Interactions between antibiotics and non - conventional antibiotics on resistant bacterial strains

(Ph.D. thesis)

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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 the treatment of bacterial infections, and particularly those caused by staphylococci and streptococci, around 1946. The period of the late 1940s and the 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 sulphonamide.

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 that complicates the management of critically ill patients.

S. aureus and enterococci are the most commonly isolated bacteria that cause nosocomial infections. Among those giving rise to therapeutic problems are methicillin-resistant staphylococci and vancomycin-resistant enterococci. 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; strains of methicillin-resistant *S. aureus* (MRSAs) were recorded even in the year of the drug's launch. MRSAs owe their behaviour to an additional, penicillin-resistant peptidoglycan transpeptidase, PBP-2', encoded by *mecA*. Their spread is clonal, with transfer of *mecA* being extremely rare. MRSAs accumulated and then declined in the 1960s and 1970s, but became re-established in the early 1980s. 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 a selection pressure. The administration of antibiotics directly selects variants resistant to different antibiotics or disinfectants. The same genetic elements (e.g. *qac* or *smr*) that confer resistance to some disinfectants are often present on the same plasmid conferring resistance to antibiotics.

2. AIMS OF THE STUDY

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

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

- The antibacterial effects of various newly synthetized calcium channel blockers, nifedipine (NP) analogues 3,5-diacetyl-1,4-dihydropyridines (AcDHPs)/G1-G11/ and 3,5-dibenzoyl-1,4-dihydropyridines (BzDHPs) /GB1-GB15/, and will be studied on different resistant *E. coli* strains from clinical specimens.
- The combinations of different resistance modifiers (AcDHPs) and (B zDHPs) with various antibiotics, such as ampicillin, erythromycin, tetracycline and 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 clinical Gram-positive strains.
- The plasmid-curing effects of resistance modifiers such as BzDHPs 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.
- Fluoro-luminometric measurements will be reported for the differentiation of bacterial growth and viability in the combined application of AMP and PZ.

3. MATERIALS AND METHODS

3.1. Materials

Antibiotics: Ampicillin (AMP) was purchased from (Beecham Research Laboratories, England), erythromycin (ERY) was from (Richter Gedeon Rt., Budapest, Hungary), and oxacillin (Oxa), tetracycline (TC) and gentamicin (GENT) were from (Chinoin, Budapest, Hungary).

Resistance modifiers: New resistance modifiers*:* fifteen substituted dihydropyridines were synthetized previously (1). The structures of the BzDHPs are reported below (GB1-GB15).

Eleven acetyldihydropyridines of NP analogues were synthetsized as previously described (2).

The standards 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), and imipramine (Melipramine, EGIS, Budapest, Hungary).

Painting: MTT: 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide) /thiazolyl blue/ (Sigma, St. Louis MO, USA).

Bacterials strains*:* ***Escherichia coli*** K12 LE 140/F'lac tsx, str, Δ lac, su, λ^{-}*ör, 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

Clinical isolates*:* ***P. aeruginosa*** (1), ***S. epidermidis*** (1), ***S. aureus met***$_{res}$ **(MRSA)** (4) and ***S. aureus met***$_{sens}$ **(MSSA)** (4).

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

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 distilled water at pH 7.2. The MTY plates contained 1.5% agar-agar.

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

LB-broth: Luria-Bertani broth (3).

3.2. Methods

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

• **Checkerboard method:** The checkerboard method is the technique used most frequently to assess antibacterial combinations *in vitro*. The results of the combined use of antibiotics and resistance modifiers were evaluated according to the literature, as synergism, additivity, indifferent or antagonism (5).

• **Time-killing method:** The colony counts of viable cell numbers were determined in various intervals in the presence of antibiotics or resistance modifiers. The antimicrobial effect was tested by a literature method (5).

• **Elimination of F'lac plasmid:** From an overnight preculture of *E. coli* K12 LE 140/F'lac, a $1:10^4$ dilution 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, the cultures were diluted in isotonic saline and 0.1 mL of each dilution was spread on EMB agar. The plates were incubated at 37 °C for 24 h (6).

• **Elimination of R-plasmid:** Growing cultures were supplemented with different concent-rations of curing agents. From tubes showing growth, different dilutions were prepared and 0.1 mL samples were plated on MTY plates in cases of R-plasmid carrying strains. The velvet replica from master plates was made on plates containing antibiotics (7).

• **Fluoro-luminometric viability analysis of *Escherichia coli* cells using an 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 (*luc*FF) of firefly *P. pyralis*. Genes were inserted into the frame with the *lac*Z initiation codon from pUC19, so that EGFP and luciferase proteins expressed from the lac promoter gene-coded tetracycline resistance (*Tet*) provided plasmid stability during the experiments (8).

• **Cultivation of bacteria for fluoro-luminometry :** Overnight cultures of bacteria were diluted (1:100) with fresh LB -broth. Equal volumes of bacterial culture and AMP, PZ or a combination of AMP and PZ (or 150 mM NaCl as a control) were mixed in wells of a 96-well plate for fluoro-luminometric analysis and optical density measurement to assess the AMP and PZ-dependent killing of the bacteria.

• **Fluoro-luminometric analysis :** Bacterial cultures (*E. coli* containing pEGFPlucTet) (100 µl) were mixed with 100 µL of various AMP and PZ dilutions. The fluorescence of the cells was measured for 0.1 s/well, using 485 nm excitation and 510 nm emission filter sets. Following the fluorescence measurements on particular wells at the 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 the bioluminescence of the cells was measured for 1 s/well (9).

4. RESULTS

4.1. Determination of minimum inhibitory concentrations (MICs) of some antibiotics in the presence of resistance modifiers

As concerns the combinations of antibiotics with resistance modifiers against Gram-negative and Gram-positive strains when MICs were measured, PZ, MB and CP alone had only limited inhibitory activity against *E. coli* K12 LE 140 F' lac strain. The combinations of PZ, MB and CP with AMP were synergistic against *E. coli* K12 LE 140 F' lac strain.

Synergism was not observed with combinations of AMP and VP. Of the four resistance modifiers employed, synergism was observed for PZ in combination with TC and ERY, and with the combination of MB and ERY on the *E. coli* K12 LE 140/F'lac strain.

The effect of the combination of GENT and MB against *P. aeruginosa* was synergistic.

PZ acted synergistically with TC and ERY. The combinations of CP with ERY and TC, MB with ERY were synergistic on *S. epidermidis*.

4.2. Antibacterial effects and interactions of antibiotics with 3,5-diacetyl-1,4-dihydro- pyridines (AcDHPs) (G1-G11) on *E. coli* strains

These features were evaluated with checkerboard and time-killing methods.

• **Effects of the combination of AcDHPs (G1-G11) with ampicillin**

Synergistic effects of seven **AcDHPs** (G1, G3, G4, G7, G8, G10 and G11) in combination with AMP were seen after 24 h on the ***E. coli* K12 LE 140 /F'lac** strain. Additive effects were observed for the combinations of G2, G5 and G6 with AMP. VP exhibited only an antagonistic effect with AMP on the ***E. coli* K12 LE 140 F'lac** strain.

AMP is in combination with G1 was antagonistic against the ***E. coli*** $\mathbf{AMP_{sens} \cdot ERY_{res}}$ strain.

As concerns the ***E. coli*** $\mathbf{AMP_{res} \cdot ERY_{res}}$ clinical isolate, the antibacterial effects of AMP in combination with G1-G11 were additive. VP exerted only an indifferent effect with AMP.

• **The combinations of AcDHPs (G1-G11) with erythromycin**

The **AcDHPs (G7-G8)** displayed synergistic effects with ERY against ***E. coli* K12 LE 140/F' lac** strain. The effects of G2, G3, G10, G11, VP and NP were additive. The actions of the other compounds (G1, G4, G5, G6 and G9) in combination with ERY were indifferent. G7 and G8 compounds in combination with ERY were synergistic.

The combinations of ERY with eleven **AcDHPs (G1-G11)** exerted additive effects on ***E. coli*** $\mathbf{AMP_{sens} \cdot ERY_{res}}$**.** VP in combination with ERY was indifferent.

Similarly, the combinations of the **AcDHPs (G1-G11)** with ERY exhibited additive effects on ***E. coli*** $\mathbf{AMP_{res} \cdot ERY_{res}}$ strain. VP and NP in combination with ERY were additive effects.

4.3. Antibacterial effects and interactions of 3,5-dibenzoyl-1,4-dihydropyridines (BzDHPs) (GB1-GB15*)*

Three different *E. coli* strains was teseted: ***E. coli* K12 LE 140/F'lac**, ***E. coli*** $\mathbf{AMP_{sens} \bullet ERY_{res}}$ and ***E. coli*** $\mathbf{AMP_{res} \cdot ERY_{res}}$.

On the ***E. coli* K12 LE 140/F'lac** strain, fifteen compounds (GB1-GB15) were studied in combination with ERY. Only GB12 with ERY gave a synergistic effect. Compounds GB2, GB5, GB8, GB9, GB11, GB13 in combination with ERY were not active.

On the ***E. coli*** $\mathbf{AMP_{sens} \cdot ERY_{res}}$ strain, GB2, GB3 and GB5 with ERY displayed synergistic effects. GB7, GB9, GB11 and GB14 showed additive activity. GB8, GB10, GB13 and GB15 in combination with ERY were indifferent.

On the ***E. coli*** $\mathbf{AMP_{res} \cdot ERY_{res}}$, the combination of GB2, GB5, GB6 with ERY had a synergistic effect. Compounds, GB8 - GB15 in the combination were indifferent. GB1, GB3, GB4 and GB7 showed additive activity.

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

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

The combination of Oxa with PZ exhibited synergy on only one of the four methicillin-resistant *S. aureus* strains. For the four methicillin-sensitive strains, only additive effects were found. The other resistance modifiers, imipramine and VP, in combination with Oxa were ineffective.

In the control experiment, the combination of Oxa and VP resulted in additive effect on the methicillin-sensitive *S. aureus* strains.

4.5. Studies on plasmid elimination

The metabolic plasmid elimination was studied with a broth dilution method on the ***E. coli* K12 LE 140 F' lac** strain. Since GB12 displayed the most effective antibacterial activity in combination with ERY, it was decided to study whether this combination with PZ induced plasmid elimination on ***E. coli* K12 LE 140/F'lac** cells. This combination of GB12: 16 μg/mL and PZ resulted in a noteworthy increase in plasmid

elimination. The F' lac plasmid elimination of PZ alone was 28.5%, whereas that of PZ with GB12 was 62 %.

The R-plasmid elimination effect of PZ at 100 μg/mL was studied by a broth dilution method on the ***S. aureus* 13137** strain plasmid-mediated methicillin resistance. The R-plasmid elimination was determined with a replica method. The antibiotic-sensitive colonies were isolated: 3.9 % for ERY, 4.4 % for TC and 3.7 % for Oxa.

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

In the presence of both AMP and PZ, the growth of *E. coli* was substantially suppressed. The results obtained by fluorescence and bioluminescence revealed that the combination of AMP and PZ functions synergistically as concerns the antibacterial effect. It is obvious that PZ in low concentrations significantly enhanced the activity of AMP against the tested *E. coli* strain. Thus, clear synergism was observed between AMP and PZ.

DISCUSSION

The direct antibacterial activity of Ca^{2+}-channel antagonists and phenothiazines against antibiotic-resistant bacteria has been studied for many years. The use of these compounds for the management of bacterial infections has not been attempted because the concentrations that inhibit bacterial growth *in vitro* are not achievable clinically. Some of these compounds are also known to produce serious side-effects. The administration of clinical doses of VP or PZ has been questioned.

Nevertheless, PZ as an adjuvant to conventional antibiotic therapy for serious paediatric bacterial infections such as chronic pyelonephritis caused by polyresistant bacteria 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 reported *in vivo* with various phenothiazines in combination with GENT and other drugs *in vitro* .

The mechanism by which phenothiazine derivatives and structurally similar compounds enhance the activity of conventional antibiotics has been postulated to involve plasmid curing and functional alterations in the plasma membrane of the bacteria, to the extent that the transport mechanisms are affected. The direct action of the phenothiazines and other resistance modifiers on the permeability of the membrane itself has also been considered.

- The *E. coli, P. aeruginosa* and *S. epidermidis* proved moderately sensitive to the direct antibacterial action of various resistance modifiers. In combinations, the various resistance modifiers were able to increase the activities of all the different antibiotics (10). Synergistic interactions were found between AMP plus PZ, or MB or CP. Similarly synergy was demonstrated between TC and PZ, ERY and PZ, or ERY and MB on an *E. coli* laboratory strain *in vitro*.

  Synergic interactions between various peptides and ERY were observed when tested against *E. coli*. A number of synergy studies have been performed on antimicrobial peptides. Similar synergistic effects were found for TC plus PZ, or TC plus CP, or ERY plus PZ, and ERY plus CP on *S. epidermidis* strains *in vitro.* Synergism was observed for the combination of GENT plus MB on the *P. aeruginosa* strain *in vitro.*

  The activities of these antibiotics and resistance modifiers at the level of the plasma membrane are modified by the chemical structures of the compounds and by the nature of the cell wall. The activity, when present, is a result of the interaction of the antibiotic and the compound external to the membrane itself.
- Synergistic or additive effects of newly synthetized AcDHPs (G1–G11) with AMP or ERY have been shown on the *E. coli* laboratory strain and the *E. coli* $AMP_{sens} \cdot ERY_{res}$ strain. These effects of synergistic or additive combinations are supported by additional experiments. The present results clearly define the effects of combinations of AcDHP analogues (G1–G11) with AMP or ERY, which are of some interest as substituted dihydropyridines in structure-activity relationships. Apparently the synergism between Ca^{2+} -channel blockers and antibiotics depends on the chemical structures of the dihydropyridines tested (11).

The results suggest that other groups of substituted dihydropyridines e.g. the BzDHPs, may contain resistance modifiers, similarly to VP or NP, the compounds may act as a Ca^{2+} -channel blockers, and hence they may inhibit the efflux of ERY or other macrolides. The enhancing effect of the BzHDPs on the antibacterial action of some macrolides may serve as an important model for drug design in the treatment of infections, as previously found for resistance modifiers based on antiplasmid effects (12).

- The interaction of different antibiotics (AMP, ERY, GENT, Oxa and TC) with resistance modifiers (PZ, imipramine, omeprasol, yohimbine, VP) were studie on 4 methicillin-resistant an 4 methicillin-sensitive *S. aureus* strains with checkerboard method. The 8 *S. aureus* strains were sensitive to GENT. The Oxa and PZ combination showed a synergistic effect on a methicillin-resistant *S. aureus* 13137 strain, it was additive in 4 instances, indifferent to 2 strains.
- The antiplasmid effects of some of the newly synthetized BzDHP compounds have been revealed. GB12 and PZ in combination enhanced F' lac plasmid elimination, which means that there is possible synergism between resistance modifiers.
- The evidence for beneficial interactions between resistance modifier and antibiotics was defined through the viability and killing of *E. coli,* measured on a real-time basis by using a fluoro-luminometric device. Bacteria were made fluorescent and bioluminescent by the expression of *gfp* and insect luciferase (*luc*FF) genes. The green fluorescent protein, which accumulates in cells during growth, and therefore governs the measured fluorescence signal, is proportional to the total number of cells. The luciferase reaction is dependent on the ATP produced by living cells, so that the bioluminescence level is a direct measure of the viable cells. Luminescence studies confirmed the strong synergistic bactericidal interactions between antibiotics and non-antibiotics in the GFP bacteria labelled *luc* gene. Fluoro-luminometric single cell analysis is a reliable method for determination of the total cell number and the number of living cells.

SUMMARY

This thesis present a brief overview on a new perspective in drug design against drug resistance phenomena, focusing on the plasmid-mediated infectious antibiotic resistance of bacteria. The resistance modifiers and their chemical structures were analysed in detail as concerns synergistic action with some antibiotics. Correlations were analysed between the synergistic effects and the chemical structures of various potential resistance reversal drugs.

- The individual activities of antibiotics such as AMP, TC, ERY and GENT in combination with compounds known to modify bacterial resistance to the given antibiotics were studied by using the checkerboard and time-killing methods. The combination of PZ with AMP, TC or ERY or the combination of MB and ERY produced significant synergistic activity against *E. coli.* The combinations of CP with either TC or ERY were synergistic. PZ and ERY or VP and AMP proved to be synergistic combinations against *S. epidermidis.* Synergy against *P. aeruginosa* was displayed by the combination of MB and GENT.
- Fifteen BzDHPs, (GB1-GB15) as NP analogues and NP as control were tested with different antibiotic sensitivities on *E. coli* strains. The compounds alone had relatively high MIC values 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 the *E. coli* ERY_{res}. AMP_{sens} strain isolated from a clinical specimen, the reductions in the MIC were similar to those observed on other *E. coli* strains. With a polyresistant clinical isolate *E. coli* strain, the MIC of ERY was slightly reduced in the presence of GB1-GB7. Compound GB12 was the most effective in enhancing the activity of ERY, and was selected for plasmid elimination studies.
- Eleven analogues of NP (3,5-diacetyl-1,4-dihydropyridines)/G1-G11/ exhibited synergistic interactions with AMP and ERY on an *E. coli* laboratory strain. The antibacterial effect of AMP was enhanced by most of these analogues. The actions of 2 of the 11 compounds (G7 and G8) with ERY were synergistic and four were additive. With an clinical isolate of *E. coli* AMP_{sens}. ERY_{res}, compound G1 antagonized the antibacterial effect of AMP. Synergistic effects was found for the combinations of ERY with G4, G5, G6 or G7.

- The antiplasmid effects os some the newly synthetized BzDHP compounds have been revealed. GB12 and PZ in combination enhanced F' lac plasmid elimination, which means that ther a possible synergism between resistance modifiers.
- The fluorescence-luminescence-based method proved very useful for various bacterial viability and killing measurements. In the presence of both AMP and PZ, the growth of *E. coli* was substantially suppressed. The results obtained by fluorescence-bioluminescence suggest that the combinations of AMP and PZ function synergistically. It is obvious that PZ enhances the activity of AMP against *E. coli* significantly.

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Interactions between antibiotics and non - conventional antibiotics on resistant bacterial strains

(Ph.D. thesis)

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Bevezetés

Az utóbbi években világszerte az antibiotikum-, illetve kemoterápiás szerekre rezisztens mikroorganizmusok elterjedését figyelték meg, különösen a nozokomiális fertőzéseket okozó mikróbák körében. Nem ritka, hogy bizonyos baktériumfajok izolátumai 70-90 %-ban rezisztensek egyes (régebben hatásos) antibiotikumokra. Az egyes antibioti-kumokra kialakult rezisztencia mellett az egyidejűleg több (szerkezetileg eltérő) gyógyszerre kialakult polyrezisztencia nemcsak mikroorganizmusoknál, de a daganatsejteknél is előfordul az ún. multidrog-rezisztencia (mdr).

A rezisztencia (R)-plazmid biztosítja a baktériumok szaporodását az antibiotikum jelenlétében. A baktérium ezt a tulajdonságát nemcsak az utódsejtjeinek örökíti át, hanem a környezetében lévő más baktériumoknak is átadhatja.

Az antibakteriális szerekkel szembeni rezisztencia típusok közül a legveszélyesebb, az ún. fertőző vagy plazmidok által közvetített rezisztencia.

A rezisztencia ellen általában új, a korábbiaktól eltérő szerkezetű és hatású gyógyszerek kifejlesztése az *egyik* kézenfekvő megoldás.

A *másik* út: bizonyos antibiotikum kombinációk célzott alkalmazása, melynek során a baktériumpopulációban egy-egy szerre spontán kialakult baktériumrezisztens mutánsok a kombinációk egy második tagjával elpusztíthatók. A kombinált kemoterápiát csak súlyos fertőzésekben alkalmazzák, mivel többszörösen rezisztens sejtek kiszelektálódásával járhat.

A *harmadik* megoldás: az antibiotikum rezisztencia teljes megszüntetése jelenleg pusztán elméleti jelentőségű.

Molnár és mtsai már az 1970-es években megállapították, hogy több különböző gyógyszernek is van antiplazmid hatása pl. chlorpromazin, promethazin, stb.

Az a tény, hogy egymástól távol álló baktériumfajok között is létrejöhet a rezisztencia-

plazmidok átvitele, különösen indokolttá tette, hogy a már ismert gyógyszerek és az újonnan szintetizált vegyületek hatását a baktériumok különböző csoportjain vizsgáljuk.

Célkitüzések

A kemoterápia újabb antibiotikumok kutatása mellett több fontos kérdés tanulmányozását tartja napirenden, többek között az új gyógyszerkombinációk alkalmazását. Kisérleteimben a modellként alkalmazott rezisztens laboratóriumi és klinikai törzsekre hatásos gyógyszerkombinációk modelljét tanulmányoztam *in vitro*.

1. A baktériumok antibiotikum (ampicillin, chloramphenicol, erythromycin, tetracyclin) érzékenységét, továbbá a rezisztencia módosítók (promethazin, clomipramin, verapamil) minimális gátló koncentrációját (MIC) meghatároztam (*E. coli, P. aeruginosa, S. aureus és S. epidermidis*) baktériumtörzseken.
2. Különböző újonnan szintetizált Ca^{2+}-csatornablokkolók, nifedipin analóg vegyületek 3,5-dibenzoyl-1,4-dihydropyridinek (BzDHP) /GB1-GB15/ és 3,5-diacetyl-1,4-dihydro-pyridinek (AcDHP) /G1-G11/ antibakteriális hatását tanulmányoztam klinikai mintákból származó rezisztens *E. coli* törzseken.
3. A különböző antibiotikumok (ampicillin, erythromycin, chloramphenicol, tetracyclin) és rezisztencia módosítók (AcDHP, BzDHP, nifedipin és verapamil) kombinációját vizsgáltam Gram-negatív és Gram-pozitív törzseken Checkerboard módszerrel. Az oxacillin hatását kombinációban promethazinnal, verapamillal vagy imipraminnal tanulmányoztam klinikai Gram-pozitiv törzseken.
4. A rezisztencia módosítók (BzDHP) és a promethazin F'-plazmid eliminàló hatását vizsgáltam az *E. coli* K12 LE 140 F'lac törzsön. A promethazin rezisztencia (R)-plazmid eliminàló hatását *S. aureus* 13137 törzsön tanulmányoztam.
5. Az ampicillin és promethazin kombináció antibakteriális hatását *E. coli* törzsön fluoro-luminometrias eljárással vizsgáltam.

Anyag és módszer

Antibiotikumok: ampicillin (AMP), erythromycin (ERY), oxacillin (Oxa), tetracyclin (TET) és gentamicin (GENT).

Rezisztenciamódosítók: verapamil (VP), nifedipin (NP), promethazin (PZ), methylénkék (MB), clomipramin (CP) és impramin.

Újonnan szintetizált (2, 3 és 4. C atomon szubsztituált dihydropyridin-származékokat vizsgáltam. Nifedipin analóg szerkezetű vegyületek: 3,5-dibenzoyl-1,4-dihydropyridinek (GB1-GB15) és 3,5-diacetyl-1,4-dihydro-pyridinek (G1-G11).

Baktériumtörzsek: Escherichia coli K12 LE 140 F' lac laboratóriumi törzs,
E. coli AMP_{sens} • ERY_{res} és E. coli AMP_{res} • $ERY_{res,}$
E. coli MC1061 klinikai izolátumok,
P. aeruginosa, S. epidermidis, (4) *S. aureus* met_{res} (MRSA),
(4) *S. aureus* met_{sens} (MSSA) klinikai izolátumok.

Táptalajok: MTY-levestáptalaj, MTY-agar, EMB-agar és LB-levestáptalaj.

Módszerek

Mikrohigításos módszerrel a minimális gátló koncentráció (MIC) meghatározást végeztem. **Checkerboard-féle és Time-killing módszer** alkalmazásával, az antibiotikumok és a rezisztenciamódosítók kölcsönhatását mutattam ki.

Az **F'lac plazmid eliminációját:** az *E. coli* K12 LE 140 F'lac baktériumtörzsön tanulmányoztam Mándi és mtsai (1975) módszerével.

Az **R-plazmid eliminációját:** a *S. aureus* 13137 törzsön tanulmányoztam Lederberg és Lederberg (1952) leírása szerint.

GFP-luciferáz kimutatása *E. coli* sejtekből fluoro-luminometrai eljárással Veal és

mtsai (2000) leirása szerint végeztem.

Eredmények

1. Antibiotikumok és rezisztencia módosítók MIC értéke és kölcsönhatása Gram-negatív és Gram-pozítiv baktériumtörzseken

Az *E. coli* K12 LE 140 F' lac törzs érzékenységet mutatott ampicillin, tetracyclin, erythromycin és gentamicinnel szemben. A promethazin, methylénkék, verapamil vagy a clomipraminnal szemben a baktériumtörzs közepes vagy magas szintű rezisztenciáját mutattam ki. A promethazin, methylénkék és clomipramin az ampicillinnel kombinációban szinergista hatást mutatott az *E. coli* K12 LE 140 F' lac törzzsel szemben.

A rezisztencia módosítók közül a promethazin tetracyclinnel vagy erythromycinnel szinergista hatást mutatott. Szinergizmus figyelhető meg a methylénkék és az erythromycin kombinációja esetén is *E. coli* K12 LE 140 F' lac törzsön. A gentamicin és methylénkék kombinációja szinergista antibakteriális hatású volt a *P. aeruginosa* törzzsel szemben. A promethazin tetracyclinnel vagy erythromycinnel, valamint a clomipramin erythromycinnel vagy tetracyclinnel komkbinációban szinergista hatást mutatott a *S. epidermidis* törzsön.

2. Antibiotikumok és 3,5-diacetyl-1,4-dihydropyridinek (AcDHP) (G1-G11) antibakeriális és és kölcsönhatása *E. coli* törzseken

Hét AcDHP / 3,5-dibenzoyl-1,4-dihydropyridinek/ G1, G3, G4, G7, G8, G10 és G11 az ampicillinnel kombinációban az *E. coli* K12 LE 140 F'lac törzsön szinergista hatású volt.

A G2, G5, és G6 vegyület az ampicillinnel kombinációban additív hatást mutattak. A G1 és az ampicillin kombinációja antagonista hatást mutatott az *E. coli* AMP_{sens} • ERY_{res} törzsön. Az ampicillin és G1-G11 vegyületek kombinációi additív hatást mutattak az *E. coli* AMP_{res} • ERY_{res} klinikai izolátumon.

Az AcDHP-nek közül G7-G8 vegyületnek szinergista hatása volt az erythromycinnel, a G2, G3, G10, G11, verapamil és nifedipin additív hatású volt az *E. coli* K12 LE 140 F' lac törzsön. Az erythromycin tizenegy AcDHP vegyülettel G1-G11 kombinációban additív hatást mutatott az *E. coli* AMP_{sens} • ERY_{res} törzsön. Hasonlóképpen az AcDHP (G1-G11) kombinációja az erythromycinnel additív hatást mutatott az *E. coli* AMP_{res} • ERY_{res} törzsön.

3. Antibiotikumok és 3,5-dibenzoyl-1,4-dihydropyridinek (BzDHP) (GB1-GB15) antibakteriális és kölcsönhatása *E. coli* törzseken

A vegyületek antibakteriális és kölcsönhatását három *E. coli* törzsön vizsgáltam: *E. coli* K12 LE 140 F'lac, *E. coli* AMP_{sens} • ERY_{res} és *E. coli* AMP_{res} • ERY_{res}.

Tizenöt vegyületet (GB1-GB15) erythromycinnel kombinációban vizsgáltam az *E. coli* K12 LE 140 F'lac törzsön. A GB12 az erythromycinnel kombinációban szinergista hatást mutatott.

A GB2, GB3 és GB5 vegyületeknek erythromycinnel szinergista hatásúk volt, valamint a GB1, GB4, GB6, GB7, GB9, GB11, GB12 és GB14 additív hatást mutatattak az *E. coli* AMP_{sens} • ERY_{res} törzsön. A GB8, GB10, GB13, és GB15 vegyületeknek erythromycinnel kombinációban indifferens hatásúk volt.

A GB2, GB5, GB6 erythromycinnel kombinációban szinergista hatást mutattak az *E. coli* AMP_{res} • ERY_{res} törzsön.

4. Antibiotikumok és rezisztencia módosítók antibakteriális és kölcsönhatása a methicillin-rezisztens (MRSA) és methicillin-érzékeny (MSSA) *Staphylococcus aureus* törzseken

Különböző antibiotikumok (ampicillin, erythromycin, gentamicin, oxacillin és tetracyclin) és rezisztencia módosítók (promethazin, imipramin, omeprazol, yohimbin és verapamil) antibakteriális és kölcsönhatását tanulmányoztam négy methicillin-rezisztens (MRSA) és négy methicillin-érzékeny *S. aureus* (MSSA) törzsön Checkerboard módszerrel. Az oxacillin és promethazin kombinációja a négy methicillin-rezisztens *S.*

aureus (MRSA) törzs közül csak egy törzsre volt szinergista antibakteriális hatással. A négy érzékeny törzs (MSSA) esetében a gyógyszerkombináció additív hatású volt. A kontroll kísérletben az oxacillin és a verapamil kombináció a methicillin-érzékeny *S. aureus* (MSSA) törzsekre additív hatású volt.

5. A promethazin és 3,5-dibenzoyl-1,4-dihydropyridin-származék GB12 hatása a plazmideliminációra

A metabolikus plazmid eliminációt leves hígításos módszerrel vizsgáltam. A promethazin F' lac plazmidelimináló hatását az *E. coli* K12 LE 140 törzsön tanulmányoztam. A promethazin önmagában 40%-ban, a GB12 vegyülettel kombinációban 60%-os F' lac plazmid eliminációt tapasztaltam. A kombináció szinergista hatással volt az F' lac plazmid eliminációra. A promethazin R-plazmid eliminációs hatását methicillin-rezisztens *S. aureus* 13137 törzsön vizsgáltam. Az R-plazmid eliminációt replika módszerrel határoztam meg. A promethazin R-plazmid elimináló hatása egy nagyságrenddel kisebb volt, mint az F' lac plazmid eliminációnál. Az antibiotikumokra érzékennyé vált (3.9 % erythromycinre, 4,4 % tetracyclinre és 3,7 % oxacillinre) telepeket izoláltam *S. aureus* 13137 MRSA törzsből.

6. Ampicilllin és promethazin kölcsönhatása fluoro-luminometriás eljárással az *Escherichia coli* törzsön

Mind az ampicillin mind a promethazin jelenlétében az *E. coli* növekedése jelentősen visszaesett. A fluoreszcenciás és lumineszcenciás vizsgálatok eredményei azt mutatták, hogy az ampicillin és promethazin kombinációja szinergista antibakteriális hatású volt. Alacsony koncentrációjú promethazin jelentősen növelte az ampicillin hatását az *E. coli*-val szemben. Az ampicillin és a promethazin között szinergista hatást figyeltem meg az *E. coli* törzsön.

Összefoglalás

Kisérleteim célja volt, felhivni a figyelmet a rezisztenciamódosítók tervezésének új lehetőségeire. Tanulmányoztam a rezisztenciamódosítók és a különböző hatásmechanizmusú antibiotikumok kölcsönhatását.

- Az antibiotikumoknak, mint az ampicillin, tetracyclin, erythromycin és gentamicin hatását módosították a dihydropyridin-származékok kombinációban, melyekről tudott a rezisztenciamódosító tulajdonságuk. A promethazin kombinációja az ampicillinnel, tetra-cylinnel vagy erythromycinnel illetve a methylénkék és az erythromycin kombinációja jelentős szinergista hatást mutatott a laboratóriumi *E. coli* K12 LE 140 törzsön. A clomipramin kombinációja a tetracyclinnel vagy az erythromycinnel szinergista hatású volt. A promethazin és az erythromycin, vagy verapamil és ampicillin kombinációk szinergista hatást mutattak a *S. epidermidis* törzsön. A methylénkék és a gentamicin kombinációban szinergista hatást mutatott a *P. aeruginosa* törzsön.
- Tizenöt 3,5-dibenzoyl-1,4-dihydropyridin-származékok (BzDHP) /GB1-GB15/, nifedipin analóg vegyület és a nifedipin (kontrollként alkalmazott) vegyületeket vizsgáltam különféle antibiotikummal kombinálva polyrezisztens klinikai *E. coli* törzseken. A vegyületekkel szemben a baktériumtörzsek rezisztensek voltak. Az erythromycin és a GB12 vegyület kombinációja szinergista hatású volt az *E. coli* K12 LE 140 törzsön. Az erythromycin és a GB1, GB3, GB4, GB6, GB7, GB13 és GB15 vegyületek

kombinációja additiv hatást mutattak a laborarótiumi *E. coli* K12 LE 140 törzsön.
Klinikai mintából származó *E coli* AMP_{sens} • ERY_{res} törzsön az erythromycin a GB2 és GB5 vegyületekkel kombinációban szinergista hatásúak voltak.
A polyrezisztens klinikai izolátum *E. coli* AMP_{res} • ERY_{res} törzsön az erythromycinnek a GB2, GB5 és GB6 vegyületekkel szinergista hatásúk volt.

- Tizenegy nifedipin analóg (3,5-diacetyl-1,4-dihydropyridin-származékok) (G1-G11) szinergista hatást mutatott ampicillinnel a G1, G3, G4, G7, G8, G10 és G11 és erythromycinnel a G7 és G8 kombinációban az *E. coli* K12 LE 140 laboratóriumi törzsön. Az ampicillin antibakteriális hatását a legtöbb diacetyl-dihydropyridin vegyület javította. A 11 vegyületből kettő G7 és G8 szinergista, négy vegyület additív hatást mutatott erythromycinnel az *E. coli* AMP_{sens} • ERY_{res} törzsön.
- A promethazin F' lac plazmideliminálló hatását fokozta a GB12 vegyület az *E. coli* K12 LE 140 törzsön. A promethazin R-plazmideliminálló hatását a *S. aureus* 13137 (MRSA) törzsön figyeltem meg.
- A fluoreszcencia-lumineszcencia módszer hasznosnak bizonyult a gyógyszer-kölcsönhatás vizsgálatához. A fluoreszcencia és lumineszcencia mérések eredményei azt mutatják, hogy az ampicillin és a promethazin kombináció szinergista hatással rendelkezett.

A promethazin jelentősen növelte az ampicillin hatását az *E. coli* esetében.

In vitro vizsgálataink hozzájárulhatnak ahhoz, hogy *in vivo* is javítani tudjuk az egyes antibiotikumok hatását különböző polyrezisztens baktériumtörzsek által okozott megbetegedések esetén, amely eredmények a gyógyszertervezésben hasznosíthatók lehetnek.