INTERACTIONS BETWEEN ANTIBIOTICS AND NON - CONVENTIONAL ANTIBIOTICS ON RESISTANT BACTERIAL STRAINS

Ph.D. THESIS

DR. GYÖNGYI GUNICS

University of Szeged Faculty of Medicine

Department of Medical Microbiology and Immunobiology

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I. INTRODUCTION

Recent years have witnessed a rapidly growing crisis in antimicrobial resistance, especially among microorganisms that cause nosocomial infections. The first antibiotic, penicillin, was discovered in 1929 by *Sir Alexander Fleming*. Penicillin became generally available for treatment of bacterial infections, and particularly those caused by staphylococci and streptococci, around 1946. The period of late 1940s and early 1950s saw the discovery and introduction of streptomycin, chloramphenicol and tetracycline, and the age of bacterial chemotherapy came into full being. These antibiotics were effective against the full array of bacterial pathogens, including Gram-positive and Gram-negative bacteria (e.g. *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*), intracellular parasites and tuberculosis bacillus. However, by 1957, during a Shigella outbreak in Japan, a strain of dysentery bacillus was isolated which was multiple drug resistant, exhibiting resistance to chloramphenicol, tetracycline, streptomycin and the sulphonamide (1).

Drug-resistant infectious agents are an increasingly important public health concern. Antimicrobial resistance is becoming a factor in virtually all hospital-acquired or nosocomial infections. Resistance to antimicrobial agents among bacteria and fungi is a persistent problem the complicates the management of cirtically ill patients (2).

Staphylococcus aureus and enterococci are most commonly isolated bacteria that cause nosocomial infections. Among those giving therapeutic problems are methicillin-resistant staphylococci and vancomycin-resistant enterococci (3).

When penicillin was introduced in 1944 over 94% of *S. aureus* isolates were susceptible; by 1950 half were resistant. By 1960, many hospitals had outbreaks of virulent multiresistant *S. aureus*. These were overcome with penicillinase-stable penicillins, but victory was brief; methicillin-resistant *S. aureus* (MRSA) were recorded in the year of the drug's launch. MRSA owe their behaviour to an additional, penicillin-resistant peptidoglican transpeptidase, PBP-2', encoded by *mecA* gene. Their spread is clonal, with transfer of *mecA* being extremely rare. MRSA accumulated and then declined in the 1960s and 1970s, but became re-established in the early 1980s. These may be more virulent than earlier MRSA, or their success may reflect changing hospital practice. Until 1996, glycopeptides were universally active against *S. aureus*; then glycopeptide-intermediate *S. aureus* (GISA) were found in Japan, France and the USA. This resistance is associated with increased wall synthesis.

Coagulase-negative staphylococci (CNS) are less pathogenic than *S. aureus* but are important in line-associated bacteraemias and prosthetic device infections. They are even more often resistant than *S. aureus*, notably to teicoplanin. Few anti-staphylococcal agents were launched from 1970 to 1995, but the situation is now improving (4).

The wide ranging application of antimicrobials in medical and veterinary practice, the usage of antibiotics in agriculture, and the common use of antiseptics and disinfectants result in selection pressure. The administration of antibiotics directly selects resistant variants to different antibiotics or disinfectants. The same genetic element (e. g. *qac* or *smr*) that confer resistance to some disinfectants are often present on the same plasmid conferring resistance to antibiotic.

2. ANTIBIOTIC RESISTANCE: PLASMIDS AND EFFLUX PUMPS

There are several nongenetic reasons for the failure of drugs to inhibit the growth of bacteria.

1. Bacteria can be walled of within an abscess cavity which the drug cannot penetrate
2. effectively. Surgical drainage is therefore a necessary adjunct to chemotherapy.

2. Bacteria can be in a resting state, ie, not growing. They are therefore insensitive to cell wall inhibitors such as penicillins and cephalosporins. Similarly, *Mycobacterium tuberculosis* can remain dormant in tissues for many years, during which time it is insensitive to drugs. If host defenses are lowered and the bacteria begin to multiply, they are again susceptible to the drugs, indicating that a genetic change did not occur.

3. Under certain circumstances, organisms that would ordinarily be killed by penicillin can lose their cell walls, survive as protoplasts, and be insensitive to cell-wall-active drugs. Later, if such organisms resynthesize their cell walls, they are fully susceptible to these drugs.

4. Several artificats can make it appear that the organisms are resistant, eg. administration of the wrong drug or the wrong dose, failure of the drug to reach the appropriate site in the body (a good example is the poor penetration into spinal fluid by several early-generation cephalosporins), or failure of the patient to take the drug (5).

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

Bacteria can develop resistance to antibiotics, e.g. bacterial populations previously sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria:
Mutation and *selection* (sometimes referred to as vertical evolution). Antibiotic resistance can be either *plasmid* mediated or maintained on bacterial *chromosome* (5).

The fundamental mechanisms of antimicrobial resistance are:

® species specific sensitivity of *Streptococcus pneumoniae* was known

® mutation alteration of bacterial proteins that are antimicrobial targets /Examples:

- erythromycin resistant organisms have an altered receptor on the 50S subunit of the ribosome that serves as a binding site in susceptible organisms;
- resistance to some penicillins may be a function of the loss, or alteration of PBPs (Penicillin Binding Proteins);
- development of an altered enzyme that can still perform its metabolic function but its much less affected by the drug than the enzyme in the susceptible organism /Example: in some sulphonamide-susceptible bacteria, the tetrahydropteroic acid synthetase has a much higher affinity for sulphonamide than for PABA (6).
- in sulphonamide-resistant mutant, the opposite is the cause/.

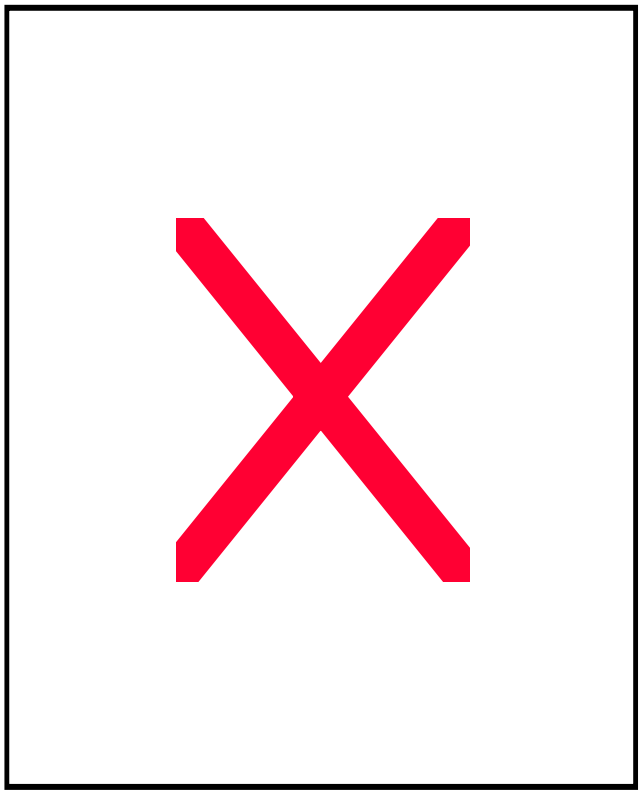


Figure 1. Five major biochemical mechanisms of antibiotic resistance

Mechanism of antibiotic resistance in bacteria: the many mechanisms that bacteria exhibit to protect themselves from antibiotics can be classified into four basic types (Figure 1).

✞infectious resistance mediated by plasmid, phage or Tn elements

- enzymatic degradation of antibacterial drugs
- changes in membrane permeability to antibiotics
- development of an altered enzyme that can still perform its metabolic function but its much less affected by the drug than the enzyme in the susceptible organism
- altered metabolic pathway that bypasses the reaction inhibited by the drugs www. bmj.com. (6).

2. 1. Mutation and resistance

Chromosomal resistance is due to a mutation in the gene that codes for either the target of the drug or the transport system in the membrane that controls the uptake of the drug. The frequency of spontaneous mutations usually ranges from 10^{-7} to 10^{-9}, which is much lower than the frequency of acquistion of resistance plasmids. Therefore, chromosomal resistance is less of a clinical problem than is plasmid-mediated resistance.

Mutation in the chromosome is a primary cause of bacterial resistance to antibiotics. Some mutations promote resistance directly (e.g. quinolone resistance mutations in genes encoding its *E. coli* targets, *gyrA* and *gyrB* (7). Other chromosomal mutations can cause mutator phenotypes that increase the likelihood of acquiring a resistance mutation (8). Mutations also ameliorate the otherwise deleterious effects on cell growth and physiology of some antibiotic resistance-conferring mutations (9). Although antibiotic resistance has been studied intensively, the mechanisms that generate resistance mutations are poorly understood.

In addition to spontaneous mutation in exponentially growing cells (growth-dependent mutation), other mutation mechanisms exist that may pertain to antibiotic (10), and adaptíve mutations produce resistance to ciprofloxacin (11, 12). For example, factors such as antibiotic concentration (13), environmental conditions (14), or other stress-inducing phenomenon that leads to resistance mutations (15, 16).

In some enterobacterial pathogens, but not in *E. coli,* loss of function, mutations in the *ampD* gene are a common route to β-lactam antibiotic resistance (17). It was constructed an assay system for studing mechanisms of enterobacterial *ampD* mutation using the well-developed genetics of *E. coli*. It was integrated the *Enterobacter ampRC* genes into the *E. coli* chromosome. These cells acquire spontaneous recombination independent β-lactam resistance mutation in *ampD*. This chromosomal system is useful for studying mutation mechanisms that promote antibiotic resistance (17).

2. 2. Infectious resistance

Exchange of genes between strains and species (sometimes called horizontal evolution) via different mechanisms.

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

Horizontal evolution is the acquisition of genes for resistance from another organism. Some bacterium develops genetic resistance through the process of mutation and selection and then donates these genes to some other bacteria through one of several processes for genetic exchange that exist in bacteria.

Bacteria are able to exchange genes in nature by four processes: *conjugation, transduction, transformation and transposition* (18, 19). *Conjugation* involves cell-to-cell contact as DNA crosses a sex pilus from donor to recipient (20). This is the commenest method by which multidrug resistance spreads among different genera of Gram-negative bacteria. Transfer of the resistance plasmids (21, 22) also occurs among some Gram-positive cocci. During *transduction*, a virus transfers the genes between mating bacteria. For example the plasmid carrying the gene for β-lactamase production can be transferred from penicillin resistant to a susceptible *Staphylococcus* spp. if carried by a suitable bacteriophage (22). Similar transduction occurs is salmonellae.

In *transformation,* DNA is acquired directly from the environment, having been released from another cell (23). Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). It is common for DNA to be transferred as *plasmids* between mating bacteria. Since bacteria usually develop their genes for drug resistance on plasmids (called resistance transfer factor; RTF), they are able to spread drug resistance to other strains and species during genetic exchange processes. *Transposition* is a transfer of short DNA sequences (transposons, transposable elements) occurs between one plasmid and another or between a plasmid and a portion of the bacterial chromosome within a bacterial cell (24, 25).

2. 3. Plasmid mediated resistance

Plasmid-mediated resistance is very important from a clinical point of view for 3 reasons:

1. it occurs in a many different species, especially Gram-negative rods;

2. plasmids frequently mediate resistance to multiple drugs;

3. plasmids have a high rate of transfer from one cell to another, usually by conjugation.

Antibiotic resistance genes eg. tetracycline, kanamycin, chloramphanicol, erythromycin are often encoded by transmissible conjugative or non-conjugative plasmids (5).

Resistance plasmids (resistance factors, R factors) are extra chomosomal, circurales double-stranded DNA molecules that carry the genes for a variety of enzymes that can degrade antibiotics and modify membrane transport systems. The most important mechanisms of resistance for several important drugs:

- Penicillins and cephalosporins: β-lactamase cleavage of β-lactam ring;
- Aminoglycosides: modifications by acetylation, adenylation, or phosphorylation
- Chloramphenicol: modification by acetylation
- Erythromycin: change in receptor by methylation of RNA
- Tetracycline: reduced uptake or increased export
- Sulphonamides: active export out of the cell and reduced affinity of enzyme.

In addition to producing drug resistance, R factors have 2 very important properties:

1**.** they can replicate independently of the bacterial chromosome, so that a cell can contain many copies;

2**.** they can be transferred not only to cells of the same species but also to other species and genera. Note that this conjugal transfer is under the control of the genes of the R plasmid and not of the F (fertility) plasmid, which governs the transfer of the bacterial chromosome (5).

R factors exist in 2 broad size categories: large plasmids with molecular weights of about 60 million dalton and small ones with molecular weights of about 10 million. The large plasmids are conjugative R factors, which contain the extra DNA to code for the conjugation process, whereas small R factors are not conjugative and contain only the resistance genes.

In addition to converying antibiotic resistance, R factors impart 2 other traits:

a. resistance to metal ions (e.g. they code for an enzyme that reduces mercuric ions to elemental mercury), and

b. resistance to certain bacterial virus by coding for restriction endonucleases that

degrade the DNA of the infecting bacteriophages (5).

2. 4. 1. Plasmids

Bacteria are hosts to small, extrachromosomal genetic elements called plasmids, which are dispensable to the cell under ordinary conditions of growth. Plasmids share many properties with bacterial viruses (phages), from which they differ chieffy in their lack of an encapsidated, extracellular phase. They are of major clinical significance, not only because they may carry genes for resistance to therapeutic drugs and for virulence factors, as discussed below, but also because many of them mediate gene transfer- a process that leads to the emergence of bacterial strains with new combination of drug resistance, antigens, and virulence mechanisms (5).

R plasmid: R plasmids were first discovered in Japan in 1957 (26). In Japan, dysentery was treated with sulphonamide until about 1950. Then more and more strains of bacteria causing dysentery became resistant to this antibiotic, rapidly rendering in ineffective. Then tetracycline, streptomycin and chloramphenicol were used against these bacteria. By 1957, 2 % of the bacteria causing dysentery were resistant to one or more of these drugs, and by 1960, 13 % were resistant. Resistance or R plasmids carry genes that confer resistance to certain antibiotics as well as to poisonous metal ions such as arsenic, silver, copper, mercury, lead, zinc and so on (27). The R plasmid usually has two types of genes:

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

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

F plasmid*:* facilities conjugation. F plasmids live in bacterium *E. coli* and were discovered 1920s (28). This can give a bacterium new genes that may help it survive in a changing environment. The F plasmid consists of 25 genes that mostly code for production of sex pili. Bacteria that have an F plasmid are F^+ or male. Those that do not have an F plasmid are F^- or female. F plasmid can behave as an *episome*-it means that it can integrate reversibily

into the bacterial chromosome. When the F^+ plasmid integrated within the bacterial chromosome, the cell is called an Hfr cell (high frequency of recombination cell). A cross over event can occur between homologous genes of the Hfr fragment and the F-DNA. The recombinant genome can be passed on to future generations (28).

***Colicin plasmids*:** The colicins of *E. coli* are members of the large and diverse family of antimicrobial toxins known as bacteriocins. The characterised colicins /over 20/ can be divided into two major classes according to their mode of action; the enzymatic and the channel-forming colicins (29). Members of both classes of colicins share a number of characteristics, but they share a low level protein sequence similarity (30, 31). Colicin gene clusters consist of three tightly linked genes: a colicin gene; an immunity gene, which encodes an immunity protein that provides specific protection against the encoded colicin a lysis gene, which encodes a protein that is involved in colicin release from the cell. Colicin gene clusters are encoded on plasmid replicons (32, 33).

***Virulence plasmids*:** virulence plasmids contain genes that make their bacterial hosts more virulent to "their" hosts. A familiar example involves the bacterium *E. coli*, which inhabits the human large intestine. Certain strains of *E. coli* contain plasmids whose genes make the *E. coli* synthesize toxins that cause diarrhoea (34, 35). These "enterotoxogenic strains" of *E. coli* are probably an important cause of diarrhoea among travellers (36). More seriously, in developing countries, diarrhoea is one of the principal causes of death among those under five (37).

VirG, a new pYV gene involved in growth restriction described. The expression and secretion of ExoS are also induced by growing *P. aeruginosa* in a medium containig a Ca^{2+}-chelating agent. The VirG and ExsB are in the synthesis or secretion of virulence proteins (38). Pathogenic bacteria of the genus *Yersinia* (*Y. pestis, Y. pseudotuberculosis*, and *Y. enterocolitica*) cause diseases in rodents and humans, with symptoms the host tissues. They harbour closely related plasmids of about 70 kb that are essential for virulence and hence are called pYP (for *Yersinia* virulence). At 37°C and in a medium deprived of Ca^{2+} ions, the pYv plasmid directs secretion of about 10 proteins called Yops. In *Y. enterocolitica* these are YopB, D, E, H, M, N, O, P, and Q and LcrV, the protective antigen described in the mid-1950s (39, 40, 41, 42).

***Metabolic plasmids*:** metabolic plasmids contain genes that let their bacterial hosts metabolise or degrade otherwise indigestible or toxic chemicals. For example, the bacterium

Pseudomonas putida is able to grow on a wide range of organic compounds that are toxic to most bacteria, including octane, camphor and naphthalene. It does this with the help of genes contained by metabolic plasmids called OCT, CAM and NAH plasmids (43).

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

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

Cosmids: cosmids are man-made circular loops of DNA containing plasmid DNA together with an arbitrary sequence of up to 45.000 base pairs of DNA. They are constructed by recombinant DNA techniques and then packaged in lambda phage protein coats. They are used to transfer genes to bacteria (44).

Phasmids: phasmids are man-made linear DNA molecules whose ends are sequences taken from the lambda phage, while the middle is a sequence taken from a plasmid, together with a seuqence of whatever DNA one wants. Like cosmids, they are constructed by recombinant DNA techniques and packaged in lambda phage protein coats, and used to transfer genes to bacteria. However, both the lambda phage and plasmid replication functions are intact. In particular, they contain the lambda phage genes for "lysis", the process where by a virus dissolves the cell membrane of its host. Depending on the conditions, the phasmid can act either like a highly defective prophage or a plasmid-hence its name (45, 46, 47).

2. 4. 2. Tn elements

Transposons are genes that are transferred either within or between larger pieces of DNA such as the bacterial chromosome and plasmids. A typical drug resistance transposon is composed of 3 genes flanked on both sides by shorter DNA sequences, usually a series of inverted repeated bases that mediate the interaction of the transposon with the larger DNA.
The 3 genes code:

1. transposase, the enzyme that catalyzes excision and reintegration of the transposons;
2. a repressor that regulates synthesis of the transposase;
3. the drug resistance gene (5).

2. 5. 1. Drug accumulation and efflux mechanisms

a. Increased production of penicillin-binding protein PBP4 is known to increase peptidoglycan cross-linking and contributes to methicillin resistance in *S. aureus*. The *pbp4* gene shares a 400-nucleotide intercistronic region with the divergently transcribed *abcA* gene, encoding an ATP-binding cassette transporter of unknown function (48).

b. The *pbp4* structural gene is separated by only 400 nucleotides from a divergently transcribed gene, *abcA*, which codes for an ATP-binding cassette (ABC) transporter-like

protein (49, 50). The ABC transporters constitute a large family of membrane transporter systems found in both procaryotic and eucaryotic cells. The contribute to the import or export of a wide range of substances such as proteins, peptides, polysaccharides, vitamins, and drugs, utilizing ATP as the source of energy (51).

Changes in membrane permeability to antibiotics /Examples: *Streptococci* have a natural permeability barrier to aminoglycosides; tetracyclines accumulate in susceptible bacteria but not in resistant bacteria, e.g. *P. aeruginosa* / (52, 53).

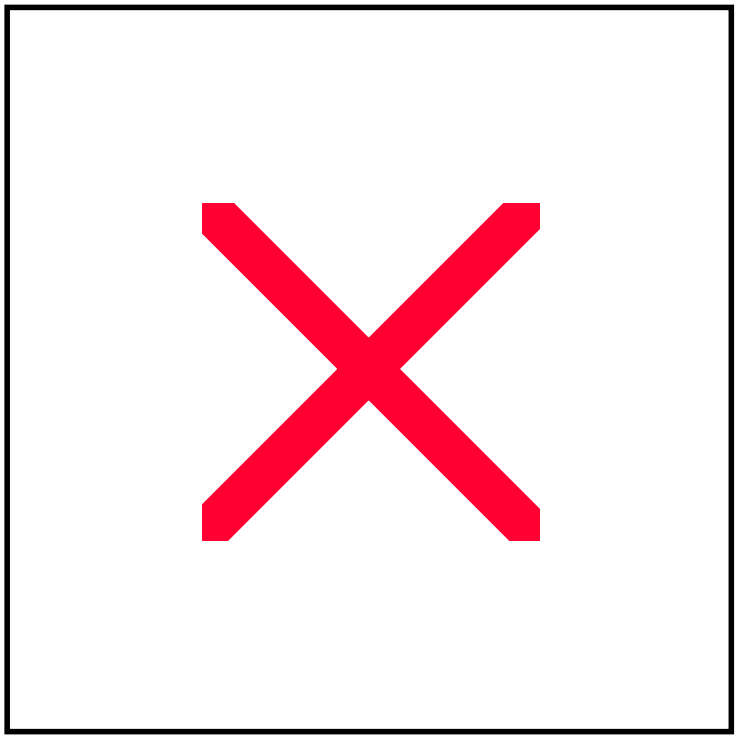


Figure 2. **GENETIC CODES FOR ANTIBIOTIC-RESISTANCE IN BACTERIA**

For example, such genes might code for "efflux" pumps that eject antibiotics from cells (**a**), or the genes might give rise to enzymes that degrade the antibiotics (**b**), or that chemically alter and inactivate drugs (**c**). Resistance genes can reside on the bacterial chromosome or, more typically, on small rings of DNA called plasmids.

Chromosome → Tn → plasmid (http://www.sciam.com) (54).

2. 5. 2. Efflux pumps and drug resistance

This efflux occurs due to the activity of membrane transporter proteins, the so-called drug efflux pumps (55). Some efflux pumps selectively extrude specific antibiotics, while others referred to as multidrug resistance (MDR) pumps, expel various structurally diverse

antibiotics. While antibiotic-specific efflux pumps are usually encoded on transmissible plasmids and tranposons, genes encoding many MDR pumps are normal constituents of bacterial chromosomes. Efflux pumps occur as either single-component or multicomponent systems. In Gram-negative bacteria, single-component efflux pumps extrude their substrates into the perisplasmic space (56). Examples of such single-component efflux pumps include the transposon-encoded tetracycline-and chloramphenicol-specific pumps TetA and CmlA, respectively (57, 58) and the MDR pump MdfA, encoded in the chromosome of *E. coli* (59). Multicomponent efflux pumps (which are found exclusively in Gram-negative bacteria) traverse both inner (IM) and outer membranes (OM). Examples include the MDR pumps AcrAB-TolC (60) and MexAB-OprM from *E. coli* and *P. aeruginosa*, respectively. Each pump contains a transporter located in the cytoplasmic membrane (61).

2. 5. 3. Efflux pumps in Gram-positive and Gram-negative bacteria

In eukaryotes, most ABC transporters are involved in multidrug resistance. However, in bacteria, the majority of drug exporters are drug-proton antiporters, including MFS-, SMR-, and RND-type transporters, while ABC transporters are usually involved in the uptake of a wide range of molecules. In Gram-positive organisms, some macrolide resistance genes code for ABC-type efflux transporter proteins. In the Gram-negative bacteria for example *E. coli* the tolerance to macrolide antibiotics is conferred by AcrAB. Expression cloning of an individual gene into an AcrAB deficient strain may be necessary to discover a potentional drug efflux transporter gene. MacB is an integral membrane protein and MacA is a periferial membrane protein. MacB is thus a novel ABC-type macrolide efflux transporter which functions by cooperating with the MFP (membrane fusion protein) MacA and the multifuntional outer membrane channel TolC. This is the first case of an experimentally identified ABC antibiotic efflux transporter in Gram-negative organisms (62).

ATP-binding cassette (ABC) transporters are the major drug efflux transporters in mammalian cells that cause multidrug resistance of cancer cells (63). On the other hand, ABC transporters are involved mainly in the uptake of a wide range of molecules (64), protein export and the efflux of toxic metal ions, such as an arsenite efflux system in bacteria (65).
LmrA is polypeptide in a Gram-positive bacterium, *Lactococcus lactis* (66) has been the only example of an experimentally identified bacterial ABC multidrug exporter. A small number of ABC single-drug exporters for macrolides and daunomycin (67, 68) are also known in Gram-positive bacteria, whereas no functional ABC multidrug exporter has been definitely established to exist in Gram-negative bacteria yet (69). The efflux-based multidrug resistance

of Gram-negative bacteria is most often conferred by RND (resistance-nodulation-cell division) family transporters (70, 71), in addition to some SMR (small multidrug resistance) transporters (72). Efflux-based single-drug resistance of Gram-negative bacteria is usually due to plasmid-encoded MFS (major facilitator superfamily) transporters such as tetracycline-H^+ antiporters (73, 74).

Active efflux of tetracycline (TC) is a resistance mechanism found in both Gram-negative and Gram-positive bacteria (75). In the Gram-negative bacterium *E.coli,* efflux resistance genes have been identified on both plasmids and transposons (the *tet* genes) and on the chromosome (76, 77). The TC resistance gene *tetA* [class B] in transposon Tn*10* (and other homologous resistance genes such as the *tetA* [class C] gene present in pBR322) codes for an integral inner membrane (IM) protein, Tet, which contains 12 transmembrane helices and belongs to a family of bacterial and eukaryotic transporters, the major facilitator family (78, 79). The Tet protein binds TC in the cytoplasm and pumps it, possibly into the periplasm, in exchage for a proton, a process that is driven by the proton motive force across the IM efflux pump (80).

Although tetracycline TC can diffuse readily through the IM bilayer (81, 82), the lipid bilayer of the OM is relatively impermeable to lipophilic solutes (83) such as TC, and TC is though to cross the OM mainly via the porin OmpF. In mutants with decreased OmpF expression (84).

Many of these pumps belong to the RND family, whose members have been known to pump out mostly lipophilic or amphiphilic molecules or toxic divalent cations (85, 86). The *E. coli* genome contains several genes coding for RND transporters. Among these, *acrB* is constitutively expressed and is largely responsible for the intrinsic resistance of *E. coli* to a tergents (including bile salts), and dyes (87, 88, 89, 90). The gene *acrF* has a high degree of similarity to *acrB* (77 % identify at the amino acid level, with no gaps) and is also expected to pump out a wide variety of lipophilic and ampiphilic agents. Indeed, AcrF overexpression strains can be isolated as suppressors of acrAB mutants and seem to have a resistance phenotype similar to that of the $acrAB^+$ wild-type strain.

However, disruption of the *acrF* gene in the wilde-type strain does not produce a drug susceptibility phenotype suggesting that *acrF* weakly expressed in wild-type *E.coli* (91).

Disruption of the *acrD* gene also did not result in hypersusceptibility to lipophilic and ampiphilic drugs. However recently RND-type transporters that pump out very hydrophilic compounds, aminoglycosides, have been observed in *P. aeruginosa* and in *Burkholderia pseudomallei* (92, 93) is an opportunistic human pathogen characterized by an innate

resistance to a variety of antimicrobial agents. Previously attributed to a highly impermeable outer membrane (94), this so-called intrisic multidrug resistance is now known to result from the synergy between broadly specific drug efflux pumps and a low degree of OM permeability (87). One such efflux system, a tripartite pump encoded by the MexAB-OprM operon (95), exports a range of antibiotics, including tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim, and β-lactams (96, 97). The β-lactam antibiotics are somewhat unique among these efflux substrates in that their cellular targets are within the periplasm. It was supposed that pumps indicating that OprM is not one or both of inner membrane-associated proteins MexA and MexB are responsible for drug recognition, including recognition of β-lactams (98).

2. 6. Proton motive forces and drug efflux

The isolation of a mutant of *S. aureus* having a phenotype consistent with the expression of a non-NorA MDR efflux transporter (99).

NorA is a proton motive force (PMF)-dependent MDR efflux pump in *S. aureus* (100). It is a member of the major facilitator superfamily and has 12 transmembrane-spanning segments (101, 102, 103). Hydrophilic fluoroquinolones and monocationic organic compounds such as acriflavine, ethidium, and tetraphenylphosphonium bromide (TPP) are substrates of this pump. Interestingly, data available from the *S. aureus* genome sequencing projects suggest that NorA is not the only *S. aureus* MDR transporter; at least 10 regions encoding polypeptides having homology with NorA can be identified (104, 105).

2. 7. Other functions of efflux pumps

Two families of ATP-binding cassette (ABC) transporters in which one or two extracytoplasmic substrate-binding domains are fused to either the N-or C-terminus of the translocator protein have been detected. The implications of multiple substrate-binding sites in close proximity to the translocator in terms of broadened substrate specificity and possible cooperative interactions between SBPs (substrate-binding proteins) and the translocator (106).

ABC transporters, found in all pro-and eucaryotic species, use the hydrolisis of ATP to translocate solutes across cellular membranes. The translocator component of ABC transportes is composed of two multi-transmembrane and two intracellular ATP-binding subunits (107), with the individual subunits expressed as separate polypeptides or fused to each other in any possible combination. In addition to these ubiquitous components,

prokaryotic ABC transporters involved in solute uptake into the cell employ a specific ligand-binding protein to capture the substrate. These SBPs, which are the main determinants as SBP-dependent ABC transporter specificity, were first identified in Gram-negative bacteria, where they reside in the periplasmic space (108). Gram-positive bacteria and Archaea, organisms without a periplasm, anchour the proteins to the outer surface of the cell membrane via an N-terminal lipid moiety (109, 110) or, in the case of Archaea, use an N-terminal transmembrane segment to anchor the protein to the cytoplasmic membrane (111).

The majority of SBPs involved in ABC transport consist of two domains (C-and N-lobes) that are connected by a flexible hinge (112, 113). This enables the SBP to assume an "open-unligaded" confirmation with a high affinity for the substrate and a "closed-unliganded" state with a low affinity. Upon binding of a substrate molecule, the two lobes of the SBP close around the ligand. The protein in its closed conformation then interacts with the translocator, which is located in the cytoplasmic membrane (113).

The ATP-binding cassette (ABC) superfamilly was defined in 1986 when homology was detected between a binding protein dependent transporter and multidrug efflux cloned from human cancer cells. In addition to the periplasmic binding protein-dependent transporters that mediate uptake bacterial cells also contain ABC transporter lacking a binding protein that mediate efflux of compounds such as lipopolysaccharides, capsular polysaccharides, antimicrobial agents, and toxins. A few ABC proteins also lack a transmembrane region and use the same architecture to perform alternative functions such as DNA repair (114).

Resistance to toxic heavy metals has been found in bacteria from clinical and environmental origins. The genetic determinants of resistance are frequently located on plasmids or transposons. Several heavy metal resistance genes have been cloned and sequenced. The mechanisms of resistance to heavy metals are commonly based on novel membrane transport systems that expel the toxic ions (including cobalt, nickel, zinc, and

copper and chromium) from the bacterial cytoplasm (115, 116, 117, 118, 119).
The function of most resistance systems is based on the energy-dependent efflux of toxic ions. Some of the efflux systems are ATPases and others are chemiosmotic cation/proton antiporters. The triple-polypeptide Czc (Cd^{2+}, Zn^{2+} and Co^{2+}) chemiosmotic efflux pump consists of inner membrane (CzcA), outer membrane (CzcC) and membrane-spanning (CzcB) proteins that together transport cations from the cytoplasm across the periplasmic space to the outside of the cell (120).

2. 8. Interactions of antibiotics with some efflux pumps and other drugs

The main concept underlying the use of antimicrobial agents is to select the single best drug whenever possible, because this minimizes side effects. Hoever, there are several instances in which 2 or more drugs are commonly given:

1. to treat serious infections before the identify of the organism is known;

2. to achieve a synergistic inhibitory effect against certain organisms;

3. to prevent the emergence of resistant organisms if bacteria become resistant to one drug.

The MRSA strains are often resistant not only to β-lactam agents but also to fluoroquinolones, chloramphenicol, clindamycin, tetracylines, aminoglycosides, and vancomycin is almost universally accepted as the drug of choice for the treatment of MRSA infections (121). However, vancomycin used alone kills staphylococci slowly resulting in delayed recovery of patients with life-threatenning infections.

Therefore the purposes of some investigation were to determine among 26 commonly available β-lactams and among 8 aminoglycosides those that display the strongest beneficial bacteriostatic effect in combination with vancomycin against MRSA strains, to evaluate the bactericidal effects diplayed by the most effective agents used in different β-lactam-vancomycin, vancomycin-aminoglycoside and β-lactam-vancomycin-aminoglycoside combinations, and to examine whether the effects of different antibiotic combinations are dependent upon the aminoglycoside susceptibility pattern of the strains (122).

The best interactions with vancomycin were observed with either imipenem, cefazolin, or netilmicin. By checkerboard studies, imipenem-vancomycin and cefazolin-vancomycin each provided a synergistic bacteriostatic effect against 22 strains; the mean fractional inhibitory concentration (FIC) indexes were 0.35 and 0.46 for imipenem-vancomycin and cefazolin-vancomycin, respectively. The vancomycin-netilmicin combination provided an indifferent effect against all the of 32 strains tested (122).

The accurate prediction of clinically relevant antibiotic synergy based upon the results of in vitro testing has been a goal of researchers for some time. There are in fact, examples of such a correlation existing. Combinations of antimicrobial agents which have been shown to be synergistic *in vitro* have been associated with a more favorable clinical outcome in neutropenic patients with Gram-negative sepsis in the treatment of enterococcal endocarditis. Increased bactericidal activity in patient serum has also been documented with antibiotic

combinations which are synergistic *in vitro* (123). Time–killing and checkerboard methods are the most widely used techniques to assass synergy but are time-consuming and labor-intensive perfomed synergy testing of *E. coli, Enterobacter cloacae, P. aeruginosa* and *S. aureus* with various combinations of cefepime or ceftazidime with tobramycin or ciprofloxacin. All antimicrobial combinations demonstrated some degree of synergy against the test organisms, and antagonism was infrequent Gram-negative bacteria of growth and viability in combination of antibiotics and resistance modifiers was studied with flow cytometric method (124).

Therapeutic options for deep-seated infection due to methicillin-resistant *S. aureus* (MRSA) are limitted (125). Although intravenously administered vancomycin is the drug of choice for the tretment of MRSA infections, treatment failures with vancomycin monotherapy of staphylococcal endocarditis have been reported (126, 122). In treating compilicated MRSA infections, many clinicians combine vancomycin with another agent, such as gentamicin, in the expectation of more rapid bacteriologic response based on a synergistic interaction between the two antibiotics. Overall, there are few data comparing vancomycin monotherapy with vancomycin in combination with aminoglycosid for the treatment of severe MRSA infections.

Among the common pathogens responsible for inefective endocarditis, the mechanism of bactericidal synergy has been delineated only for enterococci. *Enterococci* are relatively resistant to penicillin G and ampicillin with each agent yielding a bacteriostatic effect. In combination with gentamicin or streptomycin, penicillin G and ampicillin facilitate the intercellular uptake of the aminoglycoside, which causes the subsequent bactericidal effect against the enterococci. In the absence of these agents, there is little intracellular uptake of the aminoglycosides (127).

3. AIMS OF THE STUDY

The frequency among clinical isolatesof antibiotic-resistant strains, including poly-and multiresistant ones, continues to increase. These antibiotic-resistant bacteria often cause life-treatening infections. To overcome these situations, we need new antibiotics or new drug combinations with which to treat antibiotic-resistant bacterial infections. In the thesis, I will focus on *in vitro* models of combination chemotherapy against laboratory strains used as

model and antibiotic-resistant clinical isolates. According by, the following questions will be studied in detail:

- The activities of resistance modifiers in modifing bacterial sensitivity to given antibiotics (ampicillin, chloramphenicol, erythromycine, tetracycline) will be studied by using various resistance modifiers (promethazine, verapamil, clomipramine). The antibacterial effect of promethazine, verapamil and clomipramine will be studied as standard "group representative" resistance modifiers on different bacterial species (*E. coli, P. aeruginosa, S. aureus, S. epidermidis*).

- The antibacterial effects of various newly synthetized calcium channel blockers, nifedipine (NP) analogues: 3,5-dibenzoyl-1,4-dihydropyridines (BzDHPs), and 3,5-diacetyl-1,4-dihydropyridines (AcDHPs) will be studied on different resistant *E. coli* strains from clinical specimens.

- The combinations of different resistance modifiers (BzDHPs) with various antibiotics, such as ampicillin, erythromycine, tetracycline, chloramphanicol will be studied via a checkerboard method on some Gram-negative strains. Oxacillin with promethazine or verapamil or imipramine will be studied in combination on *S. aureus* strains including clinical isolates.

- The plasmid-curing effects of resistance modifiers such as BzDHP and promethazine will be studied on an *E. coli* K12 LE 140/ F'lac as model. The change in the resistance of *S. aureus* after plasmid curing will be following by the changes in antibiotic sensitivity and PCR.

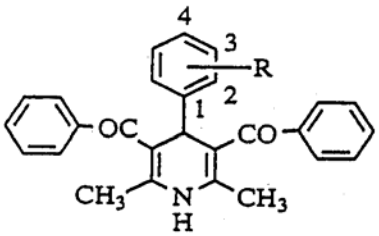
- Attemps to reduce the methicillin resistance of *S. aureus* will be studied by means of checkerboard method and PCR analysis of gene (*mecA*, *ermA and nucA*) expression.

- Flouro-luminometric measurements will be reported for the differentiation of bacterial growth and viability in combined application of antibiotics and resistance modifiers.

4. MATERIALS AND METHODS

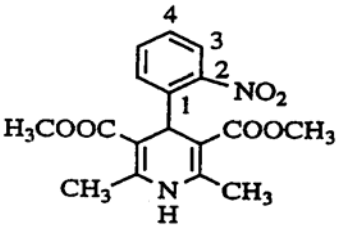
4. 1. Materials

Chemicals: Fifteen substituted dihydropyridines were synthesized previously (128). The structures of 3,5-dibenzoyl-1,4-dihydropyridines ((GB1-GB15). The structures are presented (Figure 3).

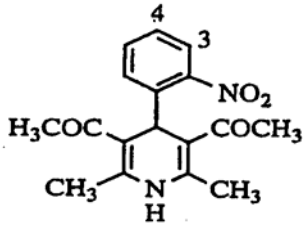


[GB1] R=H
[GB2] R=3-NO_2
[GB3] R=2-NO_2
[GB4] R=3-PhO
[GB5] R=2-CF_3
[GB6] R=3-CF_3
[GB7] R=4-CF_3
[GB8] R=4-CH_3S
[GB9] R=2-CH_3O
[GB10] R=4-CH_3O

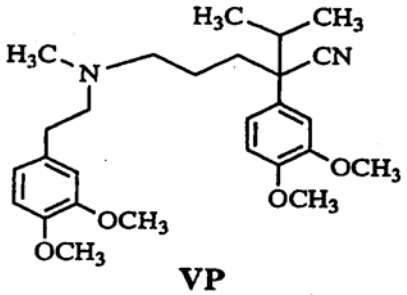
[GB11] R=2-Cl
[GB12] R=3-Cl
[GB13] R=4-Cl
[GB14] R=3-Br
[GB15] R=3,4,5-$(CH_3O)_3$



NP



G11



VP

Figure 3. Structure of 3,5-dibenzoyl-1,4-dihydropyridines [GB1-GB15], nifedipine [NP], 2'-nitro derivate of 3,5-diacetyl-1,4-dihydropyridine [G11] and verapamil [VP]

Eleven acetyldihydropyridines of NP analogues were synthesized as previously described (129).

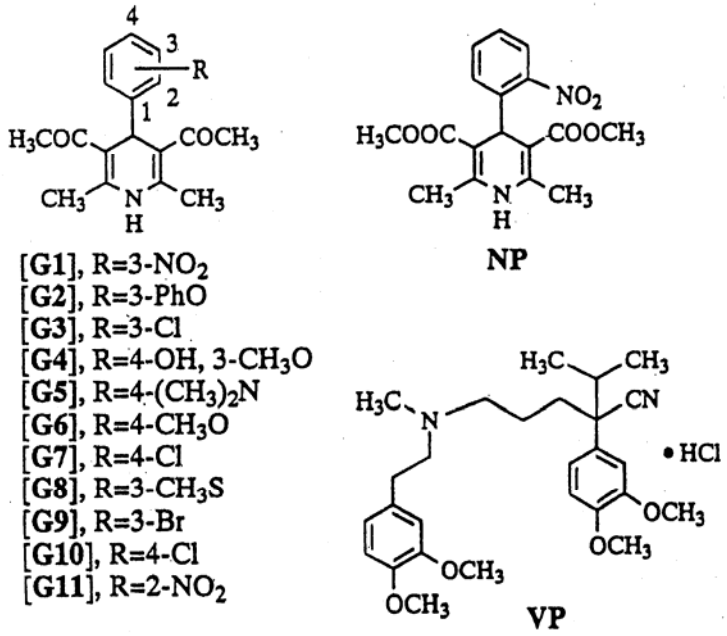


Figure 4. Structures of 3,5-diacetyl-1,4-dihydropyridines [G1-G11], nifedipine [NP], and verapamil [VP]

Antibiotics: ampicillin (AMP) was purchasned from (Beecham Research Laboratories, England, (UK), erythromycin (ERY) was from (Richter Gedeon Rt. Budapest, Hungary), tetracycline (TC) (Chinoin, Budapest, Hungary), Gentamicin (GENT) (Chinoin, Budapest, Hungary), oxacillin (Chinoin, Budapest, Hungary).

Resistance modifiers: The resistance modifiers were obtained from the indicated companies: Verapamil (VP) (Chinoin, Budapest, Hungary), nifedipine (NP) (Aldrich, N7634, St. Louis, MO. U.S.A.), promethazine (PZ) (Pipolphen, EGIS, Budapest, Hungary), methylene blue (MB) (Reanal, Budapest, Hungary), clomipramine (CP, Anaphranil) (Ciba, Geigy, Basel, Switzerland), imipramine (Melipramine, EGIS, Budapest, Hungary).

Painting: **MTT**: 3-(4,5-dimethylthiazol)-2,5-dipheniltetrazoliumbromid) /Thiazolyl blue/ (Sigma, St. Luis M.O. USA)

Bacterials strains: ***Escherichia coli*** **K12 LE 140/F'lac** tsx, str, Δ lac, su, $\lambda^{-\Downarrow r}$, mal, (F^- prime lac^+) laboratory strain and two clinical isolates ***E. coli*** **AMP_{sens} • ERY_{res}** and ***E. coli*** **AMP_{res}• ERY_{res}** were kindly provided by the Public Health Institute of Csongrad County.

E. coli **MC1061**/ cl^+ Δ (*ara leu*) 7697 Δ*lac* X74 *galU galK hsr hsm* $^+$*rpsl araD* 139 were kindly provided by the Institute of Biochemistry and Food Chemistry of University of Finland.

Clinical strains:

Pseudomonas aeruginosa: nalidix acid, ampicillin, erythromycin resistant was provided by the Public Health Institute of Csongrad County.

Staphylococcus epidermidis: ampicilin, erythromycin resistant was provided by the Public Health Institute of Csongrad County.

a. ***Staphylococcus aureus*** methicillin sensitivity **(MSSA)**: tetracycline resistant, isolated by Jenson **1906 U. K. ,** labor. number: **13137 S**

*b.****Staphylococcus aureus*** methicillin resistant **(MRSA):** penicilline, oxacilline, erythromycin, tetracycline resistant, isolated by Jenson **1906 U. K.,** labor. number: **13137 R**

c. ***Staphylococcus aureus*** **MSSA** isolated by **Dorbusch,** labor number: **DUS 4916 S**

d. ***Staphylococcus aureus*** **MRSA:** penicilline, oxacillin, tetracycline resistant, isolated by **Dorbusch,** labor. number: **DUR 4916 R**

e. ***Staphylococcus aureus*** **MRSA** : penicilline, oxacillin, tetracycline resistant, isolated by **DOTE,** labor. number: **5814 R**

f. ***Staphylococcus aureus*** **MSSA,** isolated by **DOTE**, labor. number: **5814 S**

g. ***Staphylococcus aureus*** **MSSA:** penicilline, erythromycin, tetracycline resistant, isolated by **DOTE,** labor. number: **1190 S**

h. ***Staphylococcus aureus*** **MRSA:** penicilline, oxacillin, erythromycin, tetracycline resistant, isolated by **DOTE,** labor. number: **1190 R** /The laboratory strains from a- to h/ were kindly provided by the Institute of Medical Microbiology of Semmelweis University Budapest.

Standard strains: *E. coli* (ATCC 25 922), *S. aureus* Rosenbach (ATCC 25 923), *P. aeruginosa* (ATCC 27 853).

Marker*:* D-3687 PCR Low Ladder (Sigma) containing from 100, 200 to 1000 bp.

Culture media: The antibacterial effects of the tested compounds were studied by using minimal tryptone yeast extract (**MTY**) nutrient broth, containing 1.0 g NH_4Cl, 7.0 g K_2HPO_4, 3.0 g NaH_2PO_4, 0.8 g NaCl, 1.0 g D-glucose, 10.0 g Bacto tryptone (Difco) and 1.0 g yeast extract (Difco) in 1.0 L destilled water at pH 7.2. (130).

EMB /eosin-methylene blue/ agar plates were used for the differentation of lac negative (lac^-) pink and lac positíve (lac^+) deep purple colonies.

LB: 10.0 g tryptone, 5.0 g yeast extract and 5.0 g NaCl in 1.0 L destilled water at pH 7.0.

BHI: 12.5 g Calf Brain infusion solids, 5.0 g Beef Heart infusion solids, 10.0 g Protease peptone (Oxoid L46), 5.0 g Sodium chloride, 2.0 g Dextrose, 2.5 g Disodium phosphate in 1.0 L destillated water at pH 7.4. Boiling and autoclaving at 121 °C for 15 minutes.

4. 2. Methods

4. 2. 1. Determination of minimum inhibitory concentration (MIC) : Overnight cultures of bacterial strains were diluted to (10^{-4} in 2 x MTY broth) aliquots of 50 μL transferred to a 96 well microplate, and 50 μL of different concentrations of antibiotics and resistance modifiers was then were added. The microplates were incubated at 37 °C for 24 h and minimum inhibitory concentration (MIC) values were determined with MTT (131).

4. 2. 2. Checkerboard method: The checkerboard method is technique used most frequently to asses antibacterial combinations *in vitro*. The dilutions of antibiotics and resistance modifiers were distributed in the microtiter trays. We have expressed the antimicrobial concentrations as multiples as fractions of the MIC, they are usully expressed in μg/mL. The results of the combined use of antibiotics and resistance modifiers were evaluated according to the literature (132) as synergism, additivity, indifference or antagonism.

Three bacterial strains were tested by the microdilution checkerboard technique described. Briefly, bacterial dilutions from the logaritmic-growth phase were prepared and subsequently distributed into microtiter trays containing various drug concentrations. The final inoculum size in the microtiter trays was approximately 10^5 colony forming units (cfu)/mL.

Inoculated microtiter trays were incubated at 37 °C for a period of 24 h, then the cultures in the trays were stained with 10 μL of MTT (10 mg/mL stock solution), incubated

for 6 h and read for the inhibition of bacterial growth in an microplate. In order to evaluate the outcome of the drug combination, fractional inhibitory concentration (FIC) indices were calculated as $FIC_A + FIC_B$ where FIC_A and FIC_B represent the minimum concentrations of drug A and B that inhibited growth respectively (132).

$$FIC_A = \frac{MIC_A \text{ combination}}{MIC_A \text{ alone}} \qquad FIC_B = \frac{MIC_B \text{ combination}}{MIC_B \text{ alone}} \qquad FIC_{INDEX} = FIC_A + FIC_B$$

Individual checkerboard runs were repeated twice, a mean FIC index was calculated and classified as either synergistic (≤ 0.5), additive (0.51-1.01), indifferent (1.01-4.0) or antagonistic (over 4.0).

4. 2. 3. Time killing method:

When the colony counts have been determined, the easiest way to visualize the results was to plot them on semilogarithmic paper (using the absiss for time, and the ordinate (the logarithmic scale) for the colony counts. The definitions of antimicrobial interaction with this technique was based on studied with enterococci, against which clinically acceptable concentrations of the aminoglycosides alone was generally inactíve and penicillin was only bacteriostatic.

The results were interpreted by the effect of the combination of antibiotics and resistance modifiers in comparison with the most active single drug alone. Synergism was defined as a ≥ 100-fold decrease in killing at 24 h with the combination, as compared with the most active single drug alone. Additive (or indifferent) was defined as a less than 10-fold change increase in killing at 24 h with the combination, in comparison with the most active single antimicrobial alone. These definitions assume that at least one of the drugs being were tested produces no significant inhibition or killing alone (132).

4. 2. 4. Elimination of the F'lac plasmid: The method described earlier was applied. From an overnight preculture of *E. coli* K12 LE 140/F'lac, a dilution of $1:10^4$ was prepared and 0.05 mL (approximately 5×10^3 cells) aliquots were inoculated into 5.0 mL MTY broth containing the compounds. Cultures were incubated at 37 °C for 24 h without shaking. Different dilutions were made from the tube cultures showing bacterial growth and the

cultures were diluted in isotonic saline and 0.1 mL of each dilution was spread on eosine methylene blue agar. The plates were incubated at 37 °C 24 h (133).

The differential basis of this medium involves were two indicator dyes, eosin and methylene blue, which distinguish lactose fermeting and non-lactose fermenting organisms. Acid from lactose fermentation changed the color of the colonies. The plates were counted for lac^- plasmidless (pink) and lac^+ plasmid containing (deep violet with green metallic seen) colonies. The percentage (%) of plasmid elimination was counted according to the formula given below:

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

4. 2. 5. Elimination of R-plasmid and Replica plating: An overnight preculture of methicillin-resistant *S. aureus* 13137 strains was diluted 10^{-4} fold and distributed in 0.05 mL amounts (about 5 x 10^3 bacteria) into tubes with 5.0 mL MTY broth. Cultures were supplemented with different concentrations of curing agents. The samples were then incubated without shaking at 37 °C for 48 h in the case of R-plasmid elimination. From tubes showing growth, different dilutions were prepared and 0.1 mL of samples plated on MTY plates in case of R-plasmid carrying strains (134).

The velvet replica from master plates were prepared onto plates containing erythromycin 20 μg/mL or tetracycline 20 μg/mL or oxacilline 16 μg/mL and after 24 h incubation, the master and replica plates were compared and ratio of the plasmid elimination was determined (135) and antibiotic sensitive colonia were isolated for PCR studies.

4. 2. 6. PCR analysis of *mecA* gene expression from plasmid-cured colonies of *Staphylococcus aureus*

The lyses methods that relate here are too complicated and proved too material and instrument intensive. We adopted the method recommended to lyses of *S. aureus:*

- 0,1 mL was centrifuged 16000 rpm for 30 seconds from an overnight broth of BHI
- the residuary cells were suspended in 50 μL lysostaphin solution (100 μg/mL diluted solution)
- The sample was incubated for 10 minutes at 37 °C then,

- 50 µL proteinase K (100 µg/mL) was added and then
- 150 µL puffer (0.1 M Tris pH 7.5) was added and
- finally it was boiling in water for 5 minutes
- after cooling, 10 µL was used directly to PCR analyses (136).

2.5 µL of template DNA was mixed in REDTaq™ ReadyMix™ (Sigma-Aldrich, Hungary). Preparing of the master mix was according to the manufactures's instructions. The total reaction volume was 25 µL.

Amplification with *mecA*, *nucA* primers was performed in a DNA Thermal Cycler (GeneAMP 9700) as 95°C for 3 min and for 30 cycles by denaturing at 94°C for 1 min, annealing at 55°C for 1 min, followed by polymerisation at 72 °C for 1 min and final extension 72°C for 7 min (137, 138).

Ampilification with *ermA* primers was performed in the same thermal cycler but with different thermal profile. The PCR mixtures were subjected to thermal cycling 3 min at 96 °C and then 30 cycles of 1 s at 95 °C for the denaturation step and 30 s at 55 °C for the annealing extension step (139).

Ten microliters of product was electrophoresed on 1.5% 1x Tris-acetate-EDTA agarose gel and was stained with ethidium-bromide. DNA molecular weight marker PCR Low Ladder (Sigma-Aldrich, Hungary) was used as the standard. The gels were visualized under 254-nm UV lights.

4. 2. 7. Fluoro-luminometric viability analysis of *Escherichia coli* cells using GFP-luciferase combination:

Plasmid:

pEGFPlucTet / This plasmid was a pUC19-based high-copy number plasmid which contains enhanced green fluorescent protein (*egfp)* gene and luciferase gene (lucFF) of firefly *P. pyralis* (140). Genes were inserted in the frame with the *lacZ* initiation codon from pUC19 so that EGFP and luciferase proteins were expressed from the lac promoter. Gene coded tetracycline resistance (*Tet*) provides plasmid stability during the experiments.

Cultivation of bacteria for fluoro-luminometric was studied

Plasmid-containing bacterial strains were maintained as described (141). Bacterial cells were cultivated in Luria-Bertani Broth (10 g of tryptone, 5 g of yeast extract, 5 g NaCl/L, pH 7.0) containing tetracycline (5 μg/mL) in a shaker (280 rpm) at 37 °C.

Overnight cultures of bacteria were diluted (1:100) with fresh LB-broth containing tetracycline, and further cultivated to mid-logarithmic phase. Culture was washed twice with LB-broth and the cell number was adjusted to approximately 2 x 10^8 cells per mL ($OD_{600nm} \approx 0.5$) by spectrophotometry (UV-1601 Shimadzu spectrophotometer, Shimadzu corporation, Japan). Cell number was further verified by counting with Epics XL flow cytometer (Coulter Corporation, Miami, Fla.) using fluorescent beads (diameter, 1.8 μm, Polysciences Inc., Warrington, PA) for internal calibration.

Equal volumes of bacterial culture and ampicillin or promethazine or combination of ampicillin and promethazine (or 150 mM NaCl as a control) were mixed in wells of 96-well plate for fluoro-luminometric analysis and optical density measurement to assess ampicillin and promethazine dependent killing of bacteria.

Fluoro-luminometric analysis

Bacterial cultures (*E. coli* containing pEGFPlucTet) (100 μl) were mixed with 100 μL of various ampicillin and promethazine dilutions (final concentrations of ampicillin and promethazine were 0, 20, 60, 250, 500 μg/mL and 0, 10, 30, 125 and 250 μg/mL when used in combination) in the wells of a 96-well plate (white Cliniplate, Termo Labsystems, Helsinki, Finland). The Fluoroskan Ascent FL fluoro-luminometer (Thermo Labsystems) was controlled by the Ascent software™ for Fluoroskan Ascent FL was programmed to carry out the measurement at 25 °C for a period of twenty hours. At the time points of 0 and 20 h of the incubation, the fluorescence of cells were measured for 0.1 s/well using 485 nm excitation and 510 nm emission filter sets. Following the fluorescence measurement of particular wells at indicated time points, 100 μL of luciferin solution (0.5 mM D-luciferin in 100 mM sodium citrate, pH 5.0) was automatically dispensed into the wells, and bioluminescence of cells was measured for 1 s/well (142).

5. RESULTS

5.1. Determination of minimum inhibitory concentrations (MICs) of some antibiotics and the effects of combinations with resistance modifiers against Gram-negative and Gram-positive strains

The *E. coli* K12 LE 140 F' lac strain used in this study was susceptible to AMP, TC, ERY and GENT with MIC's of 4.0, 1.0, 8.0 and 1.0 μg/mL respectively. This strain was tested for the MIC's to PZ (MIC = 128 μg/mL), MB (400 μg/mL), CP (64 μg/mL) and VP (>1250 μg/mL); the results are presented in Table 1.

The results on the combinations of antibiotics and resistance modifiers are at or below their MIC (Table 1). PZ, MB or CP alone exerts only limited inhibitory activity against *E. coli* K12 LE 140 F' lac. However the enhance the activity of AMP at concentrations that was not inhibitory. The combination of PZ, MB and CP with AMP were synergistic against *E. coli* K12 LE 140 F' lac. Synergism was not observed with combinations of AMP and VP. At concentrations equal to each MIC, the results were indifferent. The combinations of resistance modifiers and TC, ERY, GENT gave varied responses. Of the four resistance modifiers employed, synergism was observed with promethazine in combination with tetracycline and erythromycin, and with the combination of methylene blue and erythromycin.

The *P. aeruginosa* strain was susceptible only to GENT (MIC = 1.0 μg/mL). The effect of the combination of GENT and MB was synergistic against *P. aeruginosa* (Table 2).

The synergism shown by PZ against *S. epidermidis* was much less than that against *E. coli* K12 LE 140 F' lac (Table 3). PZ acted synergistically with TC and ERY. CP with ERY and TC was also synergistic against *S. epidermidis* (Table 3).

A mean FIC_{INDEX} was calculated from; $FIC_A + FIC_B$ and the interpretation was made as follows: synergistic (≤0.5), additive (0.51-1.01), indifferent (>1.01) or antagonistic (>4.0).

$$FIC_A = \frac{MIC_A \text{ combination}}{MIC_A \text{ alone}} \qquad FIC_B = \frac{MIC_B \text{ combination}}{MIC_B \text{ alone}} \qquad FIC_{INDEX} = FIC_A + FIC_B$$

Table 1. MIC of antibiotics and the effect of combinations with resistance modifiers against *Escherichia coli* K12 LE 140 strain

Antibiotics (A) + resistance modifiers (B)	**MIC (μg/mL) alone and combinations (A + B)**	**FIC_{INDEX}**	**Type of interaction**
Promethazine (PZ)	128		
Methylene blue (MB)	400		
Clomipramine (CP)	64		
Verapamil (VP)	> 1250		
Ampicillin (AMP)	**4.0**		
+ PZ	AMP (1) + PZ (16)	0.375	***synergistic***
+ MB	AMP (1) + MB (25)	0.312	***synergistic***
+ CP	AMP (1) + CP (16)	0.50	***synergistic***
+ VP	AMP (1) + VP (>1250)	1.25	indifferent
Tetracycline (TC)	**1.0**		
+ PZ	TC (0.25) + PZ (32)	0.50	***synergistic***
+ MB	TC (0.50) + MB (32)	0.58	additive
+ CP	TC (0.25) + CP (32)	0.75	additive
+ VP	TC (1) + VP (>1250)	2.0	indifferent
Erythromycin (ERY)	**8.0**		
+ PZ	ERY (1) + PZ (32)	0.375	***synergistic***
+ MB	ERY (2) + MB (50)	0.375	***synergistic***
+ CP	ERY (4) + CP (32)	1.0	additive
+ VP	ERY (4) + VP (>1250)	1.50	additive
Gentamicin (GENT)	**1.0**		
+ PZ	GENT (0.5) + PZ (64)	1.0	additive
+ MB	GENT (0.5) + MB (25)	0.562	additive
+ CP	GENT (0.5) + CP (32)	1.0	additive
+ VP	GENT (0.5) + VP (>1250)	1.5	indifferent

Table 2. MIC of antibiotics and the effect of combinations with resistance modifiers against *Pseudomonas aeruginosa* strain

Antibiotics (A) + resistance modifiers(B)	**MIC (µg/mL) alone and combinations (A + B)**	**FIC_{INDEX}**	**Type of interaction**
Promethazine (PZ)	256		
Methylene blue (MB)	400		
Clomipramine (CP)	256		
Verapamil (VP)	> 1250		
Ampicillin (AMP)	**256**		
+ PZ	AMP (128) + PZ (128)	1.0	additive
+ MB	AMP (128) + MB (50)	0.625	additive
+ CP	AMP (128) + CP (128)	1.0	additive
+ VP	AMP (256) + VP (>1250)	2.0	indifferent
Tetracycline (TC)	**16**		
+ PZ	TC (8) + PZ (64)	0.75	additive
+ MB	TC (8) + MB (50)	0.625	additive
+ CP	TC (8) + CP (128)	1.0	additive
+ VP	TC (4) + VP (>1250)	>1.25	indifferent
Erythromycin (ERY)	**64**		
+ PZ	ERY (32) + PZ (128)	1.0	additive
+ MB	ERY (32) + MB (50)	0.625	additive
+ CP	ERY (32) + CP (128)	1.0	additive
+ VP	ERY (64) + VP (>1250)	>2.0	indifferent
Gentamicin (GENT)	**1.0**		
+ PZ	GENT (0.5) + PZ (64)	0.75	additive
+ MB	GENT (0.25) + MB (25)	0.312	***synergistic***
+ CP	GENT (0.25) + CP (128)	0.75	additive
+ VP	GENT (1) + VP (>1250)	>2.0	indifferent

Table 3. MIC of antibiotics and the effect of combinations with resistance modifiers against *Staphylococcus epidermidis* strain

Antibiotics (A) + resistance modifiers (B)	MIC (μg/mL) alone and combinations (A + B)	FIC_{INDEX}	Type of interaction
Promethazine (PZ)	64		
Methylene blue (MB)	400		
Clomipramine (CP)	64		
Verapamil (VP)	> 1250		
Ampicillin (AMP)	**256**		
+ PZ	AMP (128) + PZ (32)	1.0	additive
+ MB	AMP (128) + MB (25)	0.562	additive
+ CP	AMP (64) + CP (32)	0.75	additive
+ VP	AMP (256) + VP (>1250)	2.0	indifferent
Tetracycline (TC)	**32**		
+ PZ	TC (4) + PZ (16)	0.375	***synergistic***
+ MB	TC (16) + MB (50)	0.625	additive
+ CP	TC (8) + CP (8)	0.375	***synergistic***
+ VP	TC (32) + VP (>1250)	2.0	indifferent
Erythromycin (ERY)	**64**		
+ PZ	ERY (16) + PZ (16)	0.50	***synergistic***
+ MB	ERY (16) + MB (25)	0.562	additive
+ CP	ERY (2) + CP (4)	0.093	***synergistic***
+ VP	ERY (64) + VP (>1250)	2.0	indifferent
Gentamicin (GENT)	**0.5**		
+ PZ	GENT (0.25) + PZ (16)	0.75	additive
+ MB	GENT (0.25) + MB (25)	0.562	additive
+ CP	GENT (0.25) + CP (32)	1.0	additive
+ VP	GENT (0.5) + VP (>1250)	2.0	indifferent

5.2. Antibacterial effects and interactions of antibiotics with diacetyldihydropyridines and dibenzoyldihydropyridines on *Escherichia coli* strains

Dihydropyridines are Ca^{2+}-channel antagonists (143-146) which can interact with other antibiotics (147-151). On the other hand, the antibacterial interaction of macromolecules with phenothiazines or antidepressants *in vitro* have been to be dependent on the chemical structures (152, 153). The antibacterial effects of tricyclic neuroleptics and antidepressants have been described earlier (133, 154-158). Some of the non-conventional antibiotics could also reverse the antibiotic resistance of various bacteria by an antiplasmid effect (159-161). The combinations of PZ with AMP, TC or ERY or the combination of MB and ERY produced enhanced antibacterial activity against *E. coli* (162, 155).

VP in combination with AMP reduced the activity of AMP against the *E. coli* K12 LE 140/F' lac strain (162). The new Ca^{2+}-channel antagonists may produce resistance-modifying effects. The effects of new **BzDHPs** (GB1-GB15) on *E. coli* strains were examined (Figure 3) (163).

We previously found a structure activity relationship for of **AcDHPs** (Figure 4). AcDHPs (G1-G11) contain acetyl groups, BzDHPs [GB1-GB15] contain benzoyl groups, and NP has methyl esters at positions 3 and 5, respectively (164).

5.2.1. Antibacterial effects and interactions of ampicillin and erythromycin with 3,5-diacetyl-1,4-dihydropyridines (AcDHPs) (G1-G11)

These features were tested with checkerboard and time-killing methods (Tables 4-9).
The MICs of the **AcDHPs** after 24 h were measured on three different *E. coli* strains.
No antibacterial effect was seen with the non-antibiotics up to a concentration of 100 μg/mL in the checkerboard studies (Tables 4-9).

- *Effects of combinations AcDHPs (G1-G11) with AMP*

Synergistic effects of **AcDHPs** with AMP were seen against ***E. coli* K12 LE 140 /F' lac** after 24 h. The combinations with seven **AcDHPs** (G1, G3, G4, G7, G8, G10 and G11) reduced the AMP MIC to 1 μg/mL and the MIC of NP to 2 μg/mL. Two compounds, G9 (MIC: 16 μg/mL) and VP (MIC: 32 μg/mL), were less effective than the others. The most effective compounds were G1, G3, G4, G7, G8, G10 and G11. An additive effect was exerted

by G2 with AMP (Figure 5). Additive effects observed for combinations of G5, G6, and NP with AMP.

The MIC of AMP against the clinical isolate ***E. coli*** **AMP_{sens} ∞ ERY_{res}** after 24 h in combination with G1 was antagonistic (MIC for AMP: 32 μg/mL) (Table 5). Ten other **AcDHPs (G2-G11)** (MIC: 8 μg/mL) and NP (MIC: 8 μg/mL) were indifferent and only VP (MIC: 4 μg/mL) demonstrated an additive effect.

We studied the antibacterial effect of AMP on the clinical isolate of the ***E. coli*** **AMP_{res} • ERY_{res}** strain. AMP alone had an MIC of 256 μg/mL. In combination with G1-G11, VP and NP, the MIC was 128 μg/mL (Table 6). The compounds in combination with AMP displayed additive effects, except for VP were the effect indifferent.

• *Effects of combinations of AcDHPs (G1-G11) with ERY*

The **AcDHPs (G7-G8)** displayed synergistic effects with ERY against ***E. coli*** **K12 LE 140/F' lac** after 24 h; the MICs of G7 (MIC: 2 μg/mL) and G8 (MIC: 2 μg/mL) were synergistic (Table 7). The MICs of G2, G3, G10, G11, VP and NP were additive (MIC: 4 μg/mL). G1, G4, G5, G6 and G9 were indifferent in combination with ERY against the ***E. coli*** **K12 LE 140/F' lac** strain (Table 7). G7 was the most effective of the 11 **AcDHPs (G1-G11)** (Figure 6).

The combinations of ERY with 11 **AcDHPs (G1-G11**) shown additive effects against ***E. coli*** **AMP_{sens} ∞ ERY_{res}** (MIC: 16-32 μg/mL) and NP (MIC: 32 μg/mL) had an additive effect on the MIC of ERY; VP (MIC: 32 μg/mL) exerted an indifferent effect (Table 8).

The combinations of the **AcDHPs (G1-G11)** with ERY the clinical isolate ***E. coli*** **AMP_{res} ∞ ERY_{res}** ; had additive effects VP exerted an indifferent effect (Table 9).

5.2.2. The antibacterial effects and interactions of 3,5-dibenzoyl-1,4-dihydropyridines (BzDHPs) (GB1-GB15)

Three different *E. coli* strains: *E. coli* K12 LE 140/F'lac, *E. coli* AMP_{sens}• ERY_{res} and *E. coli* AMP_{res} • ERY_{res} was tested (Table 10).

On the ***E. coli*** **K12 LE 140/F'lac** strain, the MICs of the BzDHPs after a single administration were 64 μg/mL for GB1, 32 μg/mL for GB3, 64 μg/mL for GB4 and 64 μg/mL for GB12 (Table 10).

Of the 15 compounds (8 μg/mL of each) which were used in combination with ERY against the ***E. coli*** **K12 LE 140/F'lac** strain, GB12 (MIC: 2 μg/mL) was the most synergistic, followed by GB1 (MIC : 4 μg/mL), and GB3, GB4, GB6, GB7, GB10, GB14, GB15, VP, and NP (MIC: 4 μg/mL). GB2, GB5, GB8, GB9 GB11 and GB13 (MIC: 8 μg/mL) were not active (Table 10).

On the ***E. coli*** **AMP_{sens} • ERY_{res}** strain, the MICs of the 15 GB compounds after a single administration were 16 μg/mL for GB2, GB3 and GB5 which were the most synergistic. GB1, GB4, GB6 and GB12 displayed MICs of 32 μg/mL) in combination. GB7, GB9, GB11 and GB14 (MIC: 64 μg/mL) showed additive activity. GB8, GB10, GB13 and GB15 (MIC: 128 μg/mL) were indifferent (Table 10).

On the ***E. coli*** **AMP_{res} ∞ ERY_{res}** strain, for the MIC of ERY alone was 256 μg/mL. The results combinations of 15 GB compounds (125 μg/mL of each) with ERY showed that GB5 (MIC: 8 μg/mL) was the most synergistic.

GB2, GB3, GB4 and GB6 had MICs of 16 μg/mL; and GB1 and GB7 had MICs of 32 μg/mL. The combination of GB5 exerted a synergistic effect with ERY. GB8 - GB15 had MICs of 128 μg/mL. There were no active compounds (Table 10).

Table 10. MICs of erythromycin and combination effect with 3,5-dibenzoyl-1,4-dihydropyridines [GB1-GB15] against three *E. coli* strains

Compounds	MIC (µg/mL)					
	E. coli **K12 LE 140/F'lac**		***E. coli*** **AMP_{sens} • ERY_{res}**		***E. coli*** **AMP_{res} • ERY_{res}**	
Erythromycin (ERY)	MIC value (µg/mL GB) of GB alone **(alone) 8**	MIC value (µg/mL ERY) of ERY with each 8 µg/mL GB	MIC value (µg/mL GB) of GB alone **(alone) 128**	MIC value (µg/mL ERY of ERY with each 125 µg/mL GB	MIC value (µg/mLGB) of GB alone **(alone) 256**	MIC value (µg/mL ERY) of ERY with each 125 µg/mL GB
+ GB1	64	4	128	32	256	**32**
+ GB2	1250	8	1250	**16**	1250	**16**
+ GB3	32	4	64	**16**	128	**16**
+ GB4	64	4	128	32	256	**16**
+ GB5	1250	8	1250	**16**	1250	**8**
+ GB6	128	4	256	32	512	**16**
+ GB7	256	4	512	64	512	32
+ GB8	1250	8	1250	128	1250	128
+ GB9	1250	8	1250	64	>1250	256
+ GB10	256	4	512	128	512	128
+ GB11	1250	8	>1250	64	>1250	128
+ GB12	64	**2**	128	32	256	128
+ GB13	1250	8	>1250	128	>1250	256
+ GB14	256	4	512	64	512	256
+ GB15	256	4	512	128	512	256
+ Verapamil (VP)	1250	4	>1250	1250	>1250	1250
+ Nifedipine (NP)	1250	4	>1250	1250	>1250	1250

5. 3. Antibacterial effects and interactions of antibiotics and resistance modifiers on methicillin-resistant *Staphylococcus aureus* strains

A lot of *S. aureus* strains are known to be resistant to different antibiotics as well as to methicilline (165). It is also recognised that methicillin resistance often goes together with macrolid resistance.

In our current research we studied the interaction of different antibiotics (AMP, ERY, GENT, TC) and resistance modifiers (PZ, imipramine, omeprazol, reserpine, yohimbine, VP on 4 methicillin-resistant and 4 methicillin-sensitive *S. aureus* strains with a checkerboard method .

We have found that the 8 *S. aureus* strains were sensitive to gentamicin. The grow of strains were not significantly inhibited by the resistance modifiers omeprazol, reserpine, yohimbine, VP (Table 11).

The oxacillin and PZ combination showed a synergistic antibacterial effect on a methicillin resistant *S. aureus* strain 13137 (MRSA), it was additive in 4 instances 1190 (MSSA), 4916 (MSSA), 5814 (MSSA), 13137 (MSSA), indifferent to 2 strains 4916 (MRSA), 5814 (MRSA) and 1 strain was antagonistic 1190 (MRSA) (Table 12).

We studied the effect of the oxacillin and VP in combination on methicillin-sensitive *S. aureus* strains, only additive effect was found in 3 strains of the 4 (1190 MSSA), 4916 (MSSA), 5814 (MSSA) and in 1 strain the effect was found indifferent (13137 MSSA) (Table 13).

We experienced additive effect in all cases when studying the effect of oxacillin and imipramine in combination on methicillin-resistant strains (Table 14).

5.4. Studies on plasmid elimination

F'lac plasmid elimination was studied with the broth dilution method on the ***E. coli* K12 LE 140/F'lac** strain. The lac^+ and lac^- colonies were counted on EMB lactose agar.

Since GB12 showed the most effective antibacterial activity in combination with ERY, it was decided to study this combination for PZ induced plasmid elimination. The effect of GB12 was not influenced by PZ at 20, 40, 60 and 100 μg/mL. However, 80 μg/mL PZ caused a noteworthy plasmid elimination from *E. coli* K12 LE 140/F'lac (Figure 7). The F' lac plasmid elimination of PZ alone was 28.5%, whereas that of PZ with [GB 12] was 62%.
The number of colonies examined ranged from 3700 to 4300.

The R-plasmid elimination effect of PZ was studied with the broth dilution method on the methicillin-resistant ***S. aureus*** 13137 strain. The R-plasmid elimation was determined with a replica method. The antibiotic-sensitive colonies were isolated: 3.9% for ERY, 4.4% TC and 3.7 % for oxacillin. The number of colonies examined ranged from 3900 to 4200. The plasmidless colonies were further studied by PCR.

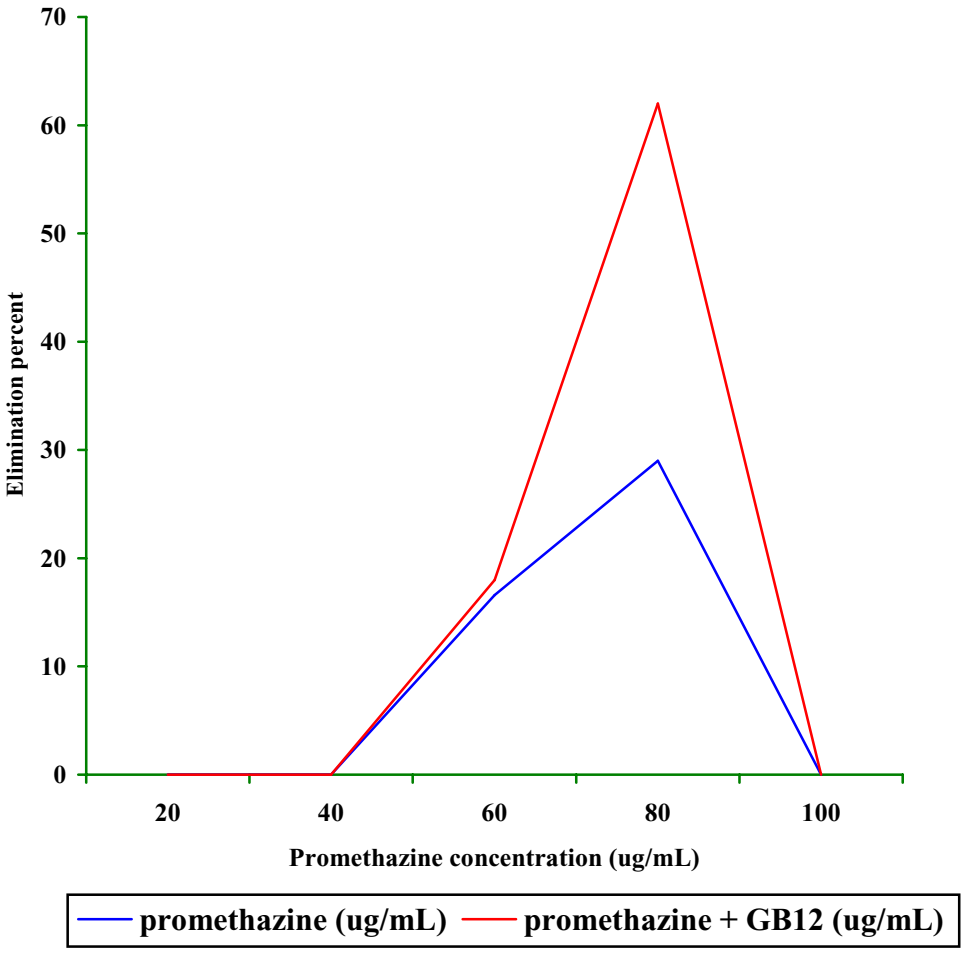


Figure 7. F' lac plasmid elimination of promethazine alone and with compound (GB12) on *E. coli* K12 LE 140 strain

5.5. Identification of *mecA, ermA* and *nucA* genes in methicillin-resistant *Staphylococcus aureus* 13137 strain after plasmid elimination by PCR

Methicillin-resistant staphylococci (MRS) are resistant to all penicillins, including semisynthetic penicillinase-resistant congeners, penems, carbapenems and cephalosporins. *S. aureus* isolates harbouring the *mecA* gene, which encodes for penicillin-binding protein 2a (PBP2a) (166), are associated with methicillin resistance (167).

The MRS typically express their resistance heterogeneously with only a few cells, 1 in 10^4or 10^6, expressing the phenotype (168). The polymerase chain reaction (PCR) assay provides a genotypic approach for the detection of *mecA* that does not depend on the unpredictable phenotypic expression of *mecA*-mediated methicillin resistance (168). Detection of this gene is considered the reference method for detecting methicillin resistance in staphylococci.

S. aureus can be distinguished from other *Staphylococcus* spp. by the presence of the *nuc* gene, which codes for an extra cellular thermostable nuclease (169). Resistance to ERY in staphylococci is usually associated with resistance to other macrolides, to the lincosamides, and to type B streptogramin (MLS). This resistance is mediated by a single alteration in the ribosome, the n6-dimethylation of an adenine residue in the 23S rRNA.

This dimethylation leads to a conformational change in the ribosome, rendering the strain resistant to most antibiotics of the MLS group. Three genes (*erm*A, *erm*B and *erm*C) encoding methylases have been found in staphylococci (170, 171). Another mechanism of inducible resistance to ERY is conferred by the gene *msr*A, which encodes an ATP-dependent efflux pump (172).

■ We used the methicillin-resistant *S. aureus* 13137 strain as standard. Its sensitive counter part was used as negative control. The clones that did not pass the substrate containing tetracycline were *mecA*-positive (4-7 lines) (Figure 8).

The clones that did not pass substrates containing ERY (11, 13, 15, 17 and 18 lines) and oxacillin became *mecA*-negative (12, 14 and 16 lines).

PZ was effective as a curing agent when the *mecA* gene was eliminated and consequently the primer could not hybridize to this sequenc and amplification did not occur (Figure 8).

■ The resistant wild strain was used as positive control (2 lines) without treatment. As a negative (3 lines) the sensitive strain was used (Figure 9).

On both substrate plates, containing TC and oxacillin, non-growing clones were positive, that is they carried the *ermA* gene, except for one clone (10 lines).

Markers are presented (1 and 18 lines) (Figure 9).

■ The presence of the *nuc* gene was identified by PCR in a control, untreated strain that was *nucA*-positive (Figure 10). We ran those clones that did not grow on substrate containing antibiotics after replication by velvet. Result: during gel treatment all the clones carried the *nucA* gene (Figure 10).

Marker: D-3687 PCR Low Ladder (Sigma) containing from 100, 200 to 1000 bp.

5.6. Fluoro-luminometric analysis of drug interaction between ampicillin and promethazine on *Escherichia coli* containing green fluorescence protein and luciferase

The effects of AMP, PZ and their combination on bacterial growth and viability measured by GFP fluorescence and luciferase bioluminescence (Table 15, Figure 11 and Figure 12). GFP accumulates in cells during growth and therefore observed GFP fluorescence is a measure of cell growth. The luciferase reaction is dependent of ATP produced by only catabolically active living cells, so that the bioluminescence is a measure of viable cells.

In the presence of AMP the growth of *E. coli* cells was suppressed as observed from the fluorescence and bioluminescence signals. According to bioluminescence most of bacteria are killed after 20 hours of incubation at AMP concentrations higher than 250 μg per mL The effect of AMP on bacteria is, however, found to be mainly "bacteriostatic" according to the fluorescence data. The fluorescence values were increased notably at each tested AMP concentrations during the incubation compared to the values at the beginning indicating that AMP mainly inhibites cell division. PZ has no antimicrobial effects at low concentrations. On the other hand, at higher concentrations the effect of PZ is similar "bactericidal" with AMP according to bioluminescence. Moreover, there were no increase in fluorescence during incubation at higher PZ concentrations which revealed that the effect of PZ is clearly bactericidic and lytic at concentrations above 250 μg/ mL. The relatíve fluorescence refers to the cell integrity. The relatíve luminoescence refers to the methabolicate living

In the presence of both AMP and PZ the growth of *E. coli* was substantially suppressed. Results obtained by fluorescence and bioluminescence suggest that the combinations of AMP and PZ function synergistically. It is obvious that PZ has limited antimicrobial effects at low concentrations but it enhances the activity of AMP against *E. coli* significantly. Thus clear synergism was observed between AMP and PZ at AMP and PZ concentrations up to 125 μg / mL.

6. DISCUSSION

The direct antibacterial activity of phenothiazines against susceptible bacteria has been studied over many years (154, 155). The use of these compounds for the management of bacterial infections has not been attempted because the concentrations that inhibit bacterial growth *in vitro* (133) are not achievable clinically. Some of these compounds are also known to produce serious side-effects. Nevertheless, the administration of clinical doses of the phenothiazine, PZ as an adjuvant to conventional antibiotic therapy for difficult paediatric bacterial infections has yielded significant success as compared with the use of the antibiotic alone. This enhancement of antibiotic activity against selected species of bacteria has been reproduced *in vivo* with various phenothiazines in combination with GENT (173) and with other drugs *in vitro* (174).

The mechanism by which phenothiazines, their derivatives and structurally similar compounds enhance the activity of conventional antibiotics has been postulated to involve plasmid curing, interaction with plasmid DNA, and functional alterations of the plasma membrane of the bacteria, to the extent that transport mechanisms are affected. The direct action of the phenothiazines on the permeability of the membrane itself has also been considered (175).

■ *E. coli, P. aeruginosa* and *S. epidermidis* are moderately sensitive to phenothiazines since the concentrations that inhibit growth exceed 60μg/mL, as shown in this and other studies (162). These organisms are well suited for studies of potential enhancement of antibiotic activity by such compounds. The results obtained in this study did not identify any combination with a given antibiotic when challenged against different species of bacteria.

Synergistic interactions were found for AMP plus PZ, AMP plus MB, AMP plus CP, TC plus PZ, ERY plus PZ and ERY plus MB on the *E. coli* K12 LE 140 F' lac strain *in vitro*.

Other authors studied the action of combinations of the synthetic peptides and antibiotics on *E. coli* (176). Neither synergy nor antagonism was revealed with antibiotics AMP or vancomycin that interact with the cell wall.

Synergic interactions between the different peptides and ERY were observed when tested against *E. coli*. Several synergy studies on antimicrobial peptides have been reported (177-180).

The macrolides are large hydrophobic antibiotic molecules that are usually ineffective against Gram-negative bacteria owing to the OM barrier or efflux of the antibiotic (181, 182). Several cationic antibacterial peptides are known to interact with the OM of Gram-negative bacteria, making this outer protective shield more permeable (183). Another possible mode of action may be a blockade of macrolide efflux pumps by the peptides. Other authors reported on synergy between a macrolide and cationic peptides, concluding that this was a complex mechanism probably involving a peptide-induced entrance of large lipophilic molecules into the cell (184).

ERY acts on the ribosomal 50S subunit, inhibiting translation by blocking either the peptide transferase action or the translocation step; whilst not entirely excluding the hypothesis of an increased uptake as a result of increased permeability of the LPS, it is possible that antibacterial peptides and ERY may inhibit sequential steps in the protein biosynthesis (185).

Similar synergistic effect were found for TC plus PZ, TC plus CP, ERY plus PZ and ERY plus CP on *S. epid*ermidis strains *in vitro*.

Synergism was observed with a combination of GENT plus MB on a *P. aeruginosa* strain *in vitro.* Another author also established sensitivity to GENT, amikacin, carbenicillin and tobramycin on *P. aeruginosa* (186).

The activities of these compounds, at the level of the plasma membrane are modified by the nature of the cell wall, and thus are species specific. The activity, when present, is a result of the interaction of the antibiotic and the compound external to the membrane itself. The latter possibility has been proposed by other workers (187). Synergistic activities demonstrable for a given combination of antibiotic and a modifier of antibiotic activity for a species of bacteria may indeed prove clinically useful if such activity is present consistently for strains of that species.

■ Synergistic or additive effects of AcDHPs (G1–G11) with AMP or ERY have been shown on *E. coli* K12 LE 140/F'lac and *E. coli* AMP_{sens} • $ERY_{res.}$. These effects of synergistic or additive combinations are supported by additional experiments, in which trimeprazine exhibited significant synergistic antimicrobial activity when combined with either trimethoprim or sulfathiazole (188, 189). The present results clearly define the effects of combinations of AcDHP analogues (G1-G11) with AMP or ERY, which are of some interest.

Apparently the synergism of Ca^{2+}-channel blockers and antibiotics depends on the chemical structures of the dihydropyridines.

The results suggest that BzDHP may be a MDR modifier, as VP or NP, may act as a Ca^{2+}-channel blocker and, due to this, may inhibit the efflux of ERY or other macrolides of leucomycin (184). The enhancing effect of the BzHDPs on the antibacterial action of some macrolides may be important in the treatment of infections, as found previously for other phenothiazines (160). The relationship between the biological effects and the chemical structures of BzDHPs is a promising field justifying further studies.

Other authors investigated the effects of 3,5-diacetyl- and 3,5-dibenzoyl-1,4-dihydro-pyridines on the vascular functions *in vitro* by comparing their mechanical and electrophysiological actions on rat aorta rings and single rat tail artery mycocytes and quantifying their MDR-reversing activity in mouse T-lymphoma cells transfected with the MDR1 gene (190).

■ PZ eliminated the F' lac plasmid of the *E. coli* K12 LE 140 strain. PZ in combination with GB12 (40 μg/mL) increased the elimination of F' lac plasmid. Other authors studied the plasmid-curing efficiency of chlorpromazine with strains of *E. coli* K12 carrying F-prime lac or the resistance factor R-144. The mechanism of plasmid curing by surface action of the drugs is suggested as an alternative to direct intercalation of chlorpromethazine into the plasmid DNA (155).

Some tricyclic psychotropic drugs are known to have plasmid-curing avtivity. Other phenothiazines, such as chlorpromethazine sulfoxide, are not able to intercalate into *E. coli* DNA. It is concluded that the plasmid-curing ability is not necessarily related to the intercalation ability (191). Other authors have established that psychotropic drugs and some related compounds lead to the elimination of the F' lac plasmid of *E. coli* K12 LE 140 (155).

The R-plasmid elimination effect of different concentration of PZ was studied on methicillin-resistant *S. aureus* 13137 strain. The plasmidless colonies were tested by PCR.

■ The *mecA*, *ermA* and *nucA* genes from the sensitive colonies were identified. The colonies that did not pass substrates containing ERY and oxacillin became *mecA*-negative. The *mecA* gene was eliminated by PZ as plasmid-curring agent and this was confirmed by PCR. The *ermA* gene was eliminated from one clone only. The *nuc* gene was identified; all the clones carried the *nuc* gene.

Other authors have used PCR assay to identify methicillin-resistant *S. aureus* from clinical samples (192).

The *mecA* gene is a 2.4-kb chromosomal determinant encoding the PBP-2' protein which is not subject to dissemination via plasmid spread among staphylococcal strains (138).

The reliability of various methods for the species identification of *S. aureus* was evaluated. When the *nuc* gene, which encodes *S. aureus* thermonuclease (TNase), was amplified in a multiple PCR, simultaneously with the *mecA* gene, which encodes for the MR-associated penicillin-binding protein 2a of staphylococci the results showes that the detection of the *nuc* gene or its TNase product is highly reliable for the identification of both MRSA and MSSA strains, while various widely used agglutination kits do not show the same reliability for the identification of MRSA strains (169).

The TNase protein has been well characterized (193) and its gene, the *nuc* gene, has been cloned and sequenced (194). An enzymatic test for TNase production is used in many laboratories for the identification of *S. aureus* isolates (195). The PCR for amplification of the *nuc* gene is of potential for the rapid diagnosis of *S. aureus* by direct testing of clinical specimens (138).

■ Luminescence studies confirmed the strong synergistic bactericidal interactions between antibiotics and non-antibiotics in the case of AMP and PZ noteworthy green fluorescence protein content of the treated bacteria.

The antibacterial effects of antibiotics AMP and antibiotics plus resistance modifiers PZ on *E. coli* strains were measured in a system containing gFP and luciferase. Such bacterial constructs give an opportunity to measure the bacterial growth via GFP fluorescence and simultaneously the viability of GFP-containing cells can be measured by luciferase bioluminescence.

Other author have made similar investigations (196). The fluoro-luminometric assay of bacterial viability and killing of a recombinant strain of *E. coli* produced via transformation is performed with plasmids containing *gfp* and *luc* genes. The total number of cells and the activity of the bacteria are measured in microtiter plate wells without user-intervention as fluorescence and bioluminescence, which makes the assay suitable for high throughput screening applications. Using the non-volatile insect luciferin as a substrate makes possible the precise initiation of the bioluminescence reaction in each well. Instead of measuring the fluorescence of single cells as in flow cytometry, in the fluoro-luminometric

assay, it is possible to measure the total fluorescence from the sample mixture, which allows estimation of the cell number, even in situations when some of the cells have lysed during incubation .

Flow cytometric single cell analysis is a reliable method with which to determine the total cell number as well as the number of fluorescence-labeled cells. However, the flow cytometer is a complex, expensive instrument which equires special operating and result interpretation expertise (197).

The viability and killing of *E. coli* was measured on a real-time basis using a fluoro-luminometric device. Bacteria were made fluorescent and bioluminescent by expression of *gfp* and insect luciferase (*lucFF*) genes. The GFP is a highly fluorescent, extremely stable protein, which accumulates in cells during growth, and therefore the measured fluorescence signal is proportional to the total number of cells. The luciferase reaction is dependent on ATP produced by living cells, so that the bioluminescence level is a direct measure of the viable cells (198).

7. SUMMARY

■ The individual activities of antibiotics such as AMP, TC, ERY and GENT in combination with compounds known to modify bacterial resistance to given antibiotics were studied by using the checkerboard and time-killing methods. The combinations of PZ with AMP, TC or ERY or the combination of MB and ERY produced significant synergistic activity against *E. coli.* VP, however, in combination with AMP reduced the activity of AMP against *E. coli***.** The combinations of CP with either TC or ERY were synergistic. PZ and ERY or VP and AMP were synergistic against *S. epidermidis* that was resistant to these antibiotics alone. The only synergy against *P. aeruginosa* was shown by the combination of MB and GENT.

■ Fifteen BzDHPs, (GB1-GB15) were tested on three different *E. coli* strains. The compounds had relatively high MICs on these strains. In combination with ERY GB1, GB3, GB4, GB6, GB7, GB10 and GB12 reduced the MIC of ERY.

When the BzDHPs were tested on an *E. coli* AMP_{sen} • ERY_{res} strain isolated from a clinical specimen, the reductions in the MIC values were similar to those obtained on other *E. coli* strains. In the polyresistant clinically isolated *E. coli* AMP_{res} • ERY_{res} strain, the MIC of ERY was slightly reduced in the presence of GB1-GB7. GB12 was the most effective in enhancing the activity of ERY, and was selected for plasmid elimination studies.

■ Eleven analogues NP (G1-G11) showed synergistic interactions with AMP and ERY on *E. coli* K12 LE 140/F'lac. The antibacterial effect of AMP was enhanced by most analogues, but G9 and VP were antagonistic. Two of the 11 compounds (G7and G8) were synergistic with ERY and 4 were additive. With an AMP-sensitive clinical isolate of *E. coli* AMP_{sens} • ERY_{res}, G1 antagonized the antibacterial effect of AMP and a synergistic effect was found for the an combinations of ERY with G4, G5, G6 or G7. None of the drugs had any effect on MDR clinical isolate of *E. coli* AMP_{sens} • ERY_{res}.

■ PZ eliminated the F' lac plasmid of the *E. coli* K12 LE 140 strain. PZ in combination with GB12 (40 μg/mL) increasedthe elimination of F' lac plasmid.

The R-plasmid elimination effect of different concentration of PZ was studied on methicillin-resistant *S. aureus* 13137 strain. The plasmidless colonies were tested by PCR.

■ The MRS were resistant to all penicillins, including semisynthetic penicillinase-resistant congeners, penems, carbapenems and cephalosporins. Resistance to ERY in staphylococci is usually associated with resistance to other macrolides, to the lincosamides,

and to type B streptogramin (MLS). We used the methicillin-resistant *S. aureus* 13137 strain as standard. The clones that did not pass the substrate containing TC were *mecA*-positive.
The clones that did not pass substrates containing ERY and oxacillin became *mecA*-negative. The *mecA* gene was eliminated by PZ as curing agent and this was confirmed by PCR.
On both substrate plates containing TC and oxacillin, non-growing clones were positive, that is they carried the *ermA* gene, except for one clone. During the gel treatment all the clones carried the *nucA* gene.

■ The fluorescence-luminescence-based method proved to be very useful for various bacterial viability and killing measurements. The assay can be performed on a real-time basis without user-intervention. Combined fluoro-luminometric measurement allows a better estimation of the total number of bacteria and the activity of bacterial cells instead of separate measurements. The fact that the cells need to be transformed with genes responsible for fluorescent and bioluminescent signals limits the applications of this method. However, the main application of the method is to monitor the effects of various bacteriostatic and bactericidal agents and not to examine the survival or killing of mixed cell populations.

A good marker for the cellular catabolic activity is bioluminescence emitted in reactions catalysed by the luciferases. The luciferases are a heterogeneous group of enzymes, with a majority of the research to date centred upon bacterial luciferases encoded by *luxAB* genes and beetle luciferases encoded by *luc* genes such as that of the firefly *P. pyralis*. Bacterial luciferases have certain unfavourable properties: they require long-chain, volatile, water-insoluble fatty aldehydes as substrates, and as heterodimetric proteins, their expression in various hosts is difficult. In contrast, insect luciferases are easily expressed in prokaryotes and mammalian cells.
The insect luciferases catalyse the following reaction:

ATP + D-luciferin + O_2 → ADP + PP_i + oxyluciferin + H_2O. D-luciferin passes through cell membranes, and the reaction is initiated without disruption of the cells. Because only catabolically active cells produce ATP, the bioluminescence emission is directly proportional to the activity of the cells, making bioluminescence a good indicator of the number of viable bacteria.

The automated analyses are very easy and fast to carry out, which allows new possibilities to follow changes in bacterial cultures, as well as to determine microbicidal effects of various chemical agents such as antibiotics and disinfectants, immunological agents

such as complement and phagocytes and physical agents such as heat and irradiation. The assay is suitable for the high throughput screening of large numbers of samplesthe search for in new drugs.

In the presence of both AMP and PZ the growth of *E. coli* was substantially suppressed. Results obtained by fluorescence and bioluminescence suggest that the combinations of AMP and PZ function synergistically. It is obvious that PZ has limited antimicrobial effects at low concentrations but it enhances the activity of ampicillin against *E. coli* significantly. Thus clear synergism was observed between AMP and PZ at AMP and PZ concentrations up to 125 μg/mL.

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LIST OF ABBREVIATIONS

AcDHPs	3,5-diacetyl-1,4-dihydropyridines /G1-G11/
BzDHPs	3,5-dibenzoyl-1,4-dihydropyridines /GB1-GB15/
CAM	camphor-degradation plasmid
COM	catechol-O-methyl-transferase
CFU	colony formit unit
CNS	coagulase negative Staphylococcus
CP	clomipramine
2,4-D	dichlorophenoxyacetic acid
EMB	eosine methylene blue
ERY	erythromycin
F-plasmid	fertility plasmid
FIC	fractional inhibitory concentration
GFP	green fluorescence protein
GISA	glycopeptide intermediate *Staphylococcus aureus*
Hfr	high frequency protein
IM	inner membrane
LB-broth	Luria -Bertani broth
MB	methylene blue
MDR	mulridrug resistance
MFP	major facilitator protein
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
MLS	macrolides lincosamid streptogramin
MRSA	methicillin-resistant *Staphyloccus aureus*
MSSA	methicillin-sensitive *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol)-2,5-diphenyltetrazoliumbromide
MTY	minimal tryptone yeast extract
NAH	naphthoic acid hydroxydase
OD	optical density
OM	outer membrane
Oxa	oxacillin

NP	nifedipine
PABA	para-aminobenzoic acid
PBP	penicillin binding protein
PMF	proton motive force
PZ	promethazine
R-plasmid	resistance plasmid
RND	resistance nodulation division
RTF	resistance transfer factor
SMR	small multidrug resistance
TC	tetracycline
TNase	termonuclease
Tn	transpozon
TPP	tetraphenylphosphoricum
VP	verapamil

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I.Tariné Gombkötő Zs, Gunics Gy, ifj. Regdon G and Selmeczi B : Antibakteriális hatású vaginális kúpok formálása és *in vitro* vizsgálata. *In vitro* membrándiffúziós és mikrobiológiai vizsgálatok.

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