APPLICATION OF SYNERGETIC SET OF TRIZ PRICIPLES FOR DEVELOPING cAMP - ACCUMULATION ACTIVATORS AND THEIR INFLUENCE ON MULTI-DRUG RESISTANCE MICROORGANISMS

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If the mountain will not come to Muhammad, then Muhammad must go to the mountain
Francis Bacon, Essays, 1625

Introduction

The fight against multi-drug resistant (MDR) nosocomial microorganisms has become increasingly important in recent years [1].

Multi-drug resistant strains have arisen over the years due to overuse of antibiotics in hospitals and in the veterinary space [2]. Recent studies on the mechanism of bacterial resistance was found to be associated with biofilm formation and their ability to be in non-growing state [3,4].

Currently available antibiotic fight against planktonic forms of microorganisms. These antibiotics are no longer effective against highly susceptible microbial strains, due to bacteria generating high degree biofilm protection [5,6]. It is important to change the paradigm to combat multi-drug resistance of microorganisms [7].

Bacterial production of toxins is observed only in starving nutrient media (not rich in carbohydrates) containing aggressive factors such as, serum, red blood cells, and extracts of brain or heart tissue. This process applies to bacteria such as *Corynebacterium diphtheriae, Pseudomonas aeruginosa, Clostridium tetani* and *Bordetella pertussis* [8]. If these bacteria were transferred to the rich, non-starvation medium without the aforementioned aggressive factors, they would begin to grow rapidly and without releasing toxins [9,10]. They would also stop synthesizing biofilm and other resistance factors [11,12]

To solve this problem we face a classic TRIZ contradiction [13,14,15,16]. In order to stop the production of virulence factors we should stop killing the microorganisms, however if they are not killed then the bacteria will harm patients. This contradiction was resolved by us using the principles of TRIZ [17,18,19,20,21] and studying bacterial growth [22,23,24].

1. TRIZ principle [25] of "Inversion" (#13, belongs to the group of methods for resolving contradictions due to structural changes within the system [26].) This principle is based on "doing the opposite" of what has been done and also known in TRIZ as "The other way around." Instead of the action dictated by the conditions of the task, we should carry out the reverse action. For example, a burn can be attained not only from extreme heat, but also from extreme cold, and expansion process can occur not only by heating, but also by freezing water. Overcoming psychological inertia allowing you to use the opposite action sometimes allows you to find novel solutions. In our case this would mean that instead of killing bacteria we should enhance them.

2. TRIZ principle of "Preliminary anti-action." (#9). This means that when you know that an undesirable situation is going to happen, you may be able to take action ahead of time. This action could either prevent the undesirable situation from happening or to reduce its' impact if it does occur. We were able to come up with solution how to synchronize bacteria to the log state of bacterial growth, which makes them sensitive and they get destroyed by antibiotic, to which bacteria were resistant.

3.TRIZ principle of "Skipping "(#21). This principle tells us to conduct a process, or certain stages (e.g. destructible, harmful or hazardous operations) at high speed.

4. TRIZ principle of "Phase transitions" (#36). This important dictum tells us that substances often go through changes, such as expanding, evaporating, cooling or changing shape. This facilitated the importance of synchronizing the bacteria from the lag to the log phase.

5. TRIZ principle of "Local quality" (#3). This principle leads to the greatest local effect, specific only for log phase.

6. TRIZ principle of "Self-service" (#25) is very close to an Ideal Final Result. When bacteria stops producing virulent factors it is reduced and at some point eliminates resistance to antibiotics.

7. TRIZ Principle of "Parameter changes" (#35). Based on the TRIZ approach we can decrease the antibiotics therapeutic dose to kill bacteria, which will be as effective as the original higher antibiotic dose. The benefit of this approach will eliminate side effects of higher dose of antibiotics and achieve same antibacterial therapeutic results.

8. TRIZ Principle of "Dynamics." (#15) Dynamicity means creating systems which are able to cope with change and intrusions from the outside. In our case by dynamically changing the bacteria, which is going through phase transitions, to help cope with the environment and inhibit toxin production.

It should be noted that, even if the application of TRIZ principles does present a solution**,** it will help you better understand the system. In our case we asked, what would happen if the bacteria are not killed, but on the contrary, we stimulate their growth? How will it change bacterial aggressiveness? How will it change virulence factor production and toxins release? It is well known that bacteria secrete aggressive factors into the external environment to "clear out" the place of residence, to destroy other microbes and tissues with toxins. If nothing needs to be "cleared out" the bacteria "feels" comfortable and it ceases toxins and virulence factors release and begins bacterial growth process.

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Through TRIZ and our research we can offer a new paradigm that instead of focusing on killing bacteria, rather than focusing on synchronizing bacterial growth.

The phenomenon ("euphoric state") based on the aforementioned research is such that if the bacteria do not fight for their existence with external aggressive factors (in our case - host immune system), they become harmless to the organism [19]. To convert them to the "euphoric" state, they needed to react as if the aggressive factors from the environment are absent, and that there is an urgent need to start active growth. We have developed non-metabolite growth promoters, which stimulate the rapid growth of almost all bacterial strains in very low doses [22,23].

These promoters work by leading to the creation of high cAMP doses in microbial cells. cAMP itself is a substrate of phosphorylation, including DNA polymerases. Their activity is increased several hundred times after phosphorylation. The rapid bacterial growth is completely incompatible with the release of the majority of acquired virulence factors (including lactamases), toxin production, as well as biofilm forming [24].

At first, bacteria "clear the area" by excreting toxins, and dividing rapidly once the "enemy" is killed. Based upon this observation, we used antibacterials *after* growth stimulation. In this case, the bacteria would lose its acquired resistance in the process of rapid growth and become harmless not only to the body but also to the immune system.

We have previously studied more than 200 cAMP activators and their various combinations in order to determine the most active one. Only one of the combinations studied showed a significant acceleration of bacterial growth. cAMP- inducers (enhancers) are the derivatives of bis-pyrimidine, isoquinoline and benzimidazole. These are activators of cAMP synthesis: the first enhancer is a cAMP- adenylate-cyclase inhibitor (benzimidazole derivative), the second and third enhancers are cAMP- phosphodiesterase inhibitors (bis- pyrimidine and isoquinoline derivatives accordingly). If the activators are applied separately from each other, they are unable to stimulate bacterial growth and have no influence on multidrug resistance.

The goal of the study was to investigate the effect of cAMP accumulation activators on the antibiotic resistance of the multidrug resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains. Other researchers have shown that these bacteria can develop multidrug resistance to two or more antimicrobial drugs [27,28]

Our strains were isolated in Kharkiv hospitals and then delivered to our research institute. They were resistant to all antibiotics and antimicrobials. Only *Pseudomonas aeruginosa* remained slightly susceptible to polymyxin in 10-fold MIC.

Materials and methods

Strains and antibiotics. In our research multi-drug resistant strains *Pseudomonas aeruginosa* MDR Kharkov IMI1, *Acinetobacter baumannii* MDR Kharkov-IMI1, and *Klebsiella pneumoniae* MDR Kharkov-IMI1 were used. The following antimicrobial agents of known potency were evaluated: ciprofloxacin (Bayer AG, Wuppertal, Germany), polymyxin B (Xellia Pharmaceuticals ApS, Denmark), amikacin (Arterium, Kiev, Ukraine). Antibiotics were dissolved in water at MICs in Mueller-Hinton broth (MHB) (table 1 for ATCC strains). All subsequent dilutions were made in cation-supplemented Mueller-Hinton broth (Difco Laboratories, USA) and prepared fresh for each experiment. The same broth, but containing 0.001% enhancers: (under patenting), was used for further passaging for MDR strains. Bacterial growth characteristics were determined in a medium compared against the control group - broth without enhancers.

In our studies, we used antibacterials at MICs for strains presented as described in table 1. All test MDR strains were resistant to all studied antimicrobials, including carbapenems, at standard MICs [29]. The inoculum was added at initial concentration of 5×10^3 CFU/ml from an exponential-phase culture. At the same time, antimicrobial agents were added to the medium. Each passage included the tubes incubated for 72 h at 37°C, after which the samples (10 µl) were transferred from tubes onto blood agar plates which were incubated for 18 h at 37°C for CFU by counting live cells. Also, cell numbers were determined from an optical density-CFU standard curve by Densi-LA-meter (ERBA Lachema, Czech Republic) after incubation for 72 h in each passage. In parallel, samples (10 µl) were transferred to the new tubes as next passage. Passage-killing curves were plotted using the techniques described above. The average value of absorbance in liquid and solid medium was used for each CFU/ml point (including live and killed microbial cells).

We chose a small initial dose so that it was possible to determine the bacterial concentration along bacterial growth process using a spectrophotometer for longer observable periods. It was also important that the bacteria adapt to growth activators and begin to change. It was necessary to have sufficient time to determine the concentration of bacteria at several time intervals as the concentration became less. When the initial concentration is less, and the measurement time is not 24 hours but 72 hours. In connection with the study of bacterial growth, several points had to be fixed to determine the bacteria's concentration and at a higher initial dose, it was difficult to study the growth in detail as the bacteria multiplied very quickly due to the presence of growth enhancers and thereafter reached the stationary growth phase after 24 hours. In connection with this, it was decided to increase the intervals to 72 hours, and the initial dose to be reduced to 10 * 3 CFU / mL

Also, multiple passaging of these clinical strains in the antimicrobials presented no effect on their growth and biological properties (TRIZ Principle #42 Multiple steps action).

Results were processed using the analysis of variance in MS Exel 2016.

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Table 1. Antibacterial activities of some antibacterials in the ATCC and MDR- strains

N	Microorganism	Antimicrobials	MIC (μg/ml)	MBC (μg/ml)
1	*Pseudomonas aeruginosa* ATCC 27853	polymyxin	0,5	3,0
2		ciprofloxacin	1,0	24,0
3		amikacin	8,0	24,0
4	*Acinetobacter baumannii* ATCC 19606	polymyxin	0,5	1,0
5		ciprofloxacin	1,0	24,0
6		amikacin	8,0	-*
7	*Klebsiella pneumoniae* ATCC 13883	polymyxin	0,5	3,0
8		ciprofloxacin	1,0	3,0
9		amikacin	8,0	24,0
10	*Pseudomonas aeruginosa* MDR Kharkov IMI1	polymyxin	>64	-*
11		ciprofloxacin	>32	-*
12		amikacin	>128	-*
13	*Acinetobacter baumannii* MDR Kharkov IMI1	polymyxin	>32	-*
14		ciprofloxacin	>32	-*
15		amikacin	>128	-*
16	*Klebsiella pneumoniae* MDR Kharkov IMI1	polymyxin	64,00	-*
17		ciprofloxacin	>32	-*
18		amikacin	>128	-*

* - antimicrobials has not bactericidal properties for the strains.

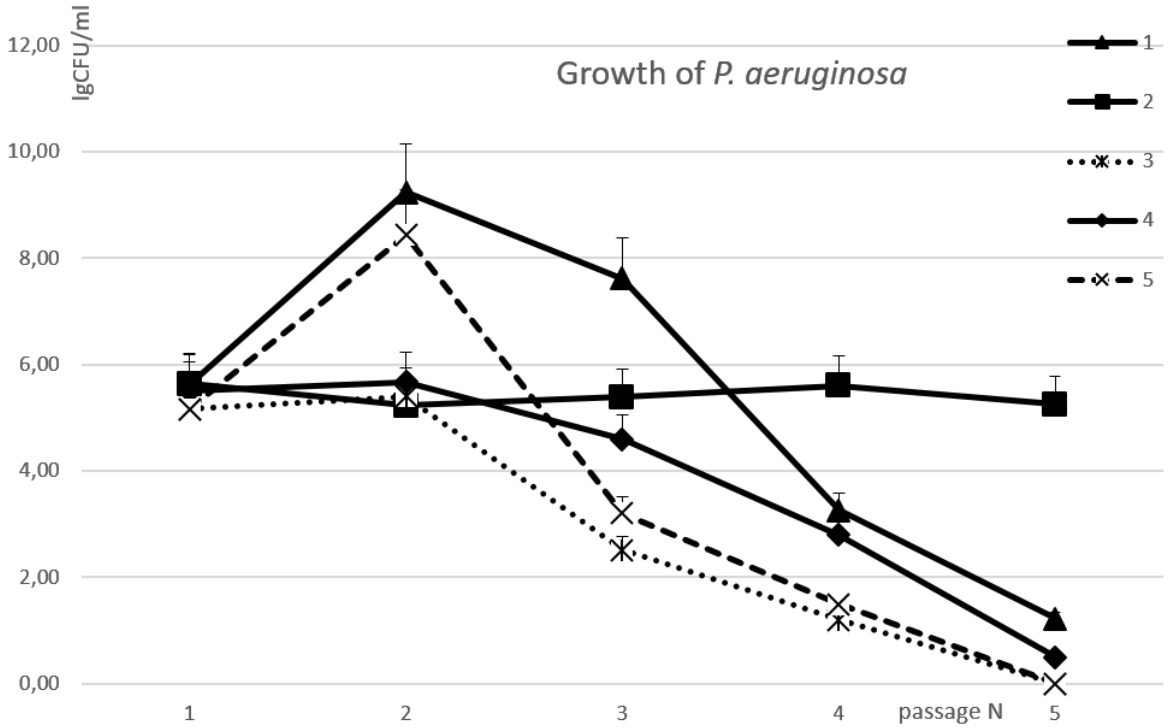

Fig.1. Relationship between the passage number of strain *P. aeruginosa* MDR Kharkov IMI1 and lg CFU/ml parameter in different media and with different antibacterials: 1 –growth on MHB with enhancers; 2 – growth on MHB without enhancers; 3 – growth on MHB with enhancers and polymyxin (0.5 μg/ml); 4 – growth on MHB with enhancers and ciprofloxacin (1.0 μg/ml); 5 – growth on MHB with enhancers and amikacin (8.0 μg/ml).

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Results and discussion

As can be seen in Fig. 1, the growth curves of *P. aeruginosa MDR Kharkov-IMI1 in* 5 passages on the medium without enhancers are almost identical (line 2): cell number increase is observed after inoculation of 10^3CFU/ml to 10^5CFU/ml at each passage. Initially low concentration of inoculum (10^3CFU/ml) was taken into consideration based on preliminary data of the potential bacterial growth within 72 hours.

P. aeruginosa Kharkov-IMI1 growth in media supplemented with enhancers is statistically different from the growth medium without enhancers: as shown in the figure (line 1), at the second passage, the CFU number was already four orders of magnitude higher versus the control; at the third passage, growth rate was markedly reduced to 10^7 CFU/ml. At passages 4-5, inhibition of bacterial growth in the presence of enhancers was observed, which required further molecular biological studies of this phenomenon. It is also not quite clear why the growth at the first passage showed almost no difference from the control 5.62 ± 0.03 lg CFU/ml with the rapid growth enhancement at the second passage.

Polymyxin addition to the medium with enhancers at concentration 0.5 μg/ml also resulted in significant changes in the microbial growth dynamics. At the first passage, the differences versus the control group were actually absent. Also, virtually no increment was observed at the second passage (line 3) (5.40 ± 0.35 lg CFU/ml). Previously multidrug resistant *P. aeruginosa Kharkov-IMI1 strain* became susceptible to polymyxin at the passage 3-4. At passage 5, no bacterial growth has been observed in the presence of polymyxin and no live bacteria were identified after blood agar inoculation. At passages 3-5, the differences from control (line 2) were statistically significant (P <0.05), as well as differences between the lines 1 and 3.

A similar growth curve was observed with the addition of 1 μg/ml ciprofloxacin to the medium. Initially, the bacterium was resistant to both polymyxin and ciprofloxacin, and did not respond to the presence of both antibacterial drugs in the medium in the recommended doses of 0.5 and 1.0 μg/ml, respectively. Growth and sensitivity to ciprofloxacin at the first passage were not statistically different from the control, whereas ciprofloxacin significantly inhibited bacterial growth already at the second passage (no statistically significant increment was observed versus the medium with enhancers). At the third passage, statistically significant (P <0.05) inhibition of bacterial growth (4.6 ± 0.13 lg CFU/ml) was observed versus both the control without enhancers (line 2), and versus the control group - the medium with enhancers (line 3). At passage 4-5, the bacterium was already highly susceptible to ciprofloxacin. Subsequent inoculation of the bacteria onto blood agar in the presence of disks with antibiotics has confirmed the loss of MDR by the bacteria and its high sensitivity to polymyxin and ciprofloxacin.

Amikacin addition in the dose 8 μg/ml to the culture medium with enhancers also led to a change in the survival curve upon passaging. The same as in the first two cases with polymyxin and ciprofloxacin, the first passage with amikacin was not different from the control – the increment of 2 orders of magnitude was the same as for the controls. The second and third passages were not statistically different from the line 1 with enhancers, whereas no bacterial growth was observed at all at passages 4 and 5, and the bacteria could not be revived on blood agar.

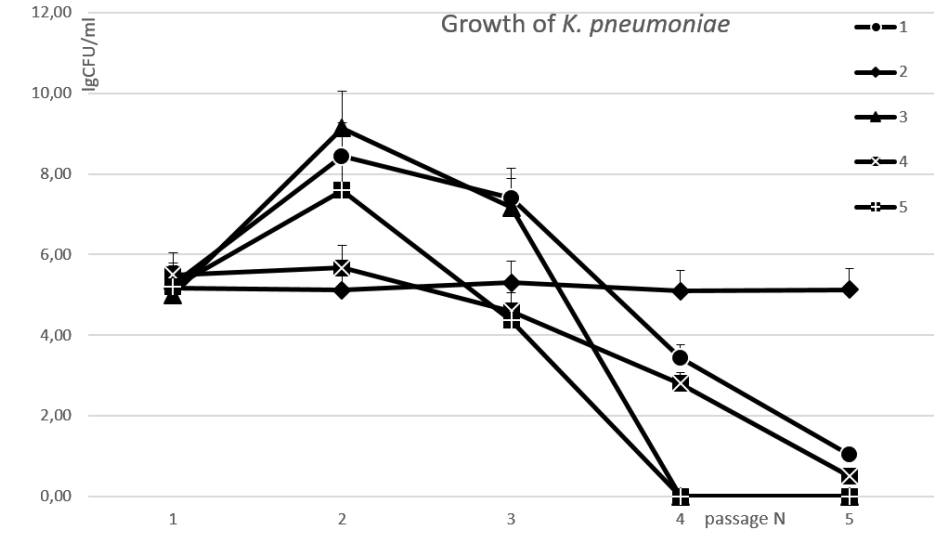

Fig. 2. Relationship between passage number of strain *K.* pneumoniae MDR Kharkov IMI1 and lg CFU/ml parameter in different media and with different antibacterials: 1 – growth on MHB with enhancers; 2 – growth on MHB without enhancers; 3 – growth on MHB with enhancers and polymyxin (0.5 μg/ml); 4 – growth on MHB with enhancers and ciprofloxacin (1.0 μg/ml); 5 – growth on MHB with enhancers and amikacin (8.0 μg/ml).

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Growth curves (Fig. 2) of the K. *pneumoniae* MDR Kharkov-IMI1 *at* 1-5 passages (without enhancers) were almost identical (line 2): increase in the cells number by 2 orders of magnitude with inoculum from 10^3 CFU/ml to 10^5 CFU/ml was observed at each passage. Growth of *K. pneumoniae* MDR Kharkov-IMI1 *in* the medium with enhancers was statistically significantly different from the growth in the medium without enhancers (8.43 ± 0.40 lg CFU/ml). As can be seen in the figure (line 1), CFU number at the second passage was already 4 orders of magnitude higher than in the control; at the third passage, the growth rate was markedly reduced to 10^7 CFU/ml. At passages 4-5, inhibition of bacterial growth was observed in the presence of enhancers; this fact requires further molecular-biological studies of this phenomenon. Addition of 0.5 μg/ml polymyxin to the culture medium with enhancers also resulted in significant changes in the growth dynamics: while the differences were absent in the first passage, the increment at the second passage (line 3) was even higher than in the control medium with enhancers (10^9 CFU/ml). *K. pneumoniae* MDR Kharkov-IMI1 *growth* at passage 3 hardly differed from the antibiotic-free control; no bacterial growth was observed in the presence of polymyxin at passages 4-5, and no live bacteria were identified after inoculation into blood agar. At passages 4-5, the differences from the control (line 2) were statistically significant ($P < 0.05$).

After 1 μg/ml ciprofloxacin addition in the medium, the pattern quite different from *P. aeruginosa* MDR Kharkov-IMI1 *was* observed. Initially, the bacterium was resistant to both polymyxin and ciprofloxacin, and did not respond to the presence of both antibacterial drugs in the recommended doses of 0.5 and 1.0 μg/ml, respectively. Microbial count increment and sensitivity to ciprofloxacin at the first passage were not statistically different from the control, whereas ciprofloxacin already significantly inhibited bacterial growth at the second and third passages ($P < 0.05$): 5.67 ± 0.37 lg CFU/ml and 4.60 ± 0.13 lg CFU/ml, respectively. The fourth passage showed statistically significant ($P < 0.05$) bacterial growth inhibition (2.80 ± 0.30 lg CFU/ml) versus both the control without enhancers (line 2) and the control group – medium with enhancers (line 3). The bacterium was already highly susceptible to ciprofloxacin, and yielded single colonies on blood agar at the passage 5. Subsequent inoculation into blood agar containing disks with antibiotics has confirmed the bacteria's MDR loss and high sensitivity to polymyxin and ciprofloxacin.

Addition of 8 μg/ml amikacin to the medium with enhancers also led to a change in the survival curve upon passaging. The first and second passages did not differ from controls. The third passage was statistically different from the line 1 with the enhancers (experimental data: 4.37 ± 0.47 lg CFU/ml), whereas in the passage 4 and 5, no bacterial growth was observed at all, and bacterial revival on blood agar was not successful. In this case, we can see the regularity typical of multi-drug resistant *P. aeruginosa* MDR Kharkov-IMI1 as well. Probably, such an abrupt transition to the bactericidal effect is related with amikacin mechanism of action.

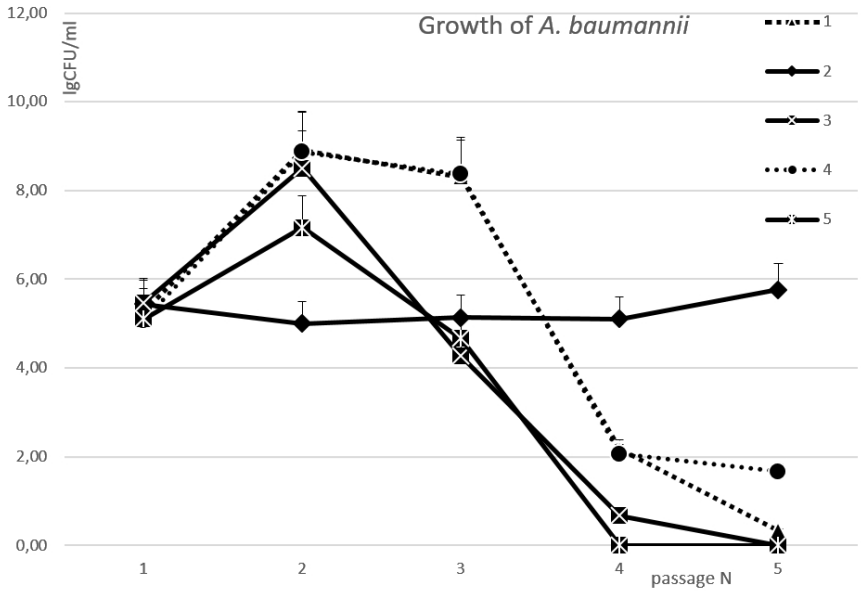

Fig. 3. Relationship between passage number for strain *A. baumannii* MDR Kharkov IMI1 and lg CFU/ml parameter in different media and with different antimicrobials: 1 – growth on MHB with enhancers; 2 – growth on MHB without enhancers; 3 – growth on MHB with enhancers and polymyxin (0.5 μg/ml); 4 – growth on MHB with enhancers and ciprofloxacin (1.0 μg/ml); 5 – growth on MHB with enhancers and amikacin (8.0 μg/ml).

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As can be seen in Figure 3, *A. baumannii* MDR Kharkov-IMI1 growth curves at 5 passages on the medium without enhancers are almost identical (line 2): increase in the number of cells by 2 orders of magnitude with inoculum from 10^3 CFU/ml to 10^5 CFU/ml was observed at each passage (Fig.3). *A. baumannii* MDR Kharkov-IMI1 growth on the medium with enhancers was statistically significantly different from the growth on the medium without enhancers (8.90 ± 0.10 lg CFU/ml). As can be seen in the figure (line 1), CFU number at the second passage was already 4 orders of magnitude higher than in the control, but at the third passage, the growth rate was significantly reduced to 10^8 CFU/ml. Bacterial growth inhibition in the presence of enhancers was observed at passages 4-5, which requires further molecular biological studies of this phenomenon. Addition of 0.5 μg/ml polymyxin to the medium with enhancers also resulted in significant changes in growth dynamics, beginning from passage 3. The growth of *A. baumannii* MDR Kharkov-IMI1 at the passage 3 was statistically significantly lower than the control (4.27 ± 0.25 lg CFU/ml); bacterial growth at passage 4 in the presence of polymyxin was decelerated (0.67 lg CFU/ml), and no growth at all was observed at passage 5. No live bacteria could be identified at passage 5 on blood agar. Differences from the control (line 2) were statistically significant ($P < 0.05$) at passages 3-5.

Addition of 1 μg/ml ciprofloxacin to the medium showed actually no statistically significant difference from the line 1, indicating *A. baumannii* MDR Kharkov-IMI1 *lack* of susceptibility to ciprofloxacin.

Addition of 8 μg/ml amikacin to the medium with enhancers led to a change in the survival curve along all passages. The first and second passages were slightly different from the control. The third passage was statistically different from the line 1 with the enhancers (experimental data: 4.67 ± 0.15 lg 4 CFU/ml), whereas no bacterial growth at all was observed at passage 4 and 5, and bacterial revival on blood agar was not successful. In this case, we observe the regularity typical of multi-drug resistant *P. aeruginosa* MDR Kharkov-IMI1 *and K. pneumoniae* MDR Kharkov-IMI1. Probably, such an abrupt transition to the bactericidal effect is related with amikacin mechanism of action.

Conclusion

By applying the TRIZ method to solve contradictions we were able pioneer new a paradigm to fight Multi Drug Resistant bacteria. Our synergistic drug combination allows us to kill what were thought to be "unkillable" antibiotics resistant bacteria. This can also be done by utilizing lower dosages of antibiotics than are currently used as standard of care.

Thus as a result of investigating the influence of cAMP accumulation activators (enhancers) on multi-drug resistant microorganisms (*P. aeruginosa* MDR Kharkov-IMI1 , *K. pneumoniae* MDR Kharkov-IMI1 , *A. baumannii* MDR Kharkov-IMI1) we have established that rapid growth stimulation by enhancers at the second passage results in the inhibition of bacterial growth at subsequent passages until complete cessation of growth at passages 4-5. We also found that enhancers contribute to a significant increase in sensitivity of multi-drug resistant bacterial strains to antimicrobials (polymyxin B, ciprofloxacin and amikacin). Changes in growth characteristics and antimicrobial sensitivity are observed only in the second passage. This demonstrates the need for further study of the molecular mechanisms of cAMP effect on the microbial cells' division and growth.

APPLICATION OF SYNERGETIC SET OF TRIZ PRICIPLES FOR DEVELOPING cAMP - ACCUMULATION ACTIVATORS AND THEIR INFLUENCE ON MULTI-DRUG RESISTANCE MICROORGANISMS

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Introduction. The control over multi-resistant nosocomial strains of microorganisms has been becoming increasingly urgent in recent years. We suggest a new paradigm that is eliminating MDR bacteria death, but makes them sensitive to antibiotics. Based on our paradigm will be decreased and suppressed future selection of resistant bacterial strains. The mechanism of action of the enhancers is caused by the activation of the cAMP high doses accumulation process in the microbial cells. cAMP itself is a substrate for phosphorylation including DNA polymerases. Applying synergetic set of TRIZ Principles from matrix of contradictions, we created pioneer new paradigm to fight multi drug resistant bacteria, which could be not only treated generally, killing "unkillable" bacteria by also it could be done by low dosage antibiotics, which is extremely important for treating patients. **Materials and methods.** MDR resistant strains *Pseudomonas aeruginosa* MDR Kharkov IMI1, *Acinetobacter baumannii* MDR Kharkov-IMI1, and *Klebsiella pneumoniae* MDR Kharkov-IMI1 were used. The following antimicrobial agents of known potency were evaluated: ciprofloxacin, polymyxin B, and amikacin. Characteristics of bacterial growth were determined in a nutrient medium compared to the control group – the broth without the enhancers. cAMP-inducers (Enhancers) are the derivatives of bis-pyrimidine, isoquinoline and benzimidazole from superfamily phosphodiesterase inhibitors. **Results and discussion.** Enhancers contribute to a significant increase in the antimicrobial sensitivity to polymyxin, ciprofloxacin and amikacin in multi-resistant strains of bacteria. During our research process changes in the growth characteristics and antimicrobial sensitivity are observed mainly in the second passage that demonstrates the need for further studies of the molecular mechanisms of the cAMP effect on the division and growth of microbial cell –

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Based on our TRIZ approach we may find solution to resolve MDR resistance in infectious disease for different types of MDR microorganisms.

Keywords: cAMP-inducers; antimicrobials; MDR strains; *Pseudomonas aeruginosa; Acinetobacter baumannii; Klebsiella pneumoniae*; bacterial growth; sensitivity to antimicrobials, TRIZ, theory of inventive problem solving, Altshuller, TRIZ in pharmaceutical industry and pharmacology, Laws of technical systems evolution, problem solving, Su-field analysis, drug-design, dynamic self-organizing, quasi live drugs, anti-cancer, antiviral, multidrug bacterial resistance, antibacterial, synergy.

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