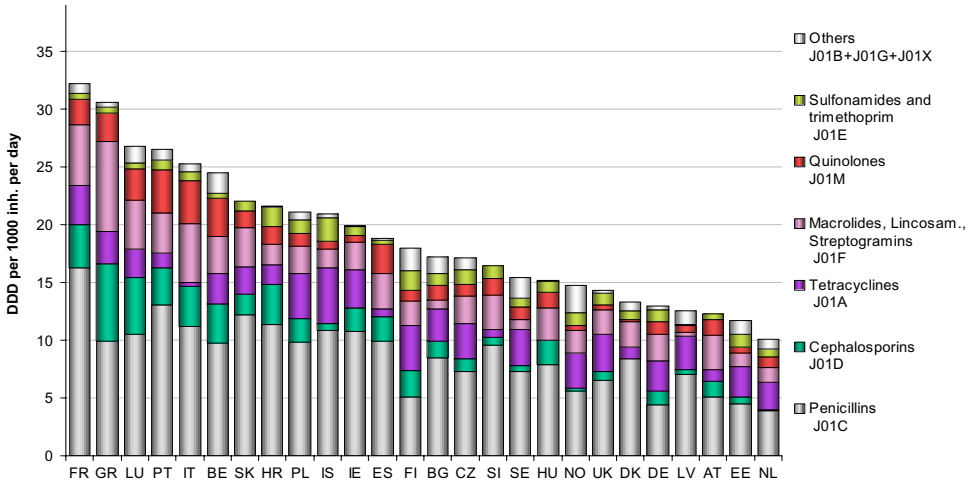

Figure 1. Total outpatient antibiotic use of 26 European countries in 2002. Legend: DDD: Defined Daily Dose; inh: inhabitant. Source of data: Reference 8.

Furthermore, the study showed higher occurrence of antibiotic resistance in high consuming countries, *e.g.* in Southern and Eastern Europe. Another potential risk factor of the emergence of resistant microorganisms is the use of personal hygiene and household products containing antibacterial agents like triclosan (2,4,4'-trichloro-2'-hydroxyphenyl-ether) [9, 10]. It was shown that bacteria with low susceptibility to triclosan can promote cross resistance[11].

Triclosan was also proven to be a substrate for MDR (Multidrug Resistance) efflux pumps, allowing for selection of pump mutations [12].

Recently it has been reported that almost half of all antibiotics produced is used for human consumption. Another half is added to animal feed for prophylaxis, treatment and growth promotion in agriculture and aquaculture [13, 14]. The life-long subtherapeutic dose of antibiotic treatment in animal husbandry promotes the selection of resistant populations, especially in the animal intestinal flora [15, 16, 17]. Vancomycin-resistant *Enterococcus faecalis* (VRE) was isolated from sewage and different animal sources in Europe [18, 19]. It was suggested that the emergence of VRE was due to the use of avoparcin (vancomycin analogue) containing animal feed additives [20]. Based on these observations, it was suggested that contaminated food products and domesticated animals are the reservoirs of VRE, which can reach humans by the food chain or direct contact [21]. In contrast, VRE is regarded as hospital acquired nosocomial pathogen in the United States where avoparcin is not a licensed feed additive.

For plant protection, streptomycin and oxytetracylin are still in regular use, although the amount of antibiotics applied for treating plant diseases is minor compared to total antibiotic use [22, 23]. Since antimicrobials for plant protection are spread in the open environment and washed into soil and ground water, they may select for resistance in commensals or even human pathogens.

Antimicrobial resistance is widespread in the environment as microorganisms carrying resistance genes can move easily among four main ecological compartments: *humans*, *animals*, *plants* and *soil-water* [2, 3]. Each ecosystem contains the selective pressure of antimicrobials, and the genetic exchange of resistance determinants can easily occur among microorganisms originating from different ecological habitats. The major driving force behind the spread of resistance is *natural selection*, which renders resistance to antimicrobials and the ability to exchange these resistance gene determinants by *horizontal gene transfer* [24, 25].

Above all, several socioeconomic and environmental factors facilitate the spread of resistant microorganisms by person-to-person transmission in the community.

- Hospital-acquired infections are caused by antibiotic-resistant pathogens selected from the patients' own flora during intensive board spectrum antibiotic treatment or from the transfer between bacteria by mobile genetic elements [26]. Subsequently, the resistant pathogens spread among patients. The risk factors enhancing the dissemination are the severity of illness, surgery, prolonged stay in intensive care unit, exposure to contaminated medical equipment, infected foreign bodies, shift of resistant bacteria by the hands of medical personnel and lack of contact isolation [27].

Recently several multiple resistant bacteria have been selected in hospitals, such as Multidrug Resistant *S. aureus* (MRSA), Vancomycin Intermediate Resistant *S. aureus* (VISA), Vancomycin-resistant *Enterococcus faecalis* (VRE) and MDR *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* [28, 29, 30]. MRSA strains recently appeared in community-acquired infections [31].

- Successful treatment protocols create an increasingly immunsupressed population in the elderly [32] as well as among patients undergoing organ transplantation, cancer and AIDS treatment. Immune disorders are also predisposing factors for infections caused by opportunistic pathogens, which are often multiresistant.
- Crowding, as it occurs in hospitals, day care centers, prisons and public transport.
- Accelerated international mass migration and trade facilitates the spread of geographically separated microorganisms [33].

1.2. Mechanisms to acquire antibiotic resistance

Mechanisms of antibiotic resistance acquisition are the evolutionary response to the selective pressure of antibiotics. It is an unstoppable phenomenon, which occurs each time new drug is introduced into practice. The emergence of antibiotic resistant phenotypes is due to mutations at different chromosomal loci or acquisition of resistance determinants by horizontal gene transfer (HGT).

1.2.1. Mutations

Several mechanisms of antibiotic resistance are based on mutational events [35]. Variations in the drug efflux and uptake systems are modified by mutations in the regulatory genes or their promoter region leading to multiple antibiotic resistances [36]. For example, mutations in *E. coli mar* gene affect the expression of 60 different genes, involving the downregulation of OmpF (porin channel) and the upregulation of AcrAB (RND efflux pump) [37]. Recently it has been found that the rate of mutation in bacteria is higher *in vivo* under stressful conditions like the presence of antibiotics, starvation and competition for novel niches during infection processes [38]. Mutation and HGT can act in a synergistic way as mutation produces new variations of the alleles and HGT introduces these new alleles into the population.

1.2.2. Horizontal gene transfer

HGT is responsible for the global and rapid dissemination of resistance genes in different bacterial species or genera within hospital and environmental settings. The transfer of

antibiotic resistance genes can occur via *conjugation*, *transformation* and *transduction* as shown by Figure 2 [2, 4, 39, 40, 41].

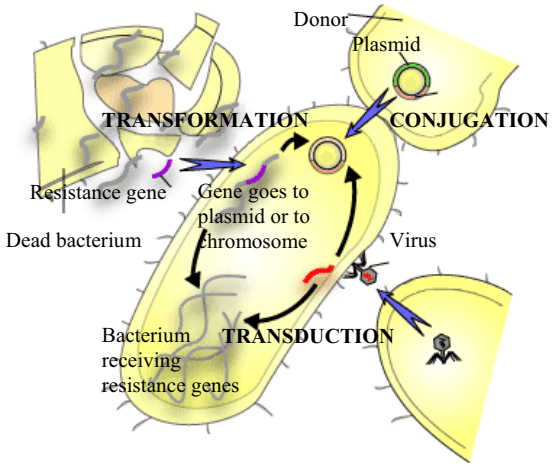

Figure 2. Genetic exchange by HGT. Figure is adapted from www.bioteach.ubc.ca

The first transferable resistance was discovered in 1959, when resistance genes of *Shigella ssp.* were transferred to *E. coli* by plasmid mediated conjugation [42]. The conjugative transfer of mobile genetic elements (plasmids, transposons, integrons and gene cassettes) provides great genetic variability as they mediate gene flux between Gram-positive and Gram-negative bacteria, referred to as *broad host range transfer* [43]. Self-transmissible plasmids and conjugative transposons are interactive elements that can mobilize other co-resident plasmids either by providing the mating apparatus (*trans* mobilization) or by forming a co-integrate (*cis* mobilization) [44, 45, 46, 47]. Retrotransfer has also been described [48]. Multiple resistant plasmids arise by acquiring several resistance genes encoded on transposons or integrons. The existence of broad host range transfer was supported by the findings that copies of the same resistant gene are detected in distantly related bacteria, which often reside in different habitats. For instance, the alleles of *ermG,* responsible for erythromycin resistance were over 95% identical in soil bacteria *Bacillus ssp*. and in the enteric bacteria *Bacteroides ssp* [49]. Such high identity could only arise by HGT and not by independent mutations. Nutrient rich environments, like matured soil and intestine are hot spots for genetic exchange between soil bacteria, the antibiotic resistant gut commensals and pathogens previously exposed to antibiotic treatment [50, 51].

New genetic elements can be acquired by transduction (viral infection) and transformation (free DNA is taken up by bacteria) referred to as *narrow host range transfer*. These types of routes occur between closely related species, as in both cases the fragments of DNA insert in the genome by homologous recombination, and in case of transduction the phage infection requires special receptors on the surface [52]. The clinically relevant *S. pneumoniae* acquires penicillin resistance by transformation in which an acquired gene creates ‘mosaic penicillin-binding protein’ (PBP), which renders low affinity to penicillins [53]. Transfer of resistance mediated by bacteriophages is frequently seen in staphylococci. It was reported that natural transformation occurs commonly among environmental isolates in soil. The free DNA

released by soil bacteria (*Bacillus ssp. and Streptomyces ssp.*) binds to sand and clay particles and become stabilized and retain a transforming ability for weeks [54]. Once bacteria become transformed by resistance genes, there can be transferred to other bacteria by gene transfer mechanisms.

1.3. Common mechanisms of antibiotic resistance

Bacteria developed different resistance mechanisms to evade antimicrobials. Types of antibiotic resistance can be classified into four main categories shown in Figure 3 [55, 56].
1. Enzymatic inactivation or modification of the active molecule. 2. Modification or protection of the antibiotic target. 3. Limited access of antibiotics and 4. Active efflux of antibiotics.

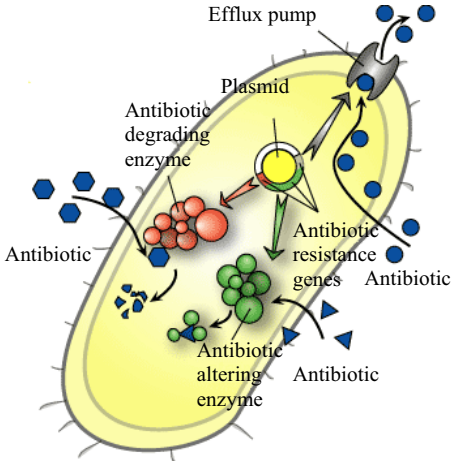

Figure 3. Mechanisms of antibiotic resistance. More than one mechanism can be present in a single bacterium. Figure is adapted from www.bioteach.ubc.ca

The first category includes enzymes (*e.g.* β-lactamase, chloramphenicol-acetyltransferase), which inactivate or modify the antibiotic molecules. Cleavage or modification in the structure of the antibiotics leads to decreased affinity to their target site[57].

The second group of resistance mechanisms involves the alteration of the target of the antibiotic. In MRSA strains, the *mecA* gene, which is located on the chromosome encodes a methicillin-inducible PBP2a. This mutant PBP shows low affinity to β-lactams to complete crosslinking of the cell wall peptidoglycans in the presence of the antibiotic [58].

The third strategy to gain protection from antimicrobials is to reduce the cell wall permeability. In case of Gram-positive bacteria the relatively thick peptidoglycan wall does not function as an effective barrier as molecules with molecular weight less than 50 kDa pass it freely. However, modification in the structure of the cell wall of VISA strains decreases the permeability of the cell wall [59]. In contrast, the envelope of Gram-negative bacteria is less effective in restricting the influx of antibiotics, than the Gram-positive cell wall. The outer membrane works as an efficient permeability barrier and renders high intrinsic resistance by the porin channels and the lipopolisaccharid (LPS) layer [60]. Porin channels drastically slow down the influx of hydrophilic antibiotics. [61]. Lipophilic and amphiphilic molecules like tetracyclines can diffuse through the phospholipid bilayer but this process is markedly slowed down by the low fluidity and negative charge of the LPS layer. In Gram-negative bacteria, the

additional contribution of MDR efflux pumps is needed to achieve high levels of intrinsic resistance [62].

In the efflux-mediated antibiotic resistance the antibiotics are expelled form the cell in an energy dependent manner to enable bacteria to survive in the toxic environment. Efflux transporters are classified as *specific transporters* referring to their narrow substrate specificity, as they mediate the extrusion of a given drug or class of drugs, like tetracycline, chloramphenicol/florfenicol efflux transporters [63, 64]. The second class of efflux proteins is the *MDR efflux pumps* that remove structurally unrelated compounds, but share physical similarities such as hydrophobicity, amphiphilicity and charge. MDR efflux systems also have a physiological role (protection of the membrane integrity), since their substrate spectrum includes bile salts, detergents, ionophores as well as antimicrobials [65, 66]. Genes encoding MDR transporters are commonly found in the chromosome, however *qacA/B* is located on plasmid in clinically relevant staphylococci [67]. Similarly the genes for specific transporters are located on plasmids and transposons that enables them exchange with other bacteria, even of foreign species. The MDR transporters are subdivided into the group of primary (ABC superfamily) and secondary transporters (H^+ antiporter). The four families of H^+ antiporters: Major Facilitator Superfamily (MFS), Resistance Nodulation Division (RND), Small Multidrug Resistance (SMR) and Multidrug and Toxic Compound Extrusion (MATE) utilize the transmembrane electrochemical gradient of protons or sodium ions to extrude drugs out of the cell. ABC transporters hydrolyze ATP for active efflux. Pumps belonging to the RND familiy render Gram-negative bacteria outstandingly high resistance, as they form a tripartite complex including outer and inner membrane proteins and a membrane fusion protein[68, 69]. In *E. coli*, the AcrAB-TolC and in *P. aeruginosa*, the MexAB-OprM [70, 71, 72, 73] efflux pump system extrudes a wide variety of compounds such as tetracycline, chloramphenicol, β-lactams, novobiocin, fusic acid, detergents, desinfectants, solvents [74, 75]. Gram-positive bacteria also possess efflux pumps that extrude numerous antimicrobial agents and desinfectants to confer clinically relevant resistance. In *S. aureus*, the MDR efflux phenotype is related to the NorA transport protein, while in *B. subtilis*, Bmr is the drug efflux protein [76].

1.4. Ways to overcome antibiotic resistance

Infections caused by mono- and MDR bacteria are difficult to manage with commonly employed antibiotics and are often incurable. One approach to combat resistance is to find new agents that possibly work in a different way than existing drugs, or to develop resistance blocking agents. The emergence of resistance to any potent drug by mutations is inevitable,

but development of a new agent is time and cost consuming. Drug resistance can be reversed by **1**. Elimination of plasmids carrying antimicrobial resistance. **2**. Enhancement of antibiotic activity by non-conventional antimicrobials such as efflux pump inhibitors[77].

1.4.1. Plasmid curing by phenothiazines

Theoretically, the curing of plasmids encoding antibiotic resistance is a way to eliminate resistance. This approach is encouraged by several *in vitro* and *in vivo* studies that show that plasmid associated-antibiotic resistance of bacteria can be reversed by selective combinations of some non-antibiotics with antibiotics to which the organism was originally resistant.

Tricyclic neuroleptics and the antidepressant phenothiazines like chlorpromazine, thioridazine, promazine, promethazine, imipramine and desipramine, were shown to have strong *in vitro* antibacterial activity against a wide variety of bacteria including mycobacteria[78, 79, 80, 81, 82]. Phenothiazines, classified as resistance modifiers were proved to reverse antibiotic resistance of some specific bacteria at sub-MIC in combination with conventional antibiotics *in vitro*. The reversal of antibiotic resistance by phenothiazine derivatives is considered to be triggered in two main ways: **1**. Promoting plasmid elimination and **2.** Inhibition of antibiotic transport functions of efflux pumps found in the plasma membrane.

Acridine orange, ethidium bromide, sodium dodecylsulfate (SDS) and phenothiazines were recognized as effective *in vitro* antibacterial and antiplasmid agents during the last three decades [83]. Acridine dyes [84] and ethidium bromide [85] were excluded from *in vivo* trials because of their mutagen effect as well as SDS [86], since it has a detergent action. A series of psychopharmacological agents without mutagenic effect were found to eliminate plasmids *in vitro*. The chemical structures of the phenothiazines are derived from methylene blue, a tricyclic compound regarded as the nucleus of the group of phenothiazine derivatives [87].
The chemical structures of phenothiazines contain a planar or non-planar heterocyclic ring system with two heteroatoms (N and S), and the basic structure can be substituted by alkyl or aromatic side chains.

The antimicrobial and antiplasmid activity of phenothiazines is a result of several intracellular target sites they act on including the bacterial membrane, plasmid DNA and different enzyme systems. Bacteria contain fewer plasmids when cultured in sub-inhibitory concentrations of phenothiazines due to inhibition of plasmid replication, partition and conjugal transfer processes [88] (Figure 4).

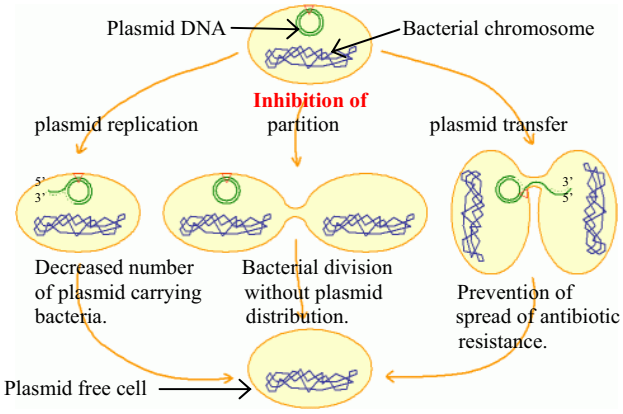

Figure 4. Schematic drawing of the inhibition of plasmid replication at three different levels. Figure is adapted from reference 83.

Laboratory experiments indicated that in mono-species cultures, promethazine markedly reduced lactose fermentation and the tetracycline, chloramphenicol, streptomycin and sulfonamide resistance of *E. coli* [89, 90], while hardly reduced antibiotic resistance of *Salmonella ssp.* [91, 92] *Y. enterocolitica* [93, 94], *S. aureus* strains [95], nodule formation of *R. meliloti* and tumor inducing ability of *A. tumefaciens* [90]. Plasmid elimination with varying frequencies can be explained by the diverse sensitivity of different bacterial species.

The inhibition of plasmid DNA replication was found to be associated with drug binding to the bacterial membrane, which changes the stability of the complex of membrane proteins with the replicating plasmid DNA, resulting in the prevention of proper plasmid replication, leading to plasmid loss [93]. The other possible target of phenothiazines is the DNA gyrase. It was assumed that drugs interfere with DNA gyrase activity and promote the relaxation of plasmid DNA to disturb plasmid replication processes.

The action of promethazine on plasmid DNA disturbs gene expression and replication leading to plasmid loss or even cell death. The heterocylic planar ring system of promethazine demonstrates high affinity binding to the covalently closed circle (CCC) form of plasmid DNA, while linear and circular forms were less sensitive as demonstrated by agarose gel electrophoresis of promethazine treated samples [96]. Recently the binding target of promethazine was defined by Miskolci *et al*. They found that promethazine may form a complex with the Guanosine-Citosine rich region of plasmid DNA. The complex formation is based on the ionic interaction between the linker of phenothiazine and the phosphate group of DNA, and stacking interaction is formed between aromatic sites of promethazine and Guanosine [97].

Antiplasmid compounds are also able to block plasmid transfer. This was proven by the inhibition of binding of the pilus-specific phages [98].

More phenothiazine derivatives were produced by chemical modification in order to find ideal plasmid curing compound. It was demonstrated that distruption of the π-electron system of the three rings significantly decreases the antiplasmid effect. Structure–activity relationship

(SAR) studies revealed that changes in molecular structures of tricyclics lead to altered antiplasmid action [99].

The mechanism by which the phenothiazines exert their effective antibacterial and antiplasmid action is still not completely explored. Hypothetically, elimination of plasmids from multiresistant bacteria results in plasmid free cells, which revert to sensitive to conventional antibiotics. Until now, the tested antiplasmid agents could eliminate plasmids only from a part of the bacterial population. If the curing efficiency does not reach 100%, the entire population becomes resistant again due to conjugational retransfer processes. Although phenothiazines have a strong antibacterial effect and are capable of enhancing the activity of antibiotics, the treatment of bacterial infections was not introduced to clinical practice as the *in vitro* activities take place well beyond the clinically achievable concentrations and has side effects.

1.4.2. Resistance reversal by inhibition of efflux pumps

The efflux pump mediated MDR has become a significant complicating factor in the chemotherapy of bacterial infections. MDR efflux pumps are responsible for extruding the structurally unrelated drugs from the cells, by which bacteria can evade the antimicrobial effect of the drug[100, 101, 102]. In Gram-positive and Gram-negative bacteria, the most extensively studied MDR pumps are NorA (MFS) in *S. aureus*, and MexAB-OprM (RND) in *P. aeuginosa*[103, 104]. Both types of efflux pump systems use the proton motive force (PMF) as the energy source for drug translocation. Bacterial genome sequencing projects showed the existence of several genes encoding putative efflux pumps. Since these pumps can complicate the treatment of bacterial infections, the development of safe and effective bacterial efflux pump inhibitors is needed. Novel inhibitors of the MexAB-OprM efflux system and the NorA pump have been identified and also been examined in *in vitro* studies[105, 106].

Inhibition of the efflux pumps can restore the activities of the antimicrobial agents. Since the search for new candidates for pump inhibitors is time and cost consuming, it is reasonable to screen drugs, which are already in clinical use, since data on their pharmacokinetics and toxicity are available. Combination of phenothiazines in subinhibitory concentrations with multiple standard antibiotics commonly result in synergistic or additive effect against several clinically relevant bacterial species (*E. coli* and *P. aeruginosa*) *in vitro*, as demonstrated by checkerboard microdilution experiments[107, 108, 109, 110]. Clinical doses of promethazine in combination with gentamycin showed fewer relapses of pyelonephritis caused by *E. coli* in pediatric patients as compared to the antibiotic treatment alone. Explanation for the successful

clinical trial is the *in vivo* synergistic action of the drugs as the administration of promethazine increased the permeability of *E. coli* cells to the antibiotic[111]. These findings suggest that clinically useful co-administration of resistance modifiers with certain antibiotics could represent new perspectives in combating antimicrobial resistance[112].

The possible mechanism by which phenothiazines potentiate the activity of the other antimicrobial agent is not clarified yet. It is supposed that the enhancement of the antimicrobial activity is associated with the block of the efflux pumps.

Thioridazine and prochlorperazine were shown to be potent inhibitors of NorA of *S. aureus* in checkerboard analysis, when combined with selected pump substrates. It was suggested that phenothiazines can physically interact with the efflux pumps, which result in the inhibition of the activity of the efflux pumps.

Chlorpromazine was shown to block potassium flux across the membrane both in *S. aureus* and in the yeast *Saccharomyces cerevisiae* and to alter the transmembrane potential in *Leismania donovani*[113, 114, 115]. In addition to the possible interaction with the pumps, the perturbation of membrane energetics by phenothiazines may also play a role in efflux pump inhibition. This effect results in the decrease of the PMF, upon which drug-proton antiporters are dependent for their function.

Unfortunately, the effective values for efflux pump inhibition are above those employed in clinical practice. Even so, chemical modification of the *in vitro* potent molecule may result in a compound with reduced central nervous system toxicity and improved bacterial efflux pump inhibitory action.

1.5. Drug resistance and its reversal in cancer cells

Resistance to cytotoxic drugs is the major reason for the failure of cancer treatment. Some cancers are intrinsically resistant to chemotherapy. Others initially respond to the treatment, but in course of time they acquire resistance to many different cytotoxic drugs with different chemical structure and mode of action. It is termed as MDR [116, 117, 118]. MDR appears to be the main reason for therapeutic failure of cancer as the combination of multiple agents with different targets is used to treat most cancers [119]. Several host factors, genetic and epigenetic alterations in the cancer cells, and alterations of the tumor environment influence the outcome of patient's response to anticancer therapy [120, 121]. Many different mechanisms of MDR have been elucidated, including alterations in cell cycle checkpoints, failure in apoptosis (mutated, non- functional p53, that is not able to trigger apoptosis) [122], repair of the damaged cellular targets (activation of detoxifying systems: cytochrome p450) and reduced drug accumulation

[123]. Of these mechanisms, reduced drug accumulation has been most studied in detail. Decreased drug influx or increased drug efflux or both can be responsible for maintaining intracellular drug concentration at a low level.

Hydrophobic drugs like vinblastine, vincristine, doxorubicin, daunorubicin, actinomycin D, etoposide, teniposide and paclitaxel can cross the membrane by simple diffusion. The only way to remove hydrophobic drugs is to expel them by the energy dependent transport systems *e.g.* ATP Binding Cassette (ABC) transporters (Figure 5). The main ABC transporter involved in MDR phenotype of human cancer is P-gp [124, 125]. Structurally P-gp, the product of the human *MDR1* gene is a 170 kDa molecular weight phosphoglycoprotein, consisting of 1280 amino acids forming two ATP binding cassettes and two transmembrane regions each containing six transmembrane domains [126]. The binding of the hydrophobic drug to the transmembrane region stimulates the ATPase activity of P-gp, which releases the substrate to either side of the membrane. The second ATP is needed to restore the drug binding conformation of the molecule [127]. P-gp is widely expressed in tissues with barrier function (epithelia of the liver, kidney, gastro intestinal tract , capillary endothelial cells of brain, testis and ovary), cancers of the gastrointestinal tract, the hematopoietic system, the genitourinary system and various childhood cancers [116, 128, 129, 130, 131, 132]. It was indicated that overexpression of P-gp predicts a poor response to chemotherapy with drugs that are transported by P-gp. P-gp can extrude a large variety of hydrophobic and amphipatic drugs including taxanes, vinca alkaloids, anthracyclines, epipodophyllotoxins, topotecan, and mitomycin. In addition to cytotoxic drugs, P-gp can extrude several exogenous compounds such as opiates, digoxin and polycyclic aromatic hydrocarbons [125].

Not all MDR cancers express P-gp, as another ABC family member called as MRP1 (Multidrug Resistance Associated Protein 1), or ABCC1 was also shown to have a broad spectrum of anticancer drug transport activity [133]. MRP1 is expressed in many tissues and at high levels in many cancers, especially leukemias, esophageal carcinomas and non-small-lung cancer. Unlike P-gp, MRP1 transports negatively charged natural drugs and drugs conjugated with glutathione or modified by glycosilation, sulfation and glucuronylation. Eight additional members of the ABCC family were discovered named MRP2 to 7. The third member of the ABC transporter superfamily is the ABCG2, also known as MXR; BCRP; ABC-P. It confers high resistance to hydrophobic anticancer drugs similar to P-gp and MRP1, including doxorubicin, daunorubicin, mitoxantrone and topotecan [134]. Other ABC family members are associated with drug resistance. For instance MDR3, sometimes called as MDR2, a phosphatidilcholine flippase, which normally transports phospholipids into the bile, but can

transport paclitaxel and vinblastine as well [135]. It has been shown that P-gp and MRP1 have a significant role in clinical resistance. Many studies strongly suggest that the high expression rate of P-gp correlates well with drug resistance in several cancers. It should be noted that MDR can be multifactorial, but in most cases it is linked to the overexpression of P-gp.

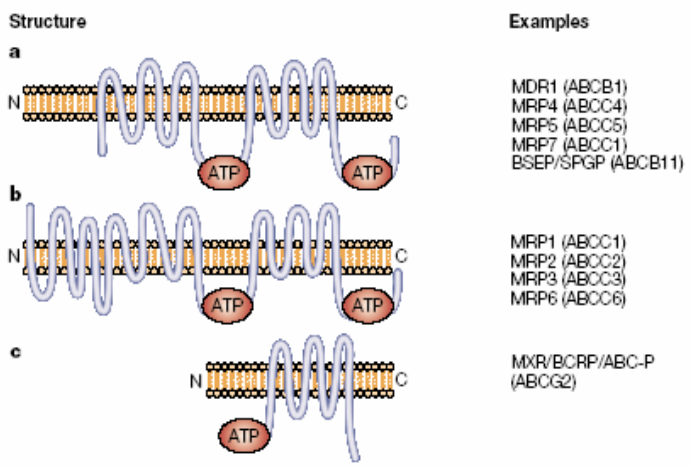

Figure 5. Structure of ABC transporters. **a:** MDR1 and MRP4 have 12 transmembrane domains and 2 ATP binding sites. **b:** MRP 1,2,3 and 6 contain 17 transmembrane domains and 2 ATP binding sites. **c:** ABCG2 contains 6 transmembrane domains and 1 ATP binding site[117].

Inhibition of P-gp is a way of reversing MDR as has been extensively studied. Several classes of MDR modulators were found: calcium channel blockers, calmoduline antagonists, cyclosporins, quinolones, anti-estrogens, HIV protease inhibitors, GF120918, *etc*. These agents appear to bind directly to the P-gp and thereby block the outward transport of the cytostatics [136, 137]. Several phenothiazines reversing bacterial resistance were proven to be also effective in resistance modification of tumor cells. The intracellular target site of phenothiazines is still not identified[138, 139, 140, 141, 142].

II. AIMS OF THE THESIS

I. One of the greatest medical concerns is the emergence of antibiotic-resistant infections and chemotherapy-resistant malignant tumors through the expression of multidrug resistance (MDR). We investigated the reversal of bacterial antibiotic resistance by phenothiazines, benzoxazoles, benzimidazols and oxazolo(4,5)pyridines in search of possible modifiers of antibiotic resistance.

1. First of all the antimicrobial effect of test compounds was determined on *E. coli* K12LE140 strain.
2. In search for effective antiplasmid compounds, various phenothiazines, benzoxazoles, benzimidazoles and oxazolo(4,5)pyridines were tested in sub-Minimum Inhibitory Concentration (MIC) on *E. coli* K12LE140 strain carrying F'lac plasmid.
3. The enhancement of antibiotic activity was tested by the combination of antibiotics and resistance reversal agents (thioridazine or prochlorperazine) on ampicillin - and vancomycin-resistant clinical isolates of *E. faecalis* strains.

II. A co-culture involving two bacterial species model was created to simulate communities of multi-species bacteria as situated in nature to examine the interaction of bacterial species influencing growth rate, viability and plasmid stability under various ecological conditions.

1. First, the growth rate of each bacterial species was analyzed in mixed culture models.
2. The antiplasmid effect of promethazine was tested in mixed culture, when *E. coli* K12LE140 strain was inoculated into the culture of numerically predominant bacterial species *B. cereus*, *S. epidermidis* or *B. fragilis* ATCC 25285.
3. Various ecological conditions were applied to investigate the influence on promethazine-induced plasmid elimination:
    a) different atmospheric conditions such as aerobe, anaerobe atmosphere and aeration created by shaking of the cultures.
    b) various temperature of incubation such as 23°C, 37°C or 39°C, respectively.

III. Based on the similarities of drug resistance in prokaryotes and eukaryotes, MDR efflux pump systems were studied in bacteria and cancer cells.

1. A possible role of proton pumps in the efflux pump system was investigated for a bacterial motion in which flagellar motility is driven by proton motive force (PMF). The effect of a novel proton pump inhibitor TF18 (trifluoro-methyl-ketone derivative) with a known antimicrobial effect was investigated for its antimotility action on two *E.*

coli strains. The combined antimicrobial effect of TF18 and clarithromycin was studied on two *H. pylori* strains.

2. As a model of ATP-Binding Cassette (ABC) transporters, the P-glycoprotein (P-gp)-based MDR was targeted by phenothiazines and cycloartanes as possible resistance reversals. Human *MDR1* gene transfected mouse lymphoma cell line and its drug sensitive subline were used as model systems.
3. Finally a possible correlation was studied between the inhibition of ABC transporters of amino alkyl substituted and benzo[a]phenothiazines and the calculated Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) energies.

III. MATERIALS AND METHODS

3.1. BACTERIAL STRAINS, CANCER CELL LINES, MEDIA AND CHEMICALS

3.1.1. Bacterial strains

3.1.1.1. Laboratory strains

- *Bacillus cereus* W12 was derived from the stock collection of the Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged.
- *Bacteroides fragilis* ATCC 25285 was provided by Dr. Edit Urbán (Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged).
- *Escherichia coli* K12LE140 (T_6^R, T_1^S, Sm^R, lac△, Su^-, λ^R, Mal^-) carrying the F'lac plasmid was kindly provided by Erzsébet Magyaródi (Department of Biochemistry, Biological Research Centre, Szeged).
- *E. coli* AG100 strain containing proton pump system and its proton pump deficient strain *E. coli* AG100A were kindly provided by Professor Hiroshi Nikaido (University of California, Berkley, USA)
- Clarithromycin susceptible *Helicobacter pylori* ATCC 700392 (CSHP) and clarithromycin resistant *H. pylori* 9447 (CRHP) were obtained from Professor Yvette Mándi (Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged).

3.1.1.2. Clinical isolates

- Strains of *Enterococcus faecalis* (A, C, D and E) were obtained from Sønderborg, Odense and Statens Serum Institut, Copenhagen, Denmark.
- *S. epidermidis* isolate was kindly provided by Dr. Edit Hajdú (Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged).

3.1.2. Cancer cell lines

The multidrug-resistant L5178 mouse T cell lyphoma cell line transfected by human *MDR1* gene and its drug sensitive subline L5178Y (PAR) were provided by Professor Adorján Aszalós (FDA, Washington, USA).

3.1.3. Growth media for bacteria

- Brain Heart Infusion (BHI) broth was applied for culturing of *H. pylori* and *B. fragilis* strains.

- Eosine-Methylene Blue (EMB) agar plate was used to differentiate between the plasmidless (Lac-) and plasmid containing (Lac+) colonies of the F'lac plasmid carrying strain of *E. coli* K12LE140.
- Hollmann's medium was used for maintaince of *B. fragilis* ATCC 25285 strain.
- Minimal-Trypton-Yeast extract (MTY) [143] nutrient broth was used for overnight culturing. The agar plates were used to determine viable counts of *E. coli, B. cereus, S. epidermidis* strains.
- Blood Agar plate: Columbia agar was supplemented by 5% sterile defibrinated sheep blood and was used for the determination of viable counts of different bacterial strains cultured under microaerophil or anaerobe conditions and for maintaince of clinical isolates of *E. faecalis* strains under aerobe conditions. For culturing the clarithromycin resistant *H. pylori* 9447 (CRHP) strain, the medium was supplemented with 2 mg/ml clarythromycin.
- Tryptic Soy Broth was used in checkerboard assay for culturing *E. faecalis* strains.
- All organisms were grown at 37°C with aeration except *H. pylori* and *B. fragilis* strains. Plates and liquid cultures containing *B. fragilis* were incubated for 48 hours under anaerobic conditions created by gas packs (bioMérieux, GENbox anaer) in anaerobic jar (Oxoid). Cultures of *H. pylori* strains were grown for 72 hours in microaerofilic atmosphere created by gas packs (bioMérieux, GENbox microaer) in anaerobic jar (Oxoid).

3.1.4. Culture media for cancer cells

MDR and PAR mouse T lymphoma cell lines were grown in McCoy's 5A medium supplemented with 10% heat inactivated horse serum and L-Glutamine and antibiotics. Further details of the medium are found in Annex II. The *MDR1* gene expressing cell line was selected by 60 ng/ml colhicine to maitain the MDR phenotype.

3.1.5. Chemicals

Phenothiazine derivatives:

Chlorpromazine, 6,9-dioxochlorpromazine, 7,8-dioxochlorpromazine, 6,9-dihydroxichlorpromazine, 5-oxo-5-H-benzo[a]phenothiazine, 6-hydroxi-5-oxo-5H-benzo[a]phenothiazine, 6-methyl-5-oxo-5H-benzo[a]phenothiazine 2-chloro-5-oxo-5H-phenothiazine were synthesized as described previously [144, 145]. Prochlorperazine, perphenazine, trifluperazine and thioridazine were purchased from Sigma Aldrich Co. and

promethazine was obtained from EGIS Works, Budapest. Perazine dimaleate was provided by Professor Masami Kawase, Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan. All solutions were prepared in sterile distilled water on the day of the experiment.

Benzoxazole derivatives:

6-methyl-2-(2-nitrophenyl)-1,3-benzoxazole (A9); 2-(4-fluorophenyl)-5-methyl-1,3-benzoxazole (A33); 2-(4-nitrobenzyl)-1,3-benzoxazole (B11); *N*-[2-(4-fluorobenzyl)-1,3-benzoxazol-5-yl]-2-phenoxyacetamide (D27); 2-(4-chlorophenoxy)-*N*-[2-(4-fluorobenzyl)-1,3-benzoxazol-5-yl]acetamide (D34); 2-(4-bromobenzyl)-5-methyl-1*H*-benzimidazole (G17); 2-(4-ethylphenyl)[1,3]oxazolo[4,5-*b*]pyridine (F1) [146, 147, 148]. Every compound was synthesized and provided by Professor Ismail Yalçin and Professor Esin Aki Şener, Faculty of Pharmacy, Ankara University, Turkey. All compounds were dissolved in DMSO.

Cycloartane triterpenes:

9,19-cycloanostane-3β24,25-triol (1); 9,19-cycloanostane-3β,26-diol (2); 3β-hydroxy-9,19-cycloanost-25-en-24-one (3); 9,19-cycloanost-23-ene-3β,25-diol (4); 24-hydroperoxy-9,19-cyloanost-25-en-3β-ol (5); 9,19-cycloanost-25-ene-3β,24-diol (6); 3β-hydroxy-4,4,14-trimethyl-26-nor-9,19-cyclolanostan-25-one (7); (*22E*)-3β-hydroxy-4,4,14-trimethyl-9,19-cyclochol-22-en-24-al (8); 3β-hydroxy-4,4,14-trimethyl-9,19-cyclocholan-24-al (9); 9,19-cycloanost-25-ene-3β,24-diacetate (10); 24-methylene-9,19-cyclolanostan-3β–ol (11); 3β-acetoxy-9,19-cycloanost-25-en-24-one (12); 3β-acetoxy-9,19-cyclolanost-23-en-25-ol (13); 9,19-cyclolanost-24-en-3β-acetate (14); 24-methylene-9,19-cyclolanostan-3β-acetate (15). Compounds 10, 12 ,13-15 were isolated from the acetone extract of *Euphorbia segetalis* L. Compounds 1-9 and 11 were isolated from the acetone extract of *Euphorbia portlandica* L. and identified by stereoscopic methods [149]. The purity of the compounds was more than 95% by HPLC analysis. Every extract was prepared and provided by Professor Maria José Umbellino Ferreira, Centro de Estudos de Ciencias Farmaceuticas, Faculdade de Farmacia da Universidade de Lisboa, Portugal. All compounds were dissolved in DMSO.

Trifluoromethyl ketone derivate:

1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone (TF18) was previously synthesized [150] and provided by Professor Masami Kawase, Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan. Stock solution was prepared in DMSO. Further dilutions were made in physiological saline.

Chemical structures of the test compounds are found in Annex I.

3.2. METHODS

3.2.1. F'lac plasmid elimination from E. coli K12LE140 in monocultures

From an overnight preculture of *E. coli* K12LE140 1 ml aliquot was added to 100 ml MTY broth, then it was separated into test tubes by 5 ml. Cultures were incubated in the presence of increasing concentration of the drug (0-120 μg/ml) for 24 hours at 23°C /37°C /39 °C. The samples showing growth were diluted and 0.1 ml from each dilution was spread onto EMB agar plates. After overnight incubation at 37°C the Lac+ (deep purple) and Lac- (pink) colonies were counted in order to calculate the plasmid curing efficiency expressed in percentage [96].

3.2.2. F'lac plasmid elimination from E. coli K12LE140 in mixed bacterial cultures

A 24 hour culture of *E. coli* K12LE140 was diluted to 10^4 and 1 ml aliquot was inoculated into 100 ml of a 24 hour culture of *B. cereus* or *S. epidermidis*. In case of anaerobe culture conditions, 5 ml of the 72 hour preculture of *B. fragilis* ATCC 25285, grown in BHI broth was inoculated by 0.05 ml aliquots of the 10^4 diluted 24 hour preculture of *E. coli.* These initial mixed cultures contained 10^7-10^8 cfu/ml *B. cereus or S. epidermidis* or *B. fragilis* ATCC 25285 and 10^2 cfu/ml of *E. coli*, respectively, as shown by colony forming units. Varying concentrations of promethazine (0-120 μg/ml) were added to 5 ml aliquots of each mixed culture and incubated at 23°C, 37°C, 39°C for 24 hours with (100 rpm) and without shaking under aerobe conditions. The mixed cultures containing *E. coli* and *B. fragilis* were grown for 24 hours, without shaking, under anaerobe atmosphere using an anaerobic jar and gas packs. Dilutions ranging from 10^2-10^5 were made from tubes showing growth and aliquots of 0.1 ml of these cultures plated on EMB and MTY agar. The plates were incubated for 24 hours, at 37°C. 0.1 ml of samples containing anaerobe bacteria were placed also onto Columbia blood agar plates and were incubated under anaerobe conditions for 72 hours at 37°C. The morphology of the colonies was used to differentiate between different bacterial species. The Lac+ plasmid containing and Lac – plasmidless colonies were counted on EMB agar and percentage of plasmid elimination was determined.

3.2.3. Co-culture experiments

These experiments were carried out as follows: a low number of *E. coli* K12 LE140 (10^2 cfu/ml) was inoculated into the 24 hour pre-culture of either *B. cereus* or *S. epidermidis* (10^8

cfu/ml), and plasmid elimination and growth rates were determined simultaneously on every third day for a total of 9 days. Plate count method was applied to determine the viable counts of each bacterial species of the mixed culture on selective media (EMB and MTY agar plates) at times indicated.

3.2.4. Checkerboard method for elucidation of combined antimicrobial effect

The checkerboard method established by Eliopoulos G. M. and Moellering R. C. is a technique to assess antibacterial combinations *in vitro* [151]. The microdilution checkerboard method was used to determine the MICs for a certain antibiotic and non antibiotic type agents separately and in combination on Enterococcal strains (*E. faecalis* A, C, D and E) and *H. pylori* strains (CRHP and CSHP). Description can be found in Hendricks O. *et al. In vitro* activity of phenothiazine derivatives on *Enterococcus faecalis* and *Enterococcus faecium. Basic Clinical Pharmacol. Toxicol.* 2005; 96(1): 33-36 and Spengler G. *et al*. Inhibitory action of a new proton pump inhibitor, trifluoromethyl ketone derivative, against the motility of clarithromycin-susceptible and-resistant *Helicobacter pylori*. *Int. J. Antimicrob. Agents* 2004; 23: 631-633.

3.2.5. Antimotility assay

Description can be found in Molnár A. *et al*. Effect of a trifluoromethyl ketone on the motility of proton pump deleted mutant of *Escherichia coli* strain and its wild type. *In Vivo* 2004; 18(4): 505-509 and Spengler G. *et al*. Inhibitory action of a new proton pump inhibitor, trifluoromethyl ketone derivative, against the motility of clarithromycin-susceptible and-resistant *Helicobacter pylori*. *Int. J. Antimicrob. Agents* 2004; 23: 631-633.

3.2.6. Rhodamine 123 uptake assay

Description can be found in Reference 152, 153 and 154.

3.2.7. HOMO and LUMO energy calculations

Geometry optimization to find the individual energy minimum of the starting structures was performed by AM1 semiempirical method. After minimiziation procedures, the Lowest Unoccupied Molecular Orbital energies (LUMO) related to the electron affinity of the molecule and the Highest Occupied Molecular Orbital energies (HOMO) related to the ionization potential of the molecule were calculated by HyperChem 6.06 version program.

IV. RESULTS

4.1. The effect of promethazine and structurally related phenothiazines on F'lac plasmid elimination

E. coli K12 LE140 strain carrying the F'lac plasmid was used to study the antiplasmid effect of certain test compounds. The curing of the F'lac plasmid encoding lactose fermentation serves as a convenient model to test the antiplasmid effect of test compounds. The plasmid loss was detected on EMB agar plate that permits the distinction between plasmidless; Lac- (with colourless to pink colouration) and plasmid-containing; Lac+ (with metallic purple colouration) colonies (Figure 6). As promethazine has one of the most potent effects on plasmid elimination, in our experimental settings promethazine treated samples were used as controls.

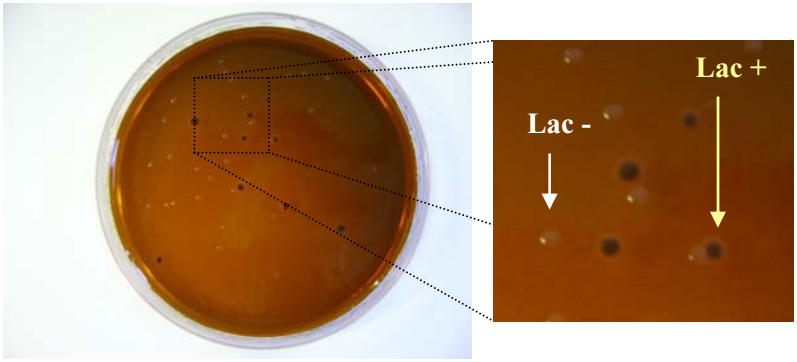

Figure 6. **Lac +** and **Lac-** colonies of promethazine treated *E. coli* K12LE140 strain on EMB agar plate.

4.1.1. Antiplasmid activity of some phenothiazine derivatives

Based on previous data with promethazine and its structurally related compounds, some phenothiazine derivatives (Perazine dimaleate, Perphenazine, 2-chloro-5-oxo-5H-phenothiazine) were tested for elimination of F'lac plasmid (Table 1). Only one compound, perazine dimaleate eliminated the F'lac plasmid of the *E. coli* strain with the extent of 27.27%, whereas the other compounds showed only slight effect in a range between 0.00-10.77%. Detailed data are presented in Table 6. in Annex III.

Table 1. F'lac plasmid curing effect of some phenothiazine derivatives on *E. coli* K12 LE140 strain.

Sample	Concentration (µg/ml)	Number of colony formers ($x10^7$ cfu/ml)	Ratio of plasmid curing (mean %)
Perazine dimaleate	20	40.92	0.002
	40	20.50	0.00
	60	19.60	0.23
	80	12.50	0.04
	100	0.001	*27.27*

4.1.2. Combined effect of promethazine and some subtituted benzoxazoles, benzimidazoles and oxazolo(4,5)pyridines on F'lac plasmid loss

It was earlier published that benzoxazoles, benzimidazoles and oxazolo(4,5)pyridines have potent antimicrobial effect against *K. pneumoniae*, *P. aeruginosa* and the yeast *C. albicans*[146, 147, 148]. In search for novel antiplasmid agents several benzoxazole derivatives (A9, A33, B11, D27, D34) benzimidazoles (G17) and oxazolo(4,5)pridine (F1) were tested on their influence on F'lac plasmid loss. Only the compound G17 was able to eliminate the F'lac plasmid with the frequency of 28.00% at 40 μg/ml concentration of the drug. Agents A33, B11, D27 and F1 did not lead to remarkable plasmid loss (0.00%-0.15%). Compounds A9, D34 and F1 were ineffective in plasmid curing (0.00%). In order to modify the antiplasmid activity of the compounds with moderate effect, they were combined with promethazine (Table 2). However, only the combination of promethazine with compound B11 showed an enhanced antiplasmid effect (89.40%) as compared to control samples.

Table 2. F'lac plasmid elimination of various benzoxazole derivatives and benzimidazoles in combination with promethazine on *E. coli* K12LE140 strain.

Samples	Concentration (μg/ml)	Number of colony formers ($x10^7$ cfu/ml)	Ratio of plasmid curing (mean %)
Control (untreated)	0	27.60	0.16
Promethazine	20	21.70	0.32
	40	8.16	5.22
	60	3.70	11.04
	80	1.01	***73.82***
	100	**MIC**	**-**
Promethazine+A33	20+10	37.00	0.03
	40+10	24.00	0.30
	60+10	12.00	4.35
	80+10	3.30	***10.15***
	100+10	0.97	0.00
	120+10	**MIC**	-
Promethazine+B11	20+10	23.00	0.20
	40+10	16.00	3.50
	60+10	5.40	26.70
	80+10	2.40	***89.40***
	100+10	**MIC**	-
Promethazine+D27	20+10	21.00	0.00
	40+10	12.00	0.40
	60+10	7.30	21.90
	80+10	3.30	***60.40***
	100+10	1.50	36.40
	120+10	0.75	1.30
Promethazine+G17	20+10	8.40	1.20
	40+10	6.80	32.00
	60+10	7.00	19.50
	80+10	5.20	***42.60***
	100+10	**MIC**	-

Legend: Benzoxazole derivatives were added in final volume of 10 μg/ml to the samples with increasing concentration of promethazine.

4.2. Antibiotic resistance reversal on clinical isolates of human pathogen E. faecalis strains

The results described above on the antiplasmid effect of the compounds suggest that elimination of plasmids is rather a complex approach. Since no ideal and universal plasmid curing agent was found up to now with efficiency of 100 %, further study is expected to design more effective antiplasmid agents. As microbes become increasingly resistant to administered antibiotics and to several drugs simultaneosly in many cases, the search is on to find new startegies to overcome antibiotic resistance. Basically there are two opportunities to combat antibiotic resistance: **1.** to find new antibacterial compounds and **2.** to use inhibitors in various steps of resistance mechanisms (resistance modifiers) to potentiate the effect of the existing antibiotics. Considering these possibilities, we decided to test the combination of phenothiazine derivatives, thioridazine (THIO) and prochlorperazine (PCP) as resistance modifiers with antibiotics, vancomycin (VAN) and ampicillin (AMP) on reversal of resistance in clinical isolates of the Gram-positive species; *E. faecalis* strains. Results are summerized by Table 3.

4.2.1. Combined effect of vancomycin with resistance modifiers on E. faecalis strains

The results obtained in the checkerboard test indicate that subinhibitory concentrations of VAN and THIO applied together had a synergystic effect on vancomycin-resistant clinical isolates of *E. faecalis* strain A and D. The susceptibility of strains A and D to VAN significanly increased in the presence of different concentartions of THIO (2 and 4 μg/ml, respectively) at the presence of subinhibitory concentration of VAN. The reduction of resistance to VAN from 192 μg/ml to 6 μg/ml was the most pronounced in the case of strain A. In case of strain C and E, only an additive effect was detected without enhanced reduction of resistance to VAN. VAN in combination with PCP showed only an additive effect on the tested enterococcal strains.

4.2.2. Combined effect of ampicillin with resistance modifiers on E. faecalis strains

Enterococcal strains A, C and E were also resistant to ampicillin and the effect of THIO and PCP on AMP resistance was also studied. In checkerboard titration studies only strains C and D demonstrated synergy when ampicillin was combined with THIO. Indifferent effect was observed in the case of strain A when AMP was combined with PCP. All other antimicrobial combinations resulted in additive effect.

Table 3. Combined effect of antibiotics and resistance modifiers in checkerboard assay on human isolates of *E. faecalis* strains.

Strains	Antibiotics + resistance modifiers	MIC (μg/ml)	Value of FIX	Type of interaction
A	Prochlorperazine (PCP)	32		
	Thioridazine (THIO)	32		
	Ampicillin (AMP)	32		
	Ampicillin (AMP) + PCP	AMP (32) + PCP (32)	2.00	Indifferent
	+ THIO	AMP (1) + THIO (16)	0.53	Additive
	Vancomycin (VAN)	192		
	Vancomycin (VAN) + PCP	VAN (96) + PCP (4)	0.63	Additive
	+ THIO	VAN (6) + THIO (2)	0.09	***Synergy***
C	Prochlorperazine (PCP)	32		
	Thioridazine (THIO)	32		
	Ampicillin (AMP)	16		
	Ampicillin (AMP) + PCP	AMP (8) + PCP (0.5)	0.51	Additive
	+ THIO	AMP (4) + THIO (4)	0.38	***Synergy***
	Vancomycin (VAN)	128		
	Vancomycin (VAN) + PCP	VAN (64) + PCP (4)	0.625	Additive
	+ THIO	VAN (64) + THIO (4)	0.625	Additive
D	Prochlorperazine (PCP)	32		
	Thioridazine (THIO)	32		
	Ampicillin (AMP)	1		
	Ampicillin (AMP) + PCP	AMP (0.25) + PCP (8)	0.5	Additive
	+ THIO	AMP (0.25) + THIO (4)	0.38	***Synergy***
	Vancomycin (VAN)	500		
	Vancomycin (VAN) + PCP	VAN (31.3) + PCP (16)	0.563	Additive
	+ THIO	VAN (125) + THIO (4)	0.375	***Synergy***
E	Prochlorperazine (PCP)	32		
	Thioridazine (THIO)	32		
	Ampicillin (AMP)	128		
	Ampicillin (AMP) + PCP	AMP (64) + PCP (16)	1.00	Additive
	+ THIO	AMP (16) + THIO (16)	0.63	Additive
	Vancomycin (VAN)	2		
	Vancomycin (VAN) + PCP	VAN (0.5) + PCP (16)	0.75	Additive
	+ THIO	VAN (1) + THIO (2)	0.56	Additive

4.3. Growth dynamics of multiple component communities of bacteria

4.3.1. The role of interactions on F'lac plasmid loss in the absence of antiplasmid agent in mixed cultures involving two bacterial species

The study was designed to observe the arising interactions between the plasmid containing newcomers and the numerically predominant inhabitants of the microbial community in regard to plasmid stability in long term incubation time at various temperatures. Presented data are the average of three independent experiments and the error bars represent standard deviations. Dateiled data are presented in Table 7. and 8. in Annex III.

4.3.1.1. Interactions in co-cultures of S. epidermidis and E. coli K12LE140 strain

After inoculation of a small number (3.1 x 10^2 CFU/ml) of the F'lac plasmid containing *E. coli* K12LE140 into the well established culture of *S. epidermidis*, continuos and remarkable decline was observed in the viable counts of the initially predominant culture of *S. epidermidis* (1.84 x 10^8 CFU/ml) at 23°C (Figure 7). At 37°C and at 39°C the viable counts of *S. epidermidis* fell down below a detectable level (10^4 CFU/ml) after the third (37°C) and first (39°C) day of the inoculation of *E. coli.* In contrast, viable counts remained in the range of 10^6 -10^8 CFU/ml in the control cultures of *S. epidermidis*. In every case the *E. coli* strain exhibited exponential kinetics and outnumbered the another, originally numerically dominant member of the community.

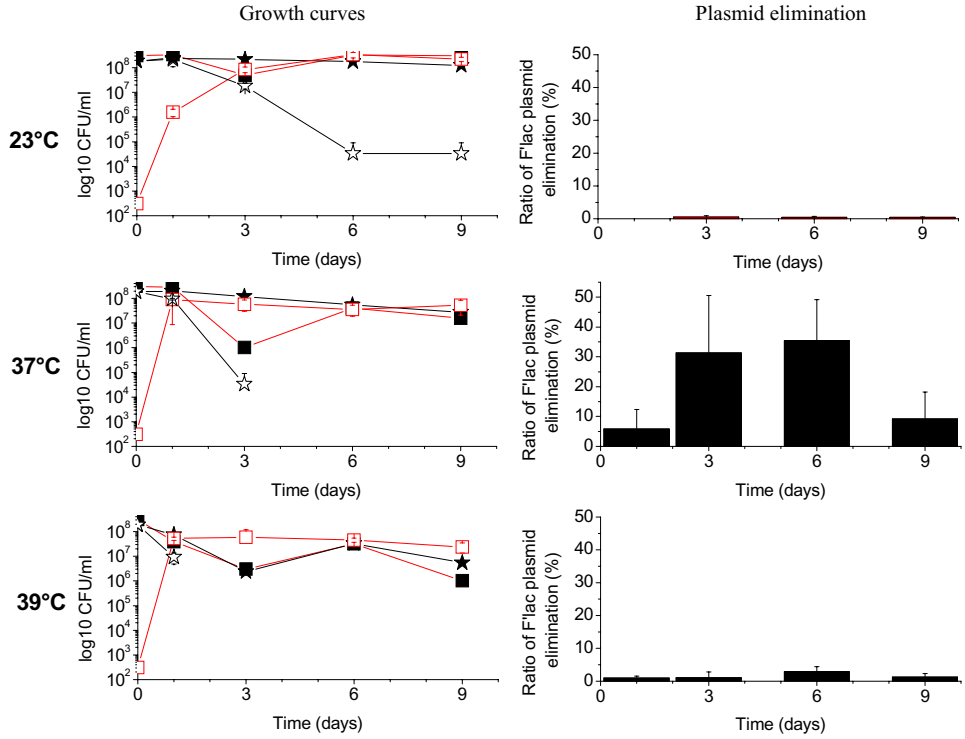

Figure 7. Growth curves of *E. coli* K12LE140 and *S. epidermidis* incubated together and alone and the ratio of F'lac plasmid elimination of *E. coli* K12 LE140 strain in the culture of *S. epidermidis* at various temperatures. Viable counts of *S. epidermidis* incubated in co-culture with (☆) and without (★) the *E. coli* strain. Viable counts of *E. coli* cultured with *S. epidermidis* (□) and without (■).

Regarding the effect of co-culturing on plasmid stability, substantial plasmid loss (13.76% on the third and 19.23% on the ninth day of incubation) was detected only when *E. coli* K12 LE140 was cultured in the presence of *S. epidermidis* at 37°C.

4.3.1.2. Interactions in co-cultures of B. cerus and E. coli K12LE140 strain

Similar experiments were performed in co-cultures with *B. cereus* and the *E. coli* strain, as shown by Figure 8.

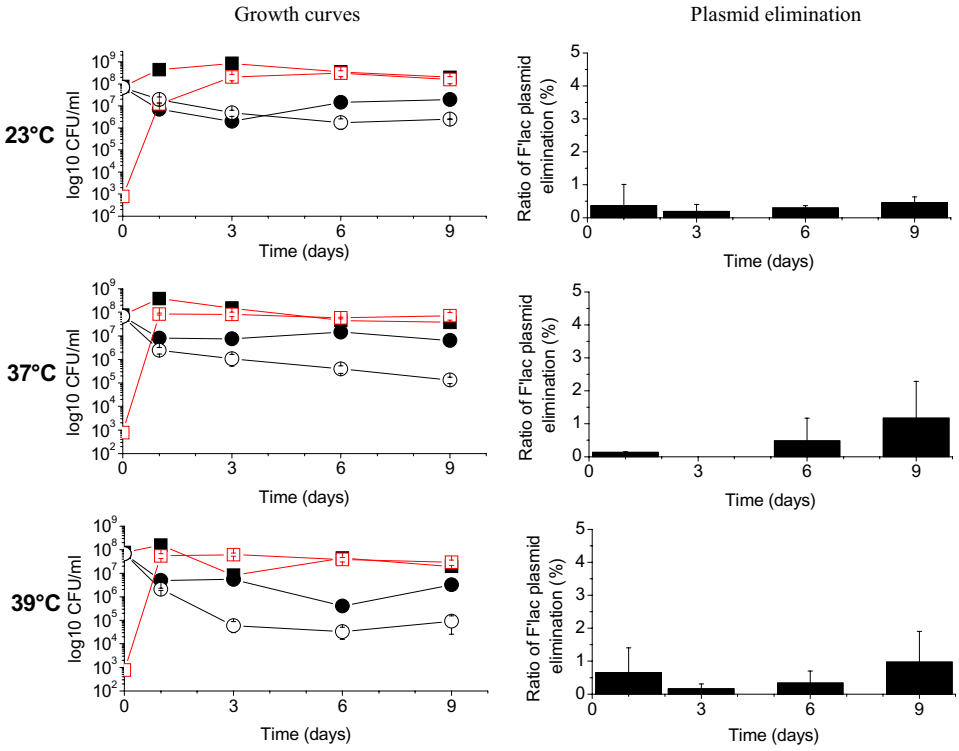

Figure 8. Growth curves of *E. coli* K12LE140 and *B. cereus* incubated together and alone and the ratio of F'lac plasmid elimination of *E. coli* K12 LE140 strain in the culture of *B. cereus* at various temperatures. Viable counts of *B. cereus* incubated in co-culture with (○) and without (●) the *E. coli* strain. Viable counts of *E. coli* cultured with *B. cereus* (□) and without (■).

At the studied temperatures, the *E. coli* strain outnumbered and lowered the survival of *B. cereus*, the originally predominant species of the bacterial consortium. Comparing the growth curves of *B. cereus* in mixed and control cultures, the same trend was observed at 37°C and at 39°C. The detected viable counts of *B. cereus* were lower in the presence of the *E. coli* strain than when cultured alone. In contrast to mixed cultrures with *S. epidermidis*, no marked F'lac plasmid loss was found (in a range between 0.00%-1.11%) in the presence of *B. cereus* at the studied temperatures.

4.4. Model experiments for F'lac plasmid curing by promethazine in mixed bacterial cultures

As a model of infections with one pathogen, the antiplasmid effect of promethazine was studied so far in cultures containing a single bacterial species. Laboratory experiments indicated that in mono-species cultures promethazine reduced or eliminated antibiotic resistance of *E. coli*, several *Salmonella ssp*., *S. aureus*, *Y. enterocolitica*, reduced lactose fermentation of *E. coli*, reduced the tumor inducing ability of *A. tumefaciens* and nodule formation of *R. meliloti*. However, in most natural habitats, bacteria form multi-species communities. This is the case in the densly populated normal florae of the oropharynx, the oral cavity, the gastrointestinal tract, the genitourinary tract and the skin. Also, polymicrobial infections of aerobic/facultative anaerobic and anaerobic bacterial species frequently occur in many clinical settings such as abcess formations and wound infections.

Since bacteria often co-exsist with multiple microbial species in natural environments, we focused to study the antiplasmid effect of promethazine in mixed cultures consisting of two bacterial species. A numerically predominant culture of *B. cereus* or *S. epidermidis* or *B. fragilis* ATCC 25285 was inoculated with a small number of the F'lac plasmid containing *E. coli* K12LE140 strain and the effect of promethazine induced plasmid elimination was studied under various ecological conditions and at three different temperatures (23°C, 37°C and 39°C). Our two component system was designed to simulate the *in vivo* situation where a small number of plasmid-carrying microbial newcomer arrives to an available niche occupied by another member of the microbiota and different concentrations of antiplasmid agent are presumably present. When various bacterial species share resources, the arising metabolic interactions may modulate the antiplasmid effect of promethazine[155]. In theory, the effective plasmid elimination may offer an alternative way to combat plasmid encoded antibiotic resistance.

To test the promethazine-induced F'lac plasmid loss in the presence of another microbial inhabitant, three different bacterial species were chosen. Each of them is typical to the following three ecological niches. *B. cereus* as an obligate aerobic species was involved to simulate external environmental sources as it is widely distributed in soil, dust and air, and may produce foodborne intoxication [156]. *S. epidermidis* as a facultative anaerobic microbe was chosen as it is a common resident of the normal flora of the skin, the gut and the upper respiratory tract, and it is also the major cause of nosocomial infections associated with implantated foreign bodies [157]. Finally, the laboratory strain of *B. fragilis* ATCC 25285 as obligate anaerobe commensal/opportunistic pathogens of human gut was used as it is a

common member of the highly anaerobic environment of the human gastrointestinal tract, and frequently occurs in surgical infections involving multispecies communities of bacteria. [158].

In our studies, three different temperatures (23°C, 37°C and 39°C), aerobic/anaerobic conditions and the effect of shaking were investigated on their influence on plasmid elimination. 23°C represented outside environmental conditions, while 37°C was applied to simulate the normal body temperature. 39°C was used to create the *in vitro* situation characteristic to feverish stages arising during infections.

Presented data values are average of three independent experiments and the error bars represent the standard deviations. Detailed data are presented in Table 9/A-B. and Table 10. in Annex III.

4.4.1. Plasmid elimination by promethazine in mixed bacterial cultures under aerobic conditions

4.4.1.1.Plasmid curing effect of promethazine in co-culture experiments without shaking

The efficiency of plasmid elimination by promethazine was studied in a microbial community containing two bacterial species: a small number of *E. coli* K12LE140 (10^2 cfu/ml) was added to the pre-existing stationary phase culture (10^6-10^8 cfu/ml) of *B. cereus* or *S. epidermidis*, and the effect of temperature without shaking under aerobic condition is presented by Figure 9. Promethazine treated monospecies culture of *E. coli* K12LE140 served as a control.

As summarised by Figure 9., in cultures of plasmid containing *E. coli* growing in the company (mixed culture) of *B. cereus* or *S. epidermidis* that are maintained at room temperature (23°C) with no shaking, there is an extremely low ratio of plasmid elimination with promethazine regardless of its concentration. The frequency of the plasmid elimination that varies from 0.00% to 0.39% is negligible because it is equal to the frequency of spontaneous plasmid loss in the absence of the phenothiazine.

At 37°C, but still maintaining the above cultures without shaking, the presence of promethazine caused the elimination of plasmids in the mono-culture of *E. coli* as well as in the mixed cultures. However, the concentration of promethazine substantially needed to increase plasmid elimination in the mono-culture of *E. coli* is above 60 μg/ml, while plasmid elimination in the mixed cultures takes place with concentrations of the phenothiazine as low as 20 to 40 μg/ml in culture with *B. cereus*. While in the culture of *S. epidermidis*, the rate of plasmid elimination was as high as 87.46% at the concentration of 60 μg/ml. At 39°C the maximum rate of plasmid elimination (97.20%) from the mono-culture of *E. coli* took place at

a concentration of 80 µg/ml of promethazine. With respect to plasmid-containing *E. coli* in mixed cultures either with *B. cereus* or *S. epidermidis* at a temperature of 39°C and no shaking, elimination of F'lac plasmid reached 96.52 % and 97.21 %, respectively. The concentrations are well below the MIC against *E. coli* K12 LE 140.

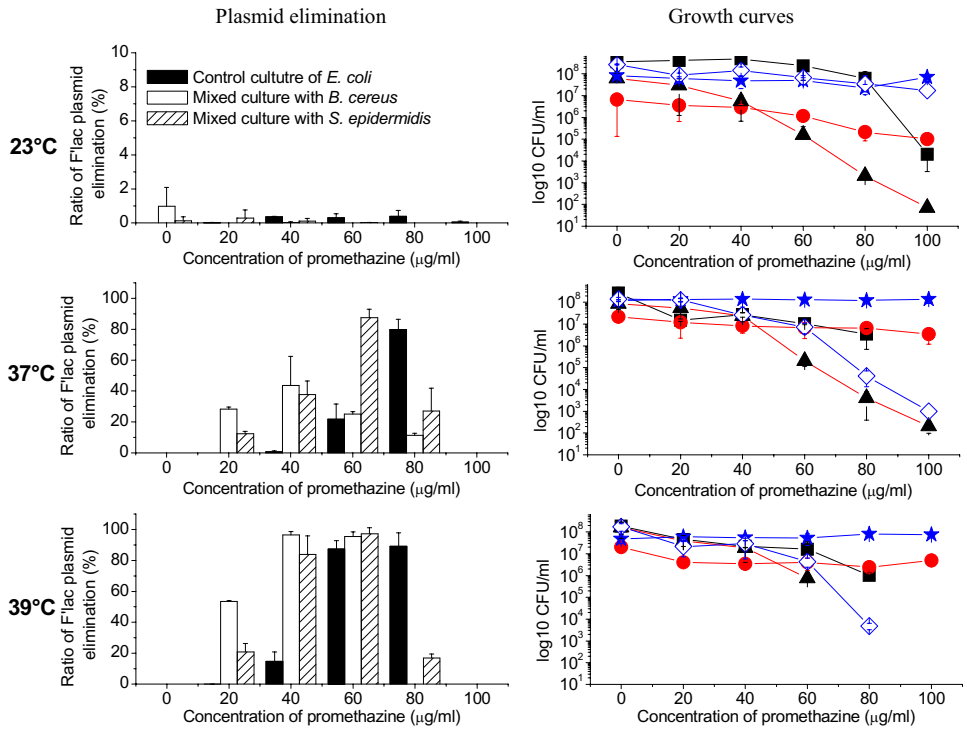

Figure 9. F'lac plasmid elimination of *E. coli* K12LE140 strain co-cultured with *B. cereus* or *S. epidermidis* and growth dynamics of each bacterial species in the presence of promethazine without shaking at various temperatures. Viable counts of *E. coli* in the control culture (■). Viable counts of *E. coli* cultured with *B. cereus* (▲) or *S. epidermidis* (◊). Viable counts of *B. cereus* (●) and *S. epidermidis* (★) cultured with *E. coli*.

4.4.1.2. Plasmid curing effect of promethazine in co-culture experiments with shaking

Furthermore, the effect of temperature and shaking of the mono- and mixed cultures on the elimination of plasmids from *E. coli* K12LE140 by different promethazine concentrations was investigated. In contrast to the data summarised by Figure 9, the shaking of the cultures reduced the plasmid elimination efficiency of the phenothiazine at the respective temperatures of 37°C and 39°C when *E. coli* K12LE140 is in single culture. Shaking of the mixed culture containing *B. cereus* also markedly reduced the F'lac plasmid elimination of promethazine at these temperatures, and to a lesser extent in the mixed culture containing *S. epidermidis.*

4.4.2. Plasmid elimination by promethazine in mixed bacterial cultures under anaerobic conditions

Our simplified model of the densly populated ecosystem of the intestinal tract included a laboratory strain of *B. fragilis* ATCC 25285 as a model for a common obligate anaerobic commensal of the gut microbiota and a small amount of F'lac plasmid containing *E. coli* K12LE140 strain as model for a microbial invader of the intestinal niche. The major goal of this study was to analyze whether the arising interactions between the co-inhabitating bacteria, changes in temperature and the anaerobic atmosphere can modulate the antiplasmid effect of promethazine (Figure 10).

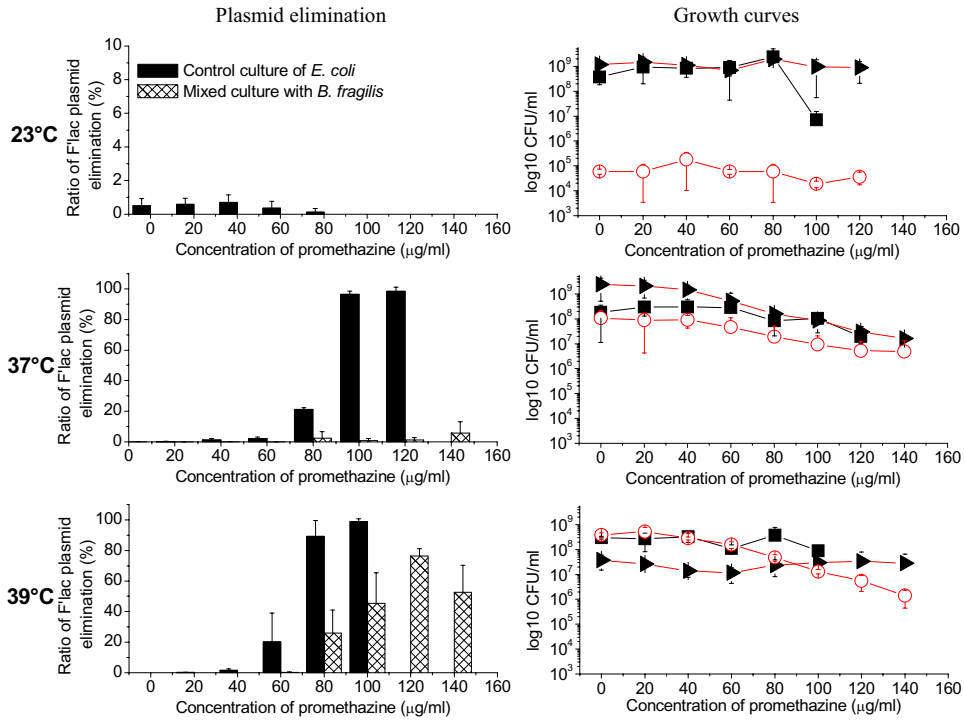

Figure 10. The effect of co-culture on F'lac plasmid elimination of *E. coli* K12LE140 with *B. fragilis* ATCC 25285 and population dynamics in the presence of different concentrations of promethazine at varying temperature. Viable counts of *E. coli* in the control culture (■). Viable counts of *E. coli* (○) cultured with *B. fragilis*. Viable counts of *B. fragilis* (▶) cultured with *E. coli*.

The efficiency of plasmid elimination in anaerobic co-culture experiments performed at room temperature (23°C) were in a range of 0.00-0.59% in the control culture, whereas in the mixed culture promethazine had no effect on plasmid loss (0.00%). At 37°C, the ratio of plasmid elimination in the monoculture of *E. coli* reached a maximum efficiency with an average of 98.33%. This stands in strong contrast to the mixed culture, where the frequency

remained as low as 2.42%. At 39°C, in case of both experimental settings the plasmid curing effect of promethazine was prominently enhanced. In case of the monoculture of *E. coli*, the maximum level of plasmid elimination of 98.97% took place at 100 μg/ml while, in the mixed culture 76.31% of elimination rate was detected at 120 μg/ml concentration of promethazine.

4.5. Inhibition of flagellar movement of bacteria by proton pump inhibitor

It was indicated that bacterial motility plays a role in structure formation of the microbial community as bacteria are able to move towards positions that allow optimal growth conditions. If we consider that gene transfer (through conjugation) between microorganisms occurs between closely associated cells, then we can assume that the environment and the arising interactions may not only determine the spatial structures of the microbial community but also can influence the spread of virulence or resistance determinants, thus affecting microbial evolution [159]. Besides the ecological role of bacterial motility within microbial communities, it is also important to consider as a virulence factor in the context of human clinical diseases, where pathogenic interactions occur between invading bacteria and its host, consequently only the bacterial partner benefits from this relationship [160]. Flagellar motility of human pathogens is an essential factor in the different phases of infection such as colonization, which is the occupation of the most favourable niches of the human host by chemothaxis. For example, *H. pylori*, whose strong motility is conferred by flagellae, colonizes the gastric mucosa and play a role in development of gastric and peptic ulcers and is even a bacterial risk factor for gastric cancer [161, 162, 163]. The uropathogenic *E. coli* strains also use flagellae to move along the urinary tract to cause ascending pyelonephritis [164].

It was previously reported that a newly synthetized trifluoro-methyl ketone derivative TF18 (2-benzoxazoyl-9-3,3,3-trifluoro-2-propanone) showed a remarkable antibacterial effect against some *E. coli* and *H. pylori* strains and it was proven that the agent exerts a proton pump inhibitory effect on *H. pylori* as well [165]. Furthermore, TF18 in combination with promethazine had a significantly synergistic effect in blocking the *E. coli* strain's proton pump system, suggesting that the compound may influence the proton motive force (PMF)[166]. Since the energy required for flagellar movement is supplied by the PMF produced by the primary proton pump system, consequently any modification in the transmembrane proton gradient results in impaired motility [166, 167, 168, 169, 170].

Detailed data are presented in Table 11. and 12. in Annex III.

4.5.1. Antimotility action of TF18 on E. coli strains

Based on these data, we intend to clarify whether TF18 (2-benzoxazoyl-9-3,3,3-trifluoro-2-propanone) modulates the flagellar motility driven by the proton motive force. To test our hypothesis two *E. coli* strains were used: *E. coli* AG100, with proton pump system and its mutant type AG100A strain with proton pump deficiency.

Different concentrations of TF18 were tested on the *E. coli* strains and the antimotility action was determined under phase contrast microscope. The applied concentartions of the drug were: 10%, 50%, 90% and 200% MIC. MICs of TF18 were determined in our preliminary experiments for both *E. coli* strains. For AG100 strain the MIC was 7.8 μg/ml and for AG100A strain the MIC was 3.9 μg/ml. The inhibition of motility was observed immediately after the addition of the different MIC values of TF18, while growth inhibition was determined after prolonged incubation with the agent. The inhibitory effect on bacterial movement was definied based on the distribution among the the amount of swimming, tumbling and non-motile cells within the population. According to our results presented in Figure 11. the *E. coli* AG100 cells were moderately motile when were measured under the phase contrast microscopy int he control sample. On the other hand, *E. coli* AG100A cells were less motile in the absence of TF18.

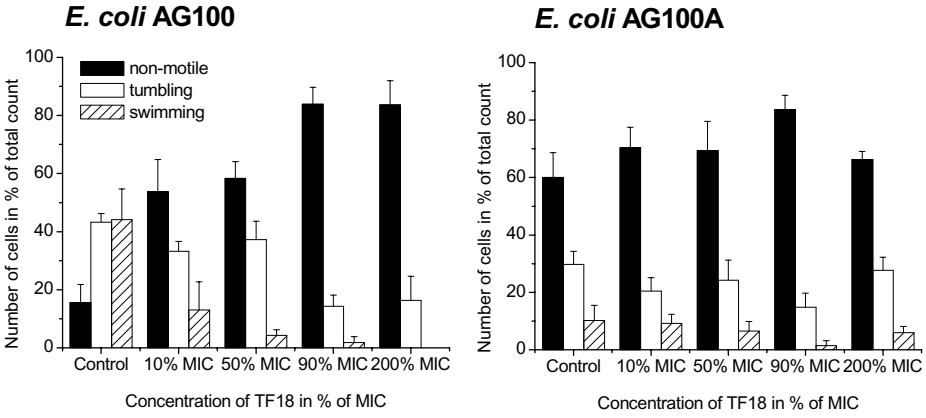

Figure 11. Inhibitory effect of TF18 on the motility of *E. coli* AG100 and *E. coli* AG 100A strains. 200 cells / field were counted in each experiment.

Inhibition of the motility of *E. coli* AG100 was observed at subinhibitory concentrations of TF18. At 10% MIC (0.78 μg/ml), the number of non-motile cells was increased and the number of the swimming cells was decreased, while tumbling was not influenced. At 50% MIC (3.9 μg/ml), the swimming cells were decreased, while the number of the tumbling and

non-motile cells did not change between 50% and 10% MIC. At 90% MIC (7.0 μg/ml), 82% of the cells were non-motile, while at 200% MIC (15.6 μg/ml) TF18 showed the strongest effect and complete inhibition of swimming under the short exposure of the drug. In case of *E. coli* AG100A strain, the swimming cells were decreased with the increased concentration of TF18, while the number of tumbling and non-motile cells were virtually unchanged between 90% (3.5 μg/ml) and 10% MIC (0.39 μg/ml).

We were also interested in the combined antibacterial action of TF18 with clarithromycin against clarithromycin-resistant *H. pylori* 9447 (CRHP) and susceptible *H. pylori* 26695 (CSHP) strains. As the number of clarithromycin resistant isolates is increasing, treatment failures to eradicate CRHP become more common [171, 172]. The development of a new class of anti-*H. pylori* agents or drug combinations has great importance.. The antibacterial activity of TF18 examined either alone or in combination with clarithromycin on CRHP and CSHP strains.

TF18 had the strongest growth inhibitory effect (MIC: 0.95 μg/ml) on CRHP strain, however; only additive effect was observed in combination with clarithromycin in case of both *H. pylori* strains.

4.6. MDR reversal in mouse lymphoma cell lines

One of the major and best studied MDR mechanism employed either by pathogenic microorganisms and cancer cells is the transmembrane pump-mediated drug efflux [173]. Since clinically acquired MDR arising during the chemotherapy results in untreatable cases, therefore there is an urgent need for developement of effective agents such as transport inhibitors. The inhibition of the efflux pump leads to increased accumulation of the drug in the tumor cell resulting in *in vitro* cytotoxicity.

4.6.1. MDR reversal in mouse lymphoma cell lines by cycloartanes

Euphorbia species are well known for their antitumor activity in traditional medicine and also proved to be effective in the treatment of warts. The latex part of these plants is rich in isoprenic compounds like tetra and pentacyclic triterpens. Among the tetracyclic triterpens, cycloartanes are found in a large amount and they have been revealed as cytotoxic agents against several tumor cells. We investigated the cycloartane triterpenoids isolated from *E. segetalis* and *E. portlandica* on their activity for reversal of resistance in mouse T lymphoma cells (MDR and PAR line). The MDR reversal experiments were studied by Rhodamine 123 uptake assay. The results obtained after Flow Cytometric analysis are presented in Table 4.

Table 4. Effect of cycloartane compounds 1-15 on the reversal of MDR in mouse lymphoma cells with MDR.

Samples	Concentration (μg/ml)	FAR	Samples	Concentration (μg/ml)	FAR
PAR+R123	-		**PAR+R123**	-	
PAR-R123	-		**PAR-R123**	-	
MDR+R123	-		**MDR+R123**	-	
Verapamil	10	3.76	**Verapamil**	10	3.76
1	4	2.39	**9**	4	1.41
	40	**46.37**		40	*toxic*
2	4	2.36	**10**	4	1.55
	40	39.25		40	1.04
3	4	0.68	**11**	4	1.65
	40	0.75		40	0.92
4	4	1.39	**12**	4	2.31
	40	**54.61**		40	*toxic*
5	4	0.85	**13**	4	1.04
	40	29.11		40	1.49
6	4	1.86	**14**	4	1.50
	40	*toxic*		40	17.38
7	4	1.34	**15**	4	1.04
	40	*toxic*		40	1.58
8	4	2.83	**DMSO**	20 μl	1.11
	40	19.99			

Legend: FAR: Fluorescence Activity Ratio, **R123:** Rhodamine 123

The reversal activity of the compounds was compared to the FAR value of verapamil, which was used as a control. From the fifteen tested compounds, compound 1 (cycloartane-3β,24,25-triol), 2 (9,19-cycloanostane-3β,26-diol), 4 (cycloartane 23-ene-3β,25-diol), 5 (24-hydroperoxy-9,19-cyloanost-25-en-3β-ol), 8 ((*22E*)-3β-hydroxy-4,4,14-trimethyl-9,19-cyclochol-22-en-24-al) and 14 (9,19-cyclolanost-24-en-3β-acetate) exhibited the highest activity in reversing MDR. Compounds 10, 11, 13 and 15 showed marginal activity. Compound 3 was found to be ineffective, and compounds 6, 7, 9 and 12 at the concentration of 40 μg/ml were toxic.

4.7. Studies in Structure Activity Relationship (SAR): analysis of P-gp interactions with several phenothiazine derivatives

Investigations showed that series of phenothiazine derivatives posess effective MDR reversal activity *in vitro*. However, their exact mode of molecular action on modulating the operation of P-gp is not explored yet. In order to uncover the type of interactions between P-gp and amino alkyl and aromatic ring substituted phenothiazines, analysis was carried out using molecular modelling techniques. We investigated structurally suggested charge transfer (CT) interactions between aromatic amino acids and phenothiazines by calculating LUMO and HOMO energies [174]. Two groups of phenothiazines were involved in the calculations: phenothiazines with benzo[a] structure and amino-alkyl substituted phenothiazines. The MDR

reversal activities of the phenothiazine derivatives were determined by Rhodamine 123 uptake assay on MDR and PAR mouse lymphoma cell lines. HyperChem6.06 program was used to calculate LUMO and HOMO energies. The comparison of the biological activity of the compounds with their calculated HOMO and LUMO energies is presented in Table 5.

Table 5. Correlation between P-gp inhibition and calculated HOMO and LUMO energies.

Compounds		E_{HOMO} (eV)	E_{LUMO} (eV)	FAR at 4 μg/ml
Benzo[a] phenothiazines	**5-oxo-5H-benzo[a]phenothiazine**	-8.347	**-1.578**	0.85
	6-hydroxi-5oxo-5H-benzo[a]phenothiazine	-7-946	**-1.594**	1.2
	6-methyl--oxo-5H-benzo[a]phenothiazine	-8.248	**-1.523**	3.44
Amino-alkyl substituted phenothiazines	**Trifluperazine**	**-7.788**	-0.644	37.67
	Promethazine	**-7.420**	0.097	2.41
	Chlorpromazine	**-7.565**	-0.307	6.31
	6,9-dihydroxichlorpromazine	**-8.120**	-1.753	0.79
	7,8-dioxochlorpromazine	**-8.520**	-1.684	0.89
	6,9-dioxochlorpromazine	**-8.053**	-0.451	0.82

Apparently there is a connection between the energies of HOMO and MDR reversing effect of the compounds meaning that electron donor properties of the compounds are responsible for creating CT complexes.

V. DISCUSSION

During the past decades emergence of antibiotic-resistant microorganisms and progression of cancer cells to chemotherapy resistant phenotype have become a challenging issue in the pharmaceutical industry[175, 176]. We investigated a group of compounds for their antiplasmid and multidrug resistant (MDR) reversal action in prokaryotic and eukaryotic models, in a pursuit of resistance modifiers for use as a possible therapeutic option.

5.1. Screening for new candidates for effective plasmid elimination, several phenothiazines were tested in the monoculture of the F'lac containing *E. coli* K12LE140 strain. We found that among the tested phenothiazines, only perazine dimaleate exhibited plasmid curing effect with the extent of 27.27%.

5.2. A set of benzoxazole, benzimidazole and oxazolo(4,5-b)pyridine derivatives were previously demonstrated to have broad spectrum antimicrobial activity, especially against Gram-negative bacteria. Recent observations also suggested that benzoxazoles and benzimidazoles have low toxicity in man, thus they may be potential candidates for chemotherapeutic drugs[146, 147, 148]. Considering the fact that many heterocyclic compounds bear antiplasmid activity, we investigated the effect of the heterocyclics on F'lac plasmid elimination.

Of the tested compounds, G17 showed remarkable activity on F'lac plasmid loss with a curing ratio of 28.00%. Compounds A9, A33, B11, D27, D34 and F1 applied alone showed marginal plasmid curing frequencies found in the range between 0.00%-0.15%. To enhance the curing ratio, the compounds were combined with promethazine. Benzoxazole compound B11, in combination with promethazine yielded a curing ratio of 89.40%, which exceeded the plasmid curing rate of the promethazine treated control culture (73.82%). No remarkable increase was observed in the combined antiplasmid effect of compounds A33, D27 and G17.

5.3. During the past years, enterococci became increasingly recognized as nosocomial pathogens that additionally acquire multiple antibiotic resistances like VRE strains. The high level of resistance of *E. faecalis* to many antimicrobial agents is presumably due to the presence of many MDR pumps. Recently Davis *et al*. reported 34 putative MDR transporters based on genome sequencing information [177,178]. However, only two MDR pumps were experimentally shown. The first one is EmeA, a NorA homolog and a member of the MFS family [179]. The other is Lsa, which confers resistance to clindamycin and dalfopristin. Lsa is an ABC protein and it seems that it is not an integral membrane protein [180].

Inhibition of efflux pumps, which contribute to clinical MDR phenotype of bacteria, is an alternative way to restore the efficacy of antibiotics that are substrates of the pumps. It is suggested that the high levels of antibiotic resistance of *E. faecalis* may be due to the presence of many MDR efflux pumps [178]. Consequently, the development of drugs capable of blocking the efflux pumps, allowing antibiotics to reach their targets, is of obvious importance. Since phenothiazines, especially the mild neuroleptic thioridazine have been shown to inhibit energy-dependent efflux pumps, we investigated whether prochlorperazine (PCP) and thioridazine (THIO) are capable of altering the susceptibility of clinical isolates of *E. faecalis* strains to vancomycin (VAN) and ampicillin (AMP).

The resistance reversal effect of THIO and PCP was tested in presence of antibiotics (AMP and VAN) in clinical isolates of *E. faecalis* strains A, C, D and E. Each isolate showed the same MIC value (32 μg/ml) for THIO and PCP applied alone. We demonstrated that THIO combined with VAN was found to have synergistic activity when tested against strains A and D in the checkerboard analysis. The resistance of the VAN resistant strain C and the VAN sensitive isolate E was not reversed significantly by a combination treatment of VAN and THIO, showing only an additive effect. Our results showed that in two of the three VAN resistant strains (A and D) synergistic effect of VAN and THIO was observed. However, the resistance to either AMP or VAN was not reduced by the presence of PCP in any of the isolates evaluated.

As THIO is known to inhibit efflux pumps, we suggest that the enhanced susceptibility of the strains A and D to VAN is related to the inhibitory action of THIO on the putative MDR efflux pumps of enterococcal strains A and D.

5.4. Our findings documented that a predominant, well-established culture of *S. epidermidis* or *B. cereus* was permissive for the growth of a small number of inoculated newcomer (*E. coli* K12LE140) in the mixed culture, which later on became the dominant member of the consortium. In other words, the second species has the ability to colonize the host efficiently and outcompetes the preexisting one.

Remarkably, F'lac plasmid loss with 19.23% was shown in the presence of *S. epidermidis* at 37°C on the ninth day of incubation. Our observations suggested that a marked increase in plasmid loss might be caused by putative metabolic interactions due to the activity of a second species of bacteria. We suggest that this is also associated with the alteration of the local environment which favors the plasmid loss.

5.5. Traditionally, antiplasmid assays use a culture of a single bacterial species. We focused on the antiplasmid effect of promethazine in mixed bacterial cultures to simulate polymicrobial consortium situated as in nature. Using mixed cultures with a plasmid bearing bacteria (*E. coli* K12LE140) we were able to simulate the *in vivo* situation of polymicrobial flora or mixed infection and to study the plasmid elimination under various conditions, with co-inhabitating bacteria (*S. epidermidis, B. cereus* and *B. fragilis* ATCC 25285), different temperatures, aeration, anaerobic atmosphere and the presence of the plasmid curing agent promethazine.

Our results indicated that F'lac plasmid elimination from *E. coli* K12 LE140 promoted by sub-inhibitory concentrations of promethazine is significantly increased in non-shaking conditions in mono-culture and mixed culture with either *S. epidermidis* at 37°C and 39°C or with *B. cereus* at 39°C.

Shaking of the cultures tends to minimise the plasmid curing effects of the phenothiazine, especially at temperatures below 39°C.

Under anerobic conditions our observations suggest that plasmid elimination from mono and mixed cultures of *E. coli* K12 LE140 is enhanced with higher temperature contributing to more effective F'lac plasmid curing efficiency either in mono- or mixed cultures of *E. coli* strain at 39°C.

The results obtained at 39°C indicate that feverish stage can favour for the plasmid elimination by promethazine as the curing efficiency of the drug reached outstanding high values under some of the aerobic and anaerobic experimental settings either in mono- or mixed cultures. Our results well correlates with data from the literature stating that supra-optimal temperature can induce plasmid destabilization leading to increased plasmid loss[88].

Furthermore, it is understood that the plasmid elimination efficiency of the phenothiazines markedly alters in the presence of another species and with different environmental parameters. Nevertheless, it cannot be excluded that phenothiazine induced elimination of plasmids is influenced by soluble secondary metabolites present in the mixed culture produced by the bacterial species. Further studies on the plasmid elimination are expected in various environmental conditions such as distinct metabolites, pH, redox potential, *etc*.

5.6. TF18 (2-benzoxazoyl-9-3,3,3-trifluoro-2-propanone) was shown to posses antimicrobial action on several Gram-negative bacteria, yeasts and also render anti-HIV action[165]. In combination with antibiotics, compound TF18 showed a synergistic effect on the *E. coli* AG100 strain, being a potent resistance reversal agent. It was also shown that TF18 possibly

acts through blocking the proton motive force (PMF) driven pumps, thus allowing the intracellular concentration of antibiotic to reach the bactericide level[166]. We studied if TF18 disrupts the PMF by flagellum motility test involving *E. coli* AG 100 and AG100A strains.

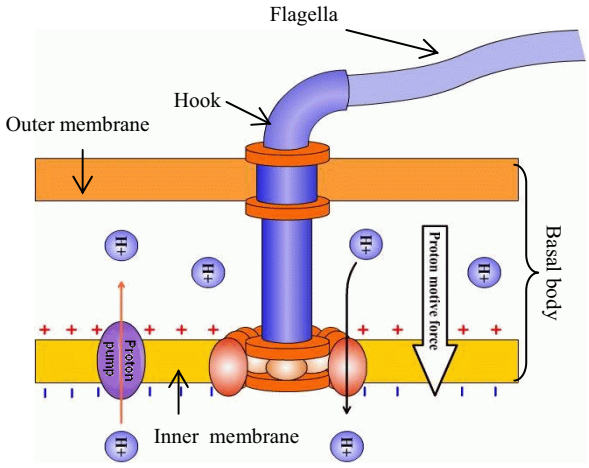

Figure 12. Schematic drawing of flagella in Gram-negative bacteria. The motor consists of two cytoplasmic proteins (MotA and MotB) forming a complex and functioning as a torque generating unit[168]. The motor can be found in two different states in counterclockwise or clockwise rotation, which make bacterial cells swimming and tumbling, respectively[169]. The energy for torque generation is obtained from PMF. The electrochemical gradient of protons is generated by primary proton pumps residing in the cytoplasmic membrane.

Our data showed that the novel TF18 compound has an anti-motility effect on the *E. coli* strain carrying the proton pump system (AG100), while it did not alter remarkably the motile behavior of the mutant type (AG100A). Subinhibitory concentration of TF18 inhibited the motile behavior of the proton pump carrying *E. coli* AG 100 strain, which was confirmed by the strong reduction in the number of swimming and in the simultaneous increase of the less motile cells. It presumes that TF18 influences the operation of the proton pump probably through the disruption of the transmembrane proton gradient, which is responsible for the motility (Figure 12.). However, the exact mechanism of antimotility action of TF18 is yet to be clarified.

Since motility plays role in the initial part of infection like adhesion and colonization and sub-MIC concentrations of TF18 weakened the motile ability of bacteria, thus TF18 may be a potential candidate for antimotility agent. We should also note here that the *in vivo* effect might be more complex than the *in vitro* situation, in which changes in the function of pili of bacteria are involved.

As earlier TF18 was proved to be a potent resistance reversal agent it was combined with clarithromycin and tested for enhancement of antibacterial activity on two *H. pylori* strains (CSHP and CRHP) in checkerboard analysis. The results obtained in this study identified additive effect in case of both *H. pylori* strains.

5.7. It is generally considered that the P-gp-mediated MDR is the most common mechanism of acquired drug resistance, which contributes to the unresponsive anticancer chemotherapy[116, 117, 118]. This observation suggests use of MDR modulators to be a strategy to reverse the emerged drug resistance. A possible strategy to prevent the emergence and reversal of drug resistance is to increase the intracellular drug concentration to a cytotoxic level by the administration of drug transport inhibitors[125]. Several *in vitro* models support the efficacy of this strategy. The agents seemed to bind directly to the P-gp to block the outward transport of the cytostatics or modify (decrease) the expression of the *MDR1* gene. Active agents are amphiphylic compounds and all of them have aromatic basic structure with one or more amino alkyl groups.

Using Rhodamine 123 accumulation tests in P-gp overexpressing cell lines, cycloartane derivatives 1, 2, 4, 5, 8 and 14 derived from plant extracts were found to be promising reversal agents *in vitro*.

It also indicates that cycloartane triterpens may be inhibitors of P-gp as demonstrated in the human P-gp overexpressing mouse lymphoma cells.

5.8. Many P-gp modulators have been discovered so far and the mode of action on P-gp has been studied. Most chemosensitisers exert their MDR reversal activities by competitively binding to the drug binding site or noncompetitively binding to the modulatory site. Some agents exhibit their inhibitory activities by interactions with the ATP binding site.

Compounds with higher LUMO energies facilitated such CT complex formation, but showed low activity as MDR reversers. Therefore, it is suggested that binding within the first loop may correlate with decreased biological activity, but it remains yet to be studied. Phenothiazines with aminoalkyl side chains showed HOMO energies in the range of MDR modulators, thus suggesting electron donor properties in CT complexes. The mechanism of binding is more complex in biological systems, compared to *in vitro*.

VI. NEW STATEMENTS

New antiplasmid compounds

6.1. Perazine dimaleate proved to have slight effect on F'lac plasmid loss. 2-cloro-5-oxo-5H-phenothiazine and perphenazine did not show remarkable plasmid curing activity.

6.2. Novel benzoxazoles, benzimidazoles and oxazolo(4,5)pyridines with known antimicrobial activity showed a plasmid curing effect to a minor degree as compared to promethazine treated samples. Among benzoxazoles; agent B11 in combination with promethazine showed an enhanced antiplasmid effect.

6.3. Checkerboard analysis revealed that thioridazine combined with vancomycin resulted in synergistic activity against *E. faecalis* strains A and D. Prochlorpromazine in combination with ampicillin or vancomycin showed an indifferent (strain A) or additive effect (strain C, D and E) on the tested clinical isolates, respectively.

Co-cultures

6.4. A numerically predominant, well established culture of *S. epidermidis* and *B. cereus* was permissive for the growth of the inoculated small population of *E. coli* K12LE140. Remarkable plasmid instability was detected in the presence of *S. epidermidis* at 37°C and reduced spontaneous plasmid loss was detected in co-cultures with *B. cereus*.

6.5. F'lac plasmid loss from *E. coli* K12LE140 was enhanced by sub-MIC concentration of promethazine in non-shaking conditions by higher temperatures in mono-cultures and mixed cultures with *B. cereus* or *S. epidermidis*.

6.6. Shaking of the cultures tended to minimize plasmid curing ratio of promethazine especially temperatures below 39°C.

6.7. Anaerobe atmosphere and increased temperature promoted the F'lac plasmid loss both in mono- and mixed-cultures with *B. fragilis* ATCC 25285.

Efflux systems

6.8. The novel proton pump inhibitor TF18 at sub-MICs remarkably inhibited the motile behavior of the proton pump carrying *E. coli* AG100 strain.

6.9. The *in vitro* combination of TF18 and clarithromycin resulted in additive effect on both *H. pylori* strains (CRHP and CSHP).

6.10. Cycloartanes (compounds: 1, 2, 4, 5, 8 and 14) can be inhibitors of P-gp as demonstrated by MDR reversal assay in human P-gp overexpressing mouse lymphoma cells.

6.11. SAR studies revealed that phenothiazines with aminoalkyl side chains showed HOMO energies in the range of MDR modulators, suggesting the importance of electron donor properties in CT-complexes.

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VIII. REFERENCES

1. Ligon BL. Penicillin: its discovery and early development Semin. Pediatr. Infect. Dis. 2004; 15(1):52-57

2. Nwosu V. C. Antibiotic resistance with particular reference to soil microorganisms. Res. Microbiol. 2001; 152:421-430.

3. Séveno NA, Kallifidas D, Smalla K, van Elsas JD, Collard JM, Karagouni AD, Wellington EMH. Occurrence and reservoirs of antibiotic resistance genes in the environment. Rev. Med. Microbiol. 2002; 13:15-27.

4. Salyers AA, Amábile-Cuaevas C F. Why are antibiotic resistance genes are so resistant to elimination? Antimicrob. Agents Chemother. 1997; 41:2321-25.

5. Patrick DM, Marra F, Hutchinson J, Monnet D, Ng H. and Bowie WR: Per capita antibiotic consumption: How does a North American jurisdiction compare with Europe? Clin. Inf. Dis. 2004; 39:11-17.

6. Adam D. Global antibiotic resistance in *Streptococcus pneumoniae* J. Antimicrob Chemother. 2002; 50:1-5.

7. World Health Organization (WHO). Overcoming antimicrobial resistance. Geneva: WHO, 2000. Available http://www.who.int/infectious-disease-report/2000/index.html Accessed 17 September 2003.

8. Goossens H, Ferech M, Stichele VR and Elseviers M and ESAC Project group. Outpatient antibiotic use in Europe with resistance: a cross-national database study. Lancet 2005; 365:579-587.

9. Aiello AE and Larson E. Antibacterial cleaning and hygiene products as emerging risk factor for antibiotic resistance in the community. Lancet Inf. Dis. 2003; 3:501-6.

10. Jones RD, Jampani HB, Newman JL, Lee AS. Triclosan: a review of effectiveness and safety in health care settings. Am. J. Infect. Control 2000; 28:184-96.

11. Schweeizer HP. Triclosan: a widely used biocide and its link to antibiotics FEMS Microbiol. Lett. 2001; 202:1-7.

12. Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP. Cross-resistanec between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. Antimicrob. Agents Chemother. 2001; 45:428-32.

13. Hoiby N. Ecological antibiotic policy. J. Antimicrob. Chemother. 2000; 46:59-62.

14. Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 2005; 84(4):634-43.

15. Angulo FJ, Baker NL, Olsen SJ, Anderson A, Barrett TJ. Antimicrobial use in agriculture: controlling the transfer of antimicrobial resistance to humans. Semin. Pediatr. Infect. Dis. 2004; 15(2):78-85.

16. Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 2004; 53(1):28-52.

17. Turnidge J. Antibiotic use in animals-prejudices, perceptions and realities. J. Antimicrob. Chemother. 2004; 53(1):26-7.

18. Bonten MJ, Willems R, Weinstein RA. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect. Dis. 2001; 1(5):314-25.

19. Klare I, Heier H, Claus G, Bohme S, Marin S, Seltmann G, Hakenbeck R, Santanassova V and Witte W. *Enterococcus faecium* strains with VanA-mediated high-level glycopeptide resistance isolated from foodstuffs and fecal samples of humans in the community. Microb. Drug Resist. 1995; 1:256-72.

20. Colligon P. Vancomycin resistant enterococci and use of avoparcin in animal feed: is there a link? Med. J. Aust. 1999; 171:144-6.
21. Gambarotto K, Ploy MC, Turlure P, Grelaud C, Martin C, Bordessoule D, Denis F. Prevalence of vancomycin-resistant enterococci in fecal samples from hospitalized patients and nonhospitalized controls in a cattle-rearing area of France. J. Clin. Microbiol. 2000; 38(2):620-4.
22. Huang TC, Burr TJ. Characterization of plasmids that encode streptomycin resistance in bacterial epiphytes of apple. J. Appl. Microbiol. 1999; 86:741-51.
23. Schnabel EL, Jones AL. Distribution of tetracycline resistance genes and transposones among phylloplane bacteria in Michigan apple orchards. Appl. Environ. Microbiol. 1999; 65:4898-907.
24. Martinez JL and Baquero F. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. 2000; 44(7): 1771-7.
25. De la Cruz F, Davies J. Horizontal gene transfer and the origins of species: lessons from bacteria. Trends Microbiol. 2000; 8:128-33.
26. Dzidic S, Bedekovic V. Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. Acta Pharmacol. Sin. 2003; 24(6):519-26.
27. Naiemi NA, Duim B, Savelkoul PH, Spanjaard L, de Jonge E, Bart A, Vandenbroucke-Grauls CM, de Jong MD. Widespread transfer of resistance genes between bacterial species in an intensive care unit: implications for hospital epidemiology. J. Clin. Microbiol. 2005; 43(9):4862-4.
28. Collignon PJ. Antibiotic resistance. Med. J. Aust. 2002; 177:325-9.
29. Goossens H. European status of resistance in nosocomial infections. Chemotherapy. 2005; 51(4):177-81.
30. Tenover FC, McDonald LC. Vancomycin-resistant staphylococci and enterococci: epidemiology and control. Curr. Opin. Infect. Dis. 2005; 18(4):300-5
31. Zetola N, Francis JS, Nuermberger EL, Bishai WR. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging threat. Lancet Infect. Dis. 2005; 5(5):275-86.
32. Hujer AM, Bethel CR, Hujer KM, Bonomo RA. Antibiotic resistance in the institutionalized elderly. Clin. Lab. Med. 2004;24(2):343-61.
33. Witte W. International dissemination of antibiotic resistant strains of bacterial pathogens. Infect. Genet. Evol. 2004; 4(3):187-91.
34. Loddenkemper R, Sagebiel D, Brendel A. Strategies against multidrug-resistant tuberculosis. Eur. Respir. J. Suppl. 2002; 36:66s-77s.
35. Blázquez J. Hypermutation as a factor contributing to the acquistition of antimicrobial resitance. Clin. Inf. Dis. 2003; 37:1201-9.
36. Poole K. Multidrug efflux pumps and antimicrobial resistance in *P. aeruginosa* and related organisms J. Mol. Microbiol. Biotechnol. 2001; 3:225-64.
37. Cohen SP, McMurry LM, Hooper DC et al. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreaseddrug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. 1989; 33:1318-25.
38. Foley I, Marsh P, Wellington MH, Smith AW and Brown MRW. General stress response master regulator rpoS is expressed in human infection: a possible role in chronicity. J. Antimicrob. Chemother. 1999; 43:164-5.
39. de la Cruz F and Davues J. Horizontal gene transfer and the origin of species: lessons from bacteria. Trends Microbiol. 2000; 8(3):128-33.
40. Koonin EV, Makarova KS, Aravid L. Horizontal gene transfer in prokaryotes: quantification and classification. Annu. Rev. Microbiol. 2001; 55: 709-42.

41. Bensasson D, Boore JL, Nielsen KM. Genes without frontiers? Heredity 2004; 92(6):483-9.
42. Hogan D. and Kolter R. Why are bacteria refractory to antimicrobials? Curr. Opin. Microbiol. 2002; 5: 472-7.
43. Courvalin P. Transfer of antibiotic resistance genes between Gram-positive and Gram-negative bacteria. Antimicrob. Agents Chemother. 1994; 38: 1447-51.
44. Salyers AA, Shoemaker NB, Li LY and Stevens AM. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. 1995; 59:579-90.
45. Francia VM. Varsaki A, Garcillán-Barcia MP, Latorre A, Drainas C, de la Cruz F. A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol. Rev. 2004; 79-100.
46. Burrus V, Waldor MK. Shaping bacterial genomes with integrative and conjugative elements. Res. Microbiol. 2004; 155(5):376-86.
47. Hastings PJ, Rosenberg SM, Slack A. Antibiotic-induced lateral transfer of antibiotic resistance. Trends Microbiol. 2004; 12(9):401-4.
48. Ankenbauer RG. Reassessing forty years of genetic doctrine: retrotransfer and conjugation. Genetics 1997; 145:543-9.
49. Salyers AA and Shoemaker NB. Resistance gene transfer in anaerobes: new insights, new problems. Clin. Infect. Dis. 1996; 23(Suppl.):S36-S43.
50. Salyers AA, Gupta A, Wang Y. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. 2004; 12(9):412-6.
51. Lilley AK, Bailey MJ. The acquisition of indigenous plasmids by a genetically marked Pseudomonad population colonizing the sugar beet phytosphere is related to local environmental conditions. Adv. Appl. Microbiol. 1997; 63:1577-83.
52. Jiang SC, Paul JH. Gene transfer by transduction in the marine environment. Appl. Environ. Microbiol. 1998; 64:2780-7.
53. Lunsford RD. Streptococcal transformation: essential features and applications of a natural gene exchange system. Plasmid 1998; 39:10-20.
54. Lorenz MG, Wackernagel W. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev. 1994; 58:563-602.
55. McKeegan KS, Ines Borges Wamsley M and Wamsley AR. Microbial and viral drug resistance mechanisms. Trends Microbiol. 2002; 10(Suppl.):S8-S14.
56. McDermott PF, Walker RD, White DG. Antimicrobials: modes of action and mechanisms of resistance. Int. J. Toxicol. 2003; 22(2):135-43.
57. Majiduddin FK, Materon IC, Palzkill TG. Molecular analysis of beta-lactamase structure and function. Int. J. Med. Microbiol. 2002; 292(2):127-37.
58. Oliviera D et al. Secrets of success of human pathogen: molecular evolution of MRSA. Lancet Infect. Dis. 2002; 2:180-9.
59. Lambert PA. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. J. Appl. Microbiol. 2002; 92(Suppl 1): 46S-54S.
60. Nikaido H. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Cell Devel. Biol. 2001; 12:215-23.
61. Sugawara E and Nikaido H. OmpA protein of *E. coli* outer membrane protein occurs in open and closed forms. J. Biol. Chem. 1994; 275:1594-600.
62. Poole K. Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol. Infect. 2004; 10(1):12-26.
63. Chopra I, Roberts MC. tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 2001;65:232-60.

64. White DG, Hudson C, Maurer JJ, Ayers S, Zhao S, Lee MD, Bolton L, Foley T, Sherwood J. Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. J. Clin. Microbiol. 2000; 38:4593-8.
65. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria. Drugs 2004 ;64(2):159-204.
66. Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. Mol. Microbiol. 2000; 37(2):219-25.
67. Butaye P, Cloeckaert A and Schwarz S. Mobile genes coding for efflux- mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. Int. J. Antimicrob. Agents 2003; 22:205-10.
68. Ryan BM, Dougherty TJ, Beaulieu D, Chuang J, Dougherty BA, Barrett JF. Efflux in bacteria: what do we really know about it? Expert Opinion Invest. Drugs 2001; 10:1409-22
69. Putman M, Veen H. W. and Konings W. N. Molecular properties of bacterial multidrug transporters. Microbiol. Mol. Biol. Res. 2000; 64(4): 672-93.
70. Zgurskaya HI. Molecular analysis of efflux pump-based antibiotic resistance. Int. J. Med. Microbiol. 2002; 292(2):95-105.
71. Yu EW. Aires JR and Nikaido H. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J. Bacteriol. 2003; 185(19):5657-64.
72. Elkins CA and Nikaido H. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominately by two large periplasmic loops. J. Bacteriol. 2002; 184(23): 6490-8.
73. Walmsley MIB and Walmsley AR. The structure and function of drug pumps. Trends Microbiol. 2001; 9(2):71-9.
74. Fernandes P, Ferreira BS and Cabral JMS. Solvent tolerance in bacteria: role of efflux pumps and cross resistance with antibiotics. Int. J. Antimicrob. Agents 2003; 22:211-7.
75. Poole K. Mechanisms of bacterial biocide and antibiotic resistance J. Appl. Microbiol. 2002; 92 (Suppl 1): S55-S64.
76. Markham PN, Neyfakh AA. Efflux-mediated drug resistance in Gram-positive bacteria. Curr. Opin. Microbiol. 2001; 4(5):509-14.
77. Molnár J, Molnár A, Spengler G and Mándi Y. Infectious plasmid resistance and efflux pump mediated resistance. Acta Microbiol. Immunol. Hung. 2004; 51(3):333-49.
78. Molnar J, Mándi Y, Holland IB, Schneider GY. Antibacterial effect, plasmid curing activity and chemical structures of some tricyclic compounds. Acta Microbiol. Acad. Sci. Hung. 1977; 24:1-6.

79. Motohasi N, Sakagami H, Kurihara T, Ferenczy L, Csuri K, Molnár J. Antimicrobial activity of phenothiazines, Benzo(a)phenothiazines and Benz(c)acridines. Anticancer Res. 1992; 12:1207-10,

80. Molnár J. Gyógyszer-rezisztencia visszafordítása baktériumoktól a daganatsejtekig (Gyógyszerkutatás előre megtervezett hatású vegyületek előállításával). Orvosi Hetilap 1999; 140:2155-60.
81. Kristiansen JE. Chlorpromazine: non-antibiotics with antimicrobial activity-new insights in managing resistance? Curr. Opin. Invest. Drugs 1993; 2:589-91.
82. Molnár J, Béládi I and Földes S. Studies on the anti-tubercular action of some phenothiazine derivatives *in vitro*. Zentralblatt fr Bacteriologie (Orig A) 239: 521-6.
83. Molnár J. A new approach to overcome bacterial resistance to antibiotics.
84. Hirota Y. Effect of acridine dyes on mating type factors in *E. coli*. Proc. Natl. Acad. Sci. USA 1960; 46:57.

85. Watanabe T, Nishida H, Ogata C, Arai T and Sato S. Episome mediated transfer of drug resistance in Enterobacteriaceae. VII. Two types of naturally occurring R-factors. J. Bacteriol. 1964; 88:716.
86. Tomoeda M, Inuzuka M, Kubo N and Nakamura S. Effective elimination of drug and sex factors in *E. coli* by sodium dodecylsulphate. J. Bacteriol. 1968; 95: 1078.
87. Amaral L, Viveiros M and Kristiansen JE. Phenothiazines: potential alternatives for the management of antibiotic resistant infections of tuberculosis and malaria in developing countries. Trop. Med. Int.Health 2001; 6(10):1-7.
88. Molnár J. Antiplasmid activity of trycyclic compounds. Meth. and Find. Exptl. Clin. Pharmacol. 1988; 10(7):467-74.
89. Molnár J, Mándi Y, Király J. Antibacterial effect of some phenothiazine compounds on their R-factor elimination by chlorpromazine. Acta Microbiol. Acad. Sci. Hung. 1976; 23:45-54.
90. Molnar J, Földeák S, Nakamura M J, Rausch H, Domonkos K, Szabó M. Antiplasmid activity: loss of bacterial resistance to antibiotics. Acta Pharmacol. Microbiol. Immunol. Scand. 1992; 100:24-31.
91. Lantos J, Marjai E. *In vitro* transfer of multiple resistance observed in vivo during a *Salmonella london* epidemic. Acta Microbiol. Acad. Sci. Hung. 1980; 27:47-53.
92. Lantos J, Fekete J, Király K. R-plasmid study of an outbreak caused by multiresistant strains of *Salmonella panama*. Acta Microbiol. Acad. Sci. Hung. 1981; 28:211-7.
93. Csiszár K, Molnár J. Mechanisms of action of tricyclic drugs on *Escherichia coli* and *Yersinia enterocolitica* plasmid maintenance and replication. Anticancer Res. 1992; 12:2267-72.
94. Molnár J, Nakamura MJ. Virulence associated plasmid and R-plasmid curing in *Yersinia enterocolitica* by some tricyclic compounds. Acta Microbiol. Hung. 1988; 35:307-12.
95. Lantos J. Transfer of erythromycin resistance in *Staphylococcus aureus*. Acta Microbiol. Acad. Sci. Hung. 1977; 24:107-13.
96. Barabás K, and Molnár J. Lack of correlation between intercalation and plasmid curing ability of some tricyclic compounds. Acta Microbiol. Acad. Sci. Hung. 1980; 27, 55-61.
97. Miskolci Cs, Labádi I, Kurihara T, Motohasi N, Molnár J. Guanine-cytosine rich regions of plasmid DNA can be the target in antiplasmid effect of phenothiazines. Int. J. Antimicrob. Agents 2000;14:243-7.
98. Mándi Y, Molnár J. Effect of chlorpromazine on conjugal plasmid transfer and sex pili. Acta Microbiol. Acad. Sci. Hung. 1981; 28:205-10.
99. Molnár J. The role of charge distribution of tricyclic compounds in the antibacterial, antiviral and antiplasmid effects. In Keyzer N, Gutmann F, Eckert G (eds), Electropharmacology CRC, 1990; 205-19.
100. Lewis K. In search of natural substrates and inhibitors of MDR pumps. J. Mol. Microbiol. Biotechnol. 2001; 3: 247-54.
101. Lomovskaya O, Watkins W. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. J. Mol. Microbiol. Biotechnol. 2001; 3(2):225-36.
102. Kaatz GW, Mougdal VV, Seo SM, Kristiansen JE. Phenothiazines and thioxanthanes inhibit multidrug efflux pump anctivity in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 2003; 47(2):719-726.
103. Kaatz GW, moudgal VV and Seo SM. Identification and characterization of a novel efflux-related multidrug resistance phenotype in *Staphylococcus aureus*. J. Antimicrob. Chemother. 2002; 50: 833-838.
104. Poole K and Srikumar. Multidrug efflux in Pseudomonas aeruginosa: components, mechanisms and clinical significance. 2001; Curr. Top. Med. Chem. 1:59-71.

105. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshio K, Ishinda H and Lee VJ. Identification and characterisation of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. 2001; 45:105-116.

106. Markham PN, Westhaus E, Klyackho K, Johnson ME and Neyfakh AA. Multiple novel inhibitors of NorA multidrug transporter of *Staphylococcus aureus.* Antimicrob. Agents Chemother. 1999; 43:2404-2408.

107. Gunics Gy, Motohashi N, Amaral L, Farkas S, Molnár J. Interaction between antibiotics and non-conventional antibiotics on bacteria. Int. J. Antimicrob. Agents 2000; 14:239-42.

108. Kristiansen MM, Leandro C, Ordway D, Martins M, Viveiros M, Pacheco T, Kristiansen JE, Amaral L. Phenothiazines alter resistance of methicillin-resistant strains of *Staphylococcus aureus* (MRSA) to oxacillin *in vitro*. Int. J. Antimicrob. Agents 2003; 22:250-3.

109. Gunics Gy, Motohashi N, Farkas S, Molnár J. Effect of some resistance modifiers on the action of ampicillin and erythromycin. J. Antimicrobial. Chemother. 1999; 44:A87.

110. Hendricks O, Butterworth TS, Kristiansen JE. The in vitro antimicrobial effect of non-antibiotics and putative inhibitors of efflux pumps on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Int. J. Antimicrob. Agents 2003; 22:262-4.

111. Molnar J, Haszon I, Bodrogi T, Martonyi E, Turi S. Synergistic effect of promethazine with gentamycin in frequently recurring pyelonephritis. Int. Urol. Nephrol. 1990; 22(5):405-11.

112. Kristiansen JE, Amaral L. The potential management of resistant infections with non-antibiotics. J. Antimicrob. Chemother. 1997; 40:319-327.

113. Kristiansen JE, Mortensen I and Nissen B. Membrane stabilizers inhibit potassium efflux from *Staphylococcus aureus* strain U2275. Biochim. Biophys. Acta 1982; 685:379-382.

114. Eliam Y. Membrane effects of phenothiazines in yeasts. Stimulation of calcium and potassium fluxes. Biochim. Biophys. Acta 1983; 733:242-248.

115. Zilberstein D, Liveanu V and Gepstein A. Tricyclic drugs reduce the proton motive force in *Leishmania donovani* promastigotes. Biochem. Pharmacol. 1990; 39:935-940.

116. Lage H. ABC transporters: implications on drug resistance from microorganisms to human cancers. Int. J. Antimicrob. Agents 2003; 22:188-99.

117. Gottesman MM. Fojo T and Bates ES. Multidrug resistance in cancer: role of ATP-dependent transporters. Nature Rev. 2002; 2:48-58.

118. Chang G. Multidrug resistance ABC transporters. FEBS Letters 2003; 102-5.

119. Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. The Oncologist 2003; 8:411-24.

120. Gottesman MM. Mechanisms of cancer drug resistance. Annu. Rev. Med. 2002; 53:615-27.

121. Pluen A, Boucher Y, Ramanujan S *et al*. Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial vs. subcutaneous tumors. Proc. Natl. Acad. Sci. USA 2001; 98:4628-33.

122. Johnstone RW. Rueffli AA and Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. Cell 2002; 108:153-64.

123. McHugh PJ, Spanswick VJ, Hartley JA. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. Lancet Oncol. 2001; 2:483-90.

124. Ambudkar SV, Kimchi-Sarfaty C, Sauna EZ and Gottesman MM. P-glycoprotein: from genomics to mechanism. Oncogene 2003; 22:7468-85.

125. Ambudkar SV, Dy S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman M. Biochemical, cellular and pharmacological aspects of the multidrug transporter. Annu. Rev. Pharmacol. Toxicol. 1999; 39:361-98.
126. Gottesman MM and Ambudkar SV. Overview: ABC transporters and human disease. J. Bioenerg. Biomembr. 2001; 33(6):453-8.
127. Sauna ZE and Ambudkar SV. Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. Proc. Natl. Acad. Sci. USA 2000; 97:2515-20.
128. Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 2005; 204(3):216-37.
129. Chan HS, Thorner PS,Haddad G, Ling V. Immunohistochemical detection of P-glycoprotein: prognostic correlation in soft tissue sarcoma of childhood. J. Clin. Oncol. 1990; 8: 689-704.
130. Pirker R, Wallner J, Geissler K *et al*. MDR1 gene expression and treatment outcome in acute myeloid leukemia. J. Natl. Cancer Inst. 1991; 83:708-12.
131. Verrelle P, Meissonnier F, Fonck Y *et al*. Clinical relevance of immunohistochemical detection of mulitdrug resistance P-glycoprotein in breast carcinoma. J. Natl. Cancer Inst. 1991; 83:111-6.
132. Arceci RJ. Clinical significance of P-glycoprotein in multidrug resistant malignancies. Blood 1993; 81:2215-22.
133. Borst P, Evers R, Kool M and Winholds J. A family of drug transporters: the multidrug-resistance-associated proteins. J. Natl. Cancer Inst. 2000; 92:1295-302.
134. Komatani H *et al*. Identification of breast cancer resistant protein/mitoxantrone resistance/placenta specific ATP-binding cassette transporter as a transporter of NB-506 and J-107088, topoisomerase I inhibitors with indolocarbazole structure. Cancer Res. 2001; 61:2827-32.
135. Borst P, Zelcer N and van Helvoort A. ABC transporters in lipid transport. Biochim. Biophys. Acta 2000; 1486:128-44.
136. Fojo T, Bates S. Strategies for reversing drug resistance. Oncogene. 2003; 20;22(47):7512-23.
137. Varma MV, Ashokraj Y, Dey CS, Panchagnula R. P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacol. Res. 2003; 48(4):347-59.
138. Szabó D. Reversal of multidrug resistance in tumor cells *in vitro* Ph.D. Thesis 2001.
139. Molnar J, Szabó D, Mándi Y, Mucsi I, Fischer J, Varga A, König S, Motohashi N. Multidrug resistance reversal in mouse lymphoma cells by heterocyclic compounds. Anticancer Res.1998; 18: 3033-38.
140. Szabó D, Keyzer H, Kaiser HE, Molnár J. Reversal of multidrug resistance of tumor cells. Anticancer Res. 2000; 20: 4261-74.
141. Molnár J, Molnár A, Mucsi I, Pintér O, Nagy B, Varga A and Motohashi N. Reversal of multidrug resistance in mouse lymphoma cells by phenothiazines. In Vivo 2003; 17: 145-150.
142. Sorile T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS *et al*. Gene expression patterns of breast carcinomas to distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. USA 2001; 98: 10869-74.
143. Alföldi L, Raskó I, Kerekes E. L-serine deaminase of *E. coli*. J. Bacteriol. 1968; 96:1512-8.
144. Motohashi N. test for antitumor activities of phenothiazines and phenoxazines. (in Japanese) Yakugaku Zassi 1983; 103:364-71.

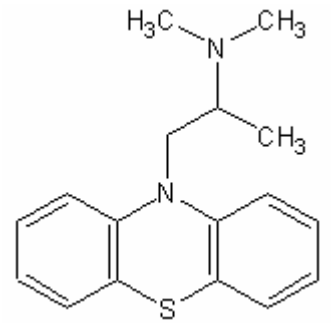
145. Hewlett I, Lee S, Molnár J, Foldeak S, Pine PS, weaver JL and Aszalos A.Inhibition of HIV infection of H9 cells by chlorprpomazine derivatives. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. 1997; 15: 16-20.
146. Ören I, Temiz Ö, Yalcin I, Sener E, Akin A and Ucartürk N. Synthesis and microbial activity of 5(or 6)-methyl-2-substituted benzoxazole and benzimidazole derivatives. Arzneimittel-Forschung/Drug Res. 1997; 47(II): 1393-7.
147. Sener E, Yalcin I, temiz Ö and Ören I. Synthesis and structure-activity relationships of some 2,5-distributed benzoxazoles and benzimidazoles as antimicrobial agents. Il Farmaco 1997; 52(2):99-103.
148. Yalcin I and Sener E. QSARs of some novel antibacterial benzimidazoles, benzoxazoles, and oxazolopyridines agains an enteric gram-negative rod; *K. pneumoniae*. Int. J. Pharmaceutics 1993; 98:1-8.
149. Madureira AM, Spengler G, Molnár A, Varga A, Molnár J, Abreu PM, Ferreira MJ. Effect of cyloartranes on reversal of multidrug resistance and apoptosis induction on mouse lymphoma cells. Anticancer Res. 2004; 24(2B): 859-64.
150. Kawase M, Saito S. convenient synthesis of 5-trifluoroacetylated imidazoles by ring transformation of mesoionic 1,3-oxazolium-5-olates. Chem. Pharm. Bull. 2000; 48:410-4.
151. Eliopoulos GM and Moellering RC. Antimicrobial combinations In V. Lorian (ed.), Antibiotics in laboratory medicine. Williams and Wilkins, Baltimore, Md. 1996; pp. 330-393.
152. Kessel D. Exploring multidrug resistance using rhodamine 123. Cancer Commun. 1989; 1:145-9.
153. Koopman G, Rentelinger CP, Kuijten Ga, Koehnen RM, Pals ST and Van Oers MH. Annexin V flow cytometric detection of phosphatydil-serine expression on B cells undergoing apoptosis. Blood 1994; 84:1115-20
154. Motohashi N, Kawase M, Kurihara T, Hevér A, Nagy Zs, Ocsovszky I, Tanaka M and Molnár J. Synthesis and antitumor activity of 1-(2-chlorethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkylureas as potential anticancer agents. Anticancer Res. 1996; 16:2525-32.
155. Van der Waaj, D, Nord CE. Development and persistence of multiresistance to antibiotics in bacteria; an analysis and new approach to this urgent problem. Int. J. Antimicrob. Agents 2000; 16:191-7.
156. Jensen GB, Hansen BM, Eilenberg J, Mahillon J. The hidden lifestyles of *Bacillus cereus* and relatives. Environ. Microbiol. 2003; 5(8):631-40.
157. von Eiff C, Peters G, Heilmann C. Pathogenesis of infections due to coagulase-negative staphylococci. Lancet Infect. Dis. 2002; 2(11):677-85.
158. Jenkins SG. Infections due to anaerobic bacteria and the role of antimicrobial susceptibility testing of anaerobes. Rev. Med. Microbiol. 2001; 12:1-12.
159. Nielsen AT, Tolker-Nielsen T, Barken KB and Molin S. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. Environ. Microbiol. 2000; 2(1): 59-68.
160. Xu J and Gordon JI. Honor thy symbionts. Proc. Natl. Acad. Sci. USA 2003; 100(18): 10452-9.
161. Lee A, Fox J and Hazell S. Pathogenicity of *Helicobacter pylori*: a perspective. Infect. Immun. 1993; 61(5):1601-10.
162. Seydel A, Tascs E, Berti D, Rappuoli R, Del Giudice G and Montecucco C. Characterization and immunogenicity of the CagF protein of the *cag* pathogenicity island of *Helicobacter pylori*. Infect. Immun. 2002; 70(11): 6468-70
163. Uemura N, Okamoto S, Yamamoto N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N and Schlemper R. *Helicobacter pylori* infection and the development of gastric cancer. N. Engl. J. Med. 2001; 345:784-9.

164. Justice SS, Hung C, Theriot JA, fletcher DA, Anderson GG, Footer MJ and Hultgren SJ. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Proc. Natl. Acad. Sci. USA 2004; 101(5):1333-8.
165. Kawase M, Harada H, Saito S, Cui J, and Tani S. *In vitro* susceptibility of *Helicobacter pylori* to trifluoromethyl ketones. Bioorg. Med. Chem. Lett. 1999; 9:193-4.
166. Kawase M, Motohashi N, Sakagami H, Kanamoto T, Nakashima H, Ferenczy L, Wolfard K, Miskolci Cs, Molnar J. Antimicrobial activity of trifluoromethyl ketones and their synergism with promethazine. Int. J. Antimicrob. Agents 2001; 18:161-5
167. Josenhans C, Suerbaum S. The role of motility as a virulence factor in bacteria. Int. J. Med. Microbiol. 2002; 291:605-14.
168. Minamino M, Imae Y, Oosawa F, Kobayashi Y and Oosawa K. Effect of intracellular pH on rotational speed of bacterial flagellar motors. J. Bacteriol. 2003; 185(4):1190-4.
169. Berg HC. Constraints on the models for the flagellar motor. Philos. Trans. R. Soc. Lond. 2000; B335:491-502.
170. Francis NR, Sosinsky GE, Thomas D and DeRosier DJ. Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. J. Mol. Biol. 1994; 235:1261-70.
171. McLoughlin RM, O'Morain CA, O'Connor HJ. Eradication of *Helicobacter pylori*: recent advances in treatment. Fundam. Clin. Pharmacol. 2005; 19(4):421-7.
172. Vaira D, Gatta L, Ricci C, Tampieri A, Cavina M, Bernabucci V, Miglioli M. Peptic ulcer and *Helicobacter pylori*: update on testing and treatment. Postgrad. Med. 2005; 117(6):17-22, 46.
173. Blackmore CG, McNaughton PA, van Veen HW. Multidrug transporters in prokaryotic and eukaryotic cells: physiological functions and transport mechanisms. Mol. Membr. Biol. 2001; 18:97-103.
174. Wang RB, Kuo CL, Lien LL and Lien EJ. Structure-activity relationship: analyses of P-glycoprotein substrates and inhibitors. J. Clin. Pharm. Ther. 2003; 28: 203-28.
175. Chang G. Multidrug resistance ABC transporters. FEBS Letters 2003; 102-105.
176. Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. The Oncologist 2003; 8: 411-424.
177. Mascini EM, Bonten MJ. Vancomycin-resistant enterococci: consequences for therapy and infection control. Clin Microbiol Infect. 2005 Jul;11 Suppl 4:43-56.
178. Davis DR, McAlpine JB, Pazoles CJ, Talbout MK, Alder EA, White C, Jonas BM, Murray BE, Weinstock GM and Rogers BL. *Enterococcus faecalis* multi-drug transporters: application for antibiotic discovery. J. Mol. Microbiol. Biotechnol. 2001; 3:179-84
179. Lee EW, Chen J, Huda N, Kuroda T, Mizushima T and Tsuchiya T. Functional cloning and expression of *emeA* and characterization of EmeA, a multidrug efflux pump from *Enterococcus faecalis*. Biol. Pharm. Bull. 2003; 26(2):266-70.
180. Lee EW, Huda N, Kuroda T, Mizushima T and Tsuchiya T. EfrAB, an ABC efflux pump in *Enterococcus faecalis*. Antimicrb. Agents Chemother. 2003; 47(12):3733-8.

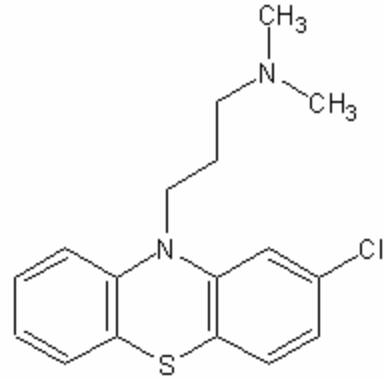
IX. ANNEX I.

CHEMICAL STRUCTURES OF TESTED COMPOUNDS

9.1. Phenothiazines



Promethazine



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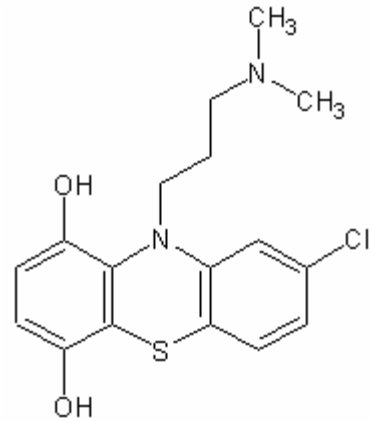
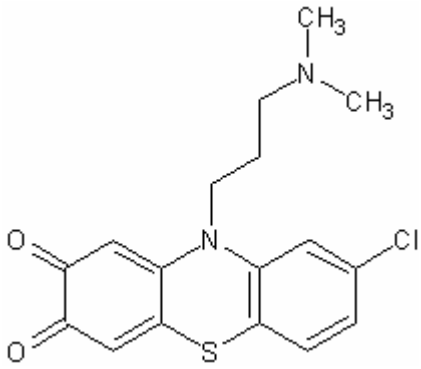


6,9-dihydroxichlorpromazine



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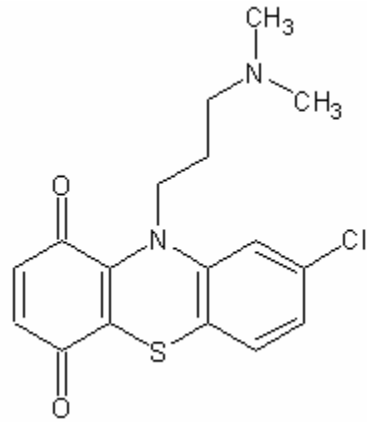

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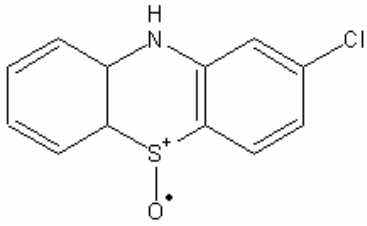
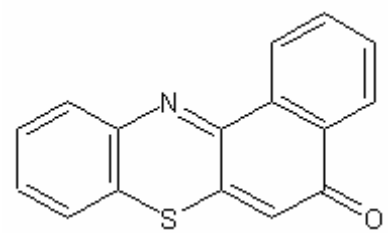
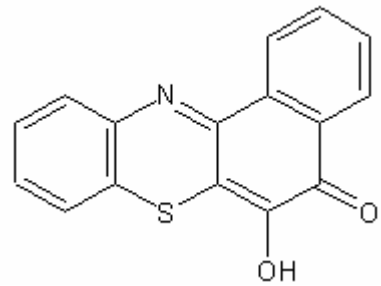


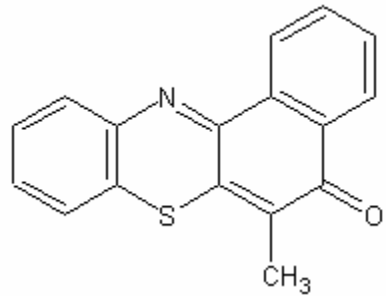
2-chloro-5-oxo-5H-phenothiazine



5-oxo-5H-benzo[a] phenothiazine



6-hydroxi-5oxo-5H-benzo[a] phenothiazine



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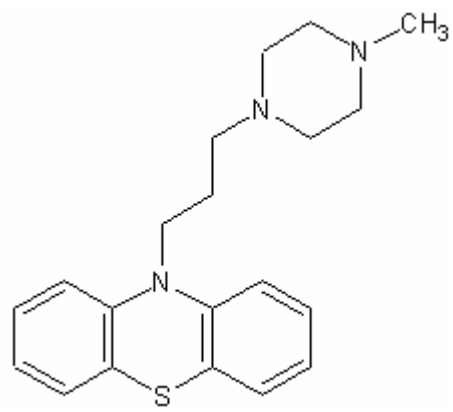
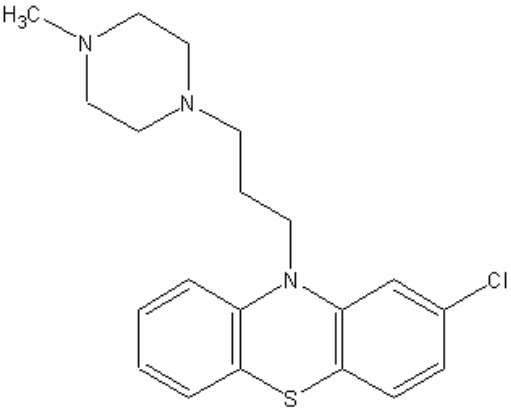
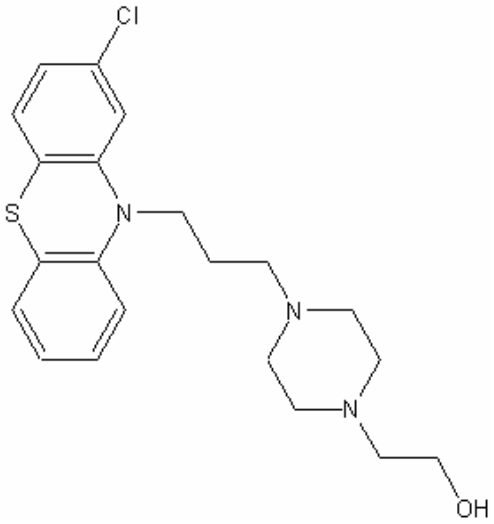


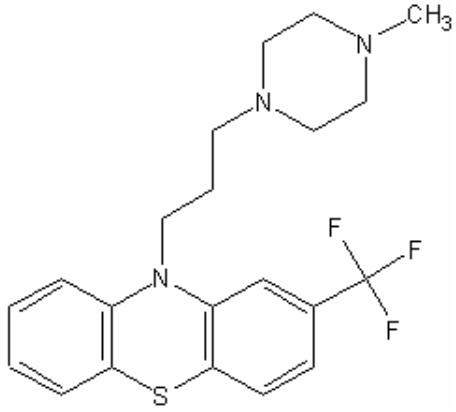
Perazine dimaleate



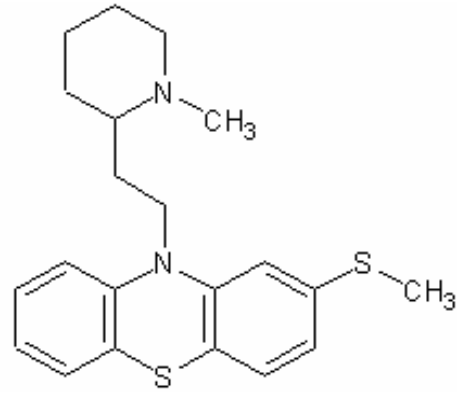
Prochlorperazine



Perphenazine

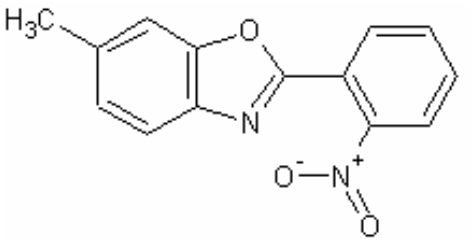


Trifluperazine

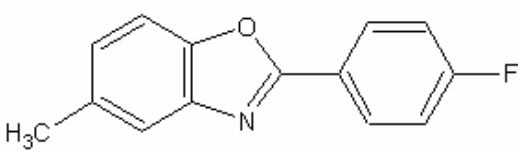


Thioridazine

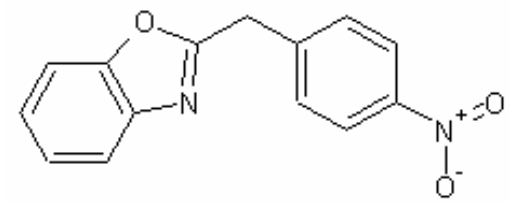
9.2. Benzoxazoles



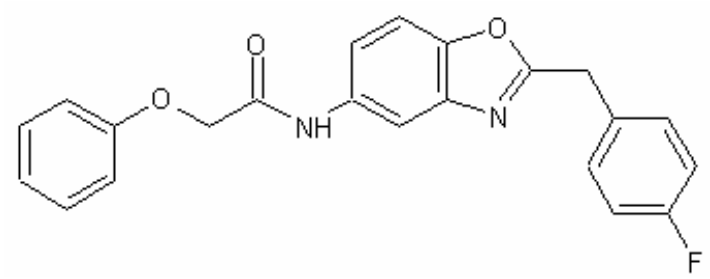
A9: 6-methyl-2-(2-nitrophenyl)-1,3-benzoxazole



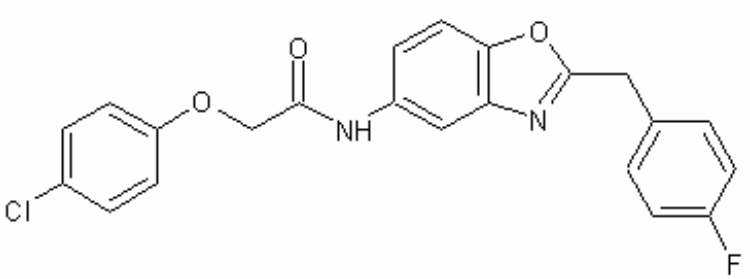
A33: 2-(4-fluorophenyl)-5-methyl-1,3-benzoxazole



B11: 2-(4-nitrobenzyl)-1,3-benzoxazole

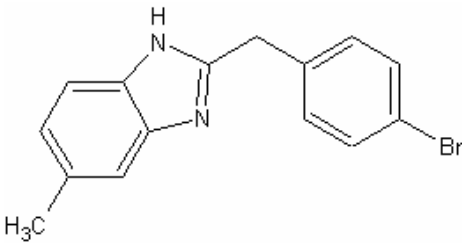


D27: *N*-[2-(4-fluorobenzyl)-1,3-benzoxazol-5-yl]-2-phenoxyacetamide



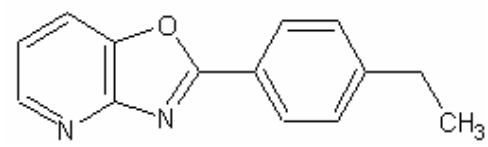
D34: 2-(4-chlorophenoxy)-*N*-[2-(4-fluorobenzyl)-1,3-benzoxazol-5-yl]acetamide

9.3. Benzimidazoles



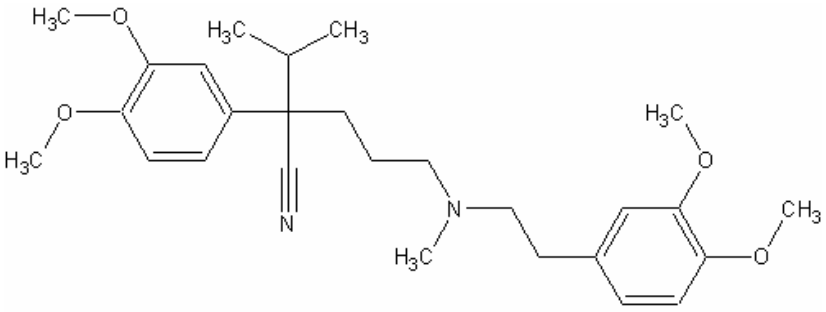
G17: 2-(4-bromobenzyl)-5-methyl-1*H*- benzimidazole

9.4. Oxazolo(4,5-b)pyridines



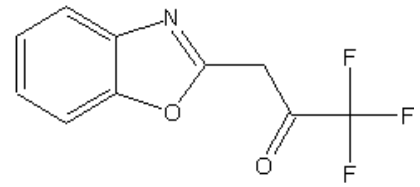
F1: 2-(4-ethylphenyl)[1,3]oxazolo[4,5-*b*]pyridine

9.5. Phenylalkylamines



Verapamil

9.6. Trifluoromethyl-ketones



TF18: 2-benzoxazoyl-9-3,3,3-trifluoro-2-propanone

X. ANNEX II.

DESCRIPTIONS OF MEDIA

10.1. Growth media for bacteria

MTY (Minimal-Tryton-Yeast extract)

NH_4Cl (Reanal)	1%
K_2HPO_4(Reanal)	7%
$NaH_2PO_4x2H_2O$ (Reanal)	3%
NaCl (Reanal)	2%
Tryptone (Reanal)	10%
Yeast Extract (Difco)	1%
Agar (Biolab)	1.5%

pH was adjusted with 10 M NaOH (Reanal).The media was autoclaved at 121°C, 0.5 MPa for 20 minutes.

Brain Heart Infusion broth (Merck), Eosine Methylene Blue agarplates (bioMérieux), Tryptic Soy Broth (Biolab) were prepared according to the instructions of the manual.
Hollmann's media was purchased from the Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged.

10.2. Media for cancer cell lines

McCoy's media modified (Gibco) for L5178Y mouse lymphoma cell line (PAR) was supplemented with:

Streptomycin (Sigma)	1%
Nystatin (Gibco)	0.1%
L-Glutamine (Gibco)	200mM
Heat inactivated horse serum (Gibco)	10%

Culture media for L4178 (MDR) was supplemented with 60 ng/ml colchicine to maintain MDR phenotype.

Samples	Concentration (μg/ml)	Number of colony formers (x10^7 CFU/ml)	Ratio of plasmid curing (mean %)
Control (untreated)	0	17.30	0.00
Promethazine	20	1.64	0.00
	40	2.07	1.05
	60	0.96	17.90
	80	0.21	88.50
	100	MIC	-
Perazine dimaleate	20	40.90	0.02
	40	20.50	0.00
	60	19.60	0.23
	80	12.50	0.04
	100	0.001	27.27
2-chloro-5-oxo-5H-phenothiazine	20	27.80	0.03
	40	31.10	0.00
	60	24.60	0.004
	80	17.20	0.25
	100	0.003	0.00
Perphenazine	20	10.20	0.12
	40	8.98	10.77
	60	4.63	1.05
	80	0.20	0.00
	100	$<10^3$	0.00

Table 7. Detailed data of co-culture experiments.

	Time (days)	Mean count of *S. epidermidis* with *E. coli* K12LE140 (x10^{6}CFU/ml)	Sd(yEr±) (x10^6)	Mean count of *S. epidermidis* in the control culture (x10^{6}CFU/ml)	Mean count of *E. coli* with *S. epidermidis* (x10^{6}CFU/ml)	Sd(yEr±) (x10^6)	Mean count of *E. coli* K12LE140 in the control culture (x10^{6}CFU/ml)	Mean of F'lac plasmid curing rate	Sd(yEr±)
23°C	0	184	0.00	184	0.0003	0.00		0.00	0.00
	1	218	111	244	1.55	0.51	335	0.00	0.00
	3	18.1	4.69	214	83.60	21.50	50.00	0.61	0.35
	6	0.33	0.06	176	332	80.90	330	0.48	0.25
	9	0.33	0.06	122	222	46.10	304	0.48	0.15
37°C	0	184	0.00	184	0.0003	0.00	310	0.00	0.00
	1	9.33	7.09	198	90.30	81.70	285	5.86	6.48
	3	0.33	0.06	121	57.70	28.10	1.00	31.30	19.20
	6	0.00	0.00	54.40	35.50	16.80	40.60	35.40	13.80
	9	0.00	0.00	27.20	53.10	31.80	15.60	9.22	8.96
39°C	0	184	0.00	184	0.0003	0.00	310	0.00	0.00
	1	9.20	4.33	75.60	51.60	7.25	39.40	0.91	0.54
	3	0.00	0.00	2.40	59.10	60.90	3.00	1.01	1.76
	6	0.00	0.00	32.40	45.30	8.53	31.60	2.87	1.55
	9	0.00	0.00	5.50	23.90	10.30	1.00	1.21	1.11

Table 8. Detailed data of co-culture experiments.

	Time (days)	Mean count of *B. cereus* with*E. coli* K12LE140 (x10^{6}CFU/ml)	Sd(yEr±) (x10^6)	Mean count of *B. cereuss* in the control culture (x10^{6}CFU/ml)	Mean count of *E. coli* with *B. cereus* (x10^{6}CFU/ml)	Sd(yEr±) (x10^6)	Mean count of *E. coli* K12LE140 in the control culture (x10^{6}CFU/ml)	Mean of F'lac plasmid curing rate	Sd(yEr±)
23°C	0	68.00	0.00	68.00	0.0077	0.00	77.00	0.00	0.00
	1	19.30	5.87	7.00	12.20	1.30	443	0.37	0.64
	3	4.83	1.50	2.00	2.04	65.30	844	0.19	0.21
	6	1.73	0.86	14.70	3.05	93.60	347	0.30	0.07
	9	2.53	0.10	19.40	1.57	54.70	199	0.45	0.18
37°C	0	68.00	0.00	68.00	0.0077	0.00	77.00	0.00	0.00
	1	2.45	0.75	8.00	84.70	7.90	380	0.13	0.03
	3	1.07	0.55	7.40	82.20	17.70	151	0.00	0.00
	6	0.39	0.15	14.30	57.40	5.01	44.90	0.49	0.69
	9	0.13	0.39	6.40	72.20	25.80	37.20	1.18	1.11
39°C	0	68.00	0.00	68.00	0.0077	0.00	77.00	0.00	0.00
	1	2.08	0.23	5.00	54.30	12.60	158	0.65	0.75
	3	0.04	0.03	5.40	62.40	10.20	8.10	.16	0.15
	6	0.03	0.02	0.41	38.10	8.15	43.60	0.33	0.37
	9	0.09	0.07	3.20	29.00	7.72	19.70	0.97	0.92

Table 9/A. Detailed data of promethazine induced F'lac plasmid elimination with non-shaking and with shaking (**B**) under aerobic conditions.

A	**Concentration of promethazine (µg/ml)**	**Mean value of F'lac plasmid curing rate in the control culture of *E. coli* K12LE140**	**Sd(yEr±)**	**Mean value of F'lac plasmid curing rate in mixed culture with *B. cereus***	**Sd(yEr±)**	**Mean value of F'lac plasmid curing rate in mixed culture with *S. epidermidis***	**Sd(yEr±)**
23°C	0	0.00	0.00	0.99	1.09	0.133	0.23
	20	0.01	0.01	0.00	0.00	0.28	0.49
	40	0.36	0.03	0.03	0.05	0.10	0.17
	60	0.29	0.23	0.00	0.00	0.01	0.02
	80	0.39	0.34	0.00	0.00	0.00	0.00
	100	0.05	0.06	0.00	0.00	0.00	0.00
37°C	0	0.00	0.00	0.00	0.00	0.00	0.00
	20	0.00	0.00	28.27	1.43	12.43	1.50
	40	0.80	0.52	43.65	18.73	37.63	8.86
	60	21.76	9.85	25.16	1.63	87.47	5.46
	80	79.81	6.54	11.33	1.53	27.07	14.53
	100	0.00	0.00	0.00	0.00	0.00	0.00
39°C	0	0.00	0.00	0.00	0.00	0.00	0.00
	20	0.03	0.02	53.33	0.61	20.81	5.46
	40	14.76	6.07	96.52	2.13	83.87	11.98
	60	87.49	5.19	95.53	2.87	97.21	3.98
	80	89.12	8.64	0.00	0.00	16.84	2.59
	100	0.00	0.00	0.00	0.00	0.00	0.00

B	**Concentration of promethazine (µg/ml)**	**Mean value of F'lac plasmid curing rate in the control culture of *E. coli* K12LE140**	**Sd(yEr±)**	**Mean value of F'lac plasmid curing rate in mixed culture with *B. cereus***	**Sd(yEr±)**	**Mean value of F'lac plasmid curing rate in mixed culture with *S. epidermidis***	**Sd(yEr±)**
23°C	0	0.08	0.00	0.00	0.00	0.00	0.00
	20	0.06	0.00	0.00	0.00	0.13	0.00
	40	0.03	0.00	0.00	0.00	0.00	0.00
	60	0.19	0.00	0.00	0.00	0.08	0.00
	80	0.30	0.00	0.00	0.00	0.22	0.00
	100	0.00	0.00	0.00	0.00	0.00	0.00
37°C	0	0.33	0.46	0.00	0.00	0.00	0.00
	20	0.22	0.30	0.00	0.00	0.20	0.00
	40	0.00	0.00	0.00	0.00	0.57	0.00
	60	1.26	1.12	0.00	0.00	60.77	0.00
	80	77.62	22.22	0.00	0.00	0.00	0.00
	100	0.00	0.00	0.00	0.00	0.00	0.00
39°C	0	0.09	0.12	0.00	0.00	0.00	0.00
	20	0.36	0.51	0.00	0.00	0.00	0.00
	40	0.16	0.22	12.40	0.00	1.40	1.98
	60	10.20	13.44	51.80	0.00	68.95	38.82
	80	28.57	0.79	8.40	0.00	97.295	3.67
	100	98.00	2.83	0.00	0.00	0.00	0.00

Table 10. Detailed data of promethazine induced F'lac plasmid elimination under anerobic conditions.

	Concentration of promethazine (µg/ml)	Mean value of F'lac plasmid curing rate in the control culture of *E. coli* K12LE140	Sd(yEr±)	Mean value of F'lac plasmid curing rate in mixed culture with *B. fragilis* ATCC 25285	Sd(yEr±)
23°C	0	0.49	0.44	0.00	0.00
	20	0.59	0.36	0.00	0.00
	40	0.70	0.44	0.00	0.00
	60	0.36	0.41	0.00	0.00
	80	0.12	0.22	0.00	0.00
	100	0.00	0.00	0.00	0.00
37°C	0	0.00	0.00	0.04	0.07
	20	0.25	0.23	0.02	0.03
	40	1.20	0.97	0.08	0.09
	60	2.22	0.89	0.04	0.04
	80	21.24	1.07	2.41	4.19
	100	96.48	2.17	0.88	1.20
	120	98.33	2.89	1.11	1.68
39°C	0	0.00	0.00	0.00	0.00
	20	0.21	0.21	0.00	0.00
	40	1.64	0.86	0.00	0.00
	60	20.22	18.82	0.27	0.32
	80	89.10	10.44	26.01	15.04
	100	98.97	1.77	45.29	20.03
	120	0.00	0.00	76.31	4.81
	140	0.00	0.00	52.58	17.79

Table 11. Detailed data of TF18 treated *E. coli* AG100 cells.

Concentration of TF18 in % of MIC	Mean value of non-motile cells	Sd(yEr±)	Mean value of tumbling cells	Sd(yEr±)	Mean value of swimming cells	Sd(yEr±)
Control	15.76	6.21	43.25	2.94	44.18	10.53
10%	53.70	11.08	33.23	3.48	13.10	9.61
50%	58.33	5.83	37.25	6.30	4.33	1.87
90%	83.90	5.75	14.33	3.87	1.78	2.08
200%	83.66	8.31	16.33	8.31	0.00	0.00

Table 12. Detailed data of TF18 treated *E. coli* AG100 A cells.

Concentration of TF18 in % of MIC	Mean value of non-motile cells	Sd(yEr±)	Mean value of tumbling cells	Sd(yEr±)	Mean value of swimming cells	Sd(yEr±)
Control	60.00	8.69	29.78	4.54	10.28	5.27
10%	70.33	7.13	20.48	4.62	9.20	3.18
50%	69.30	10.30	24.18	7.06	6.53	3.39
90%	83.68	4.92	14.78	4.98	1.50	1.73
200%	66.30	2.83	27.68	4.56	6.00	2.14