СИНЕРГЕТИЧЕСКАЯ КОМБИНАЦИИ S-ОБРАЗНОЙ КРИВОЙ И ПЕРЕХОДА В НАДСИСТЕМУ ДЛЯ БАКТЕРИЙ С МНОЖЕСТВЕННОЙ ЛЕКАРСТВЕННОЙ УСТОЙЧИВОСТЬЮ

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Аннотация

В статье мы описали наш подход синергетической комбинации S-кривой и перехода к системе более высокого уровня для спасения человечества от бактерий с множественной лекарственной устойчивостью. Проблема в том, что антибиотики не борются с бактериями с множественной лекарственной устойчивостью. Мы создали метод и провели исследования и разработки в нашей лаборатории для борьбы с MDR. Применяя метод ТРИЗ для разрешения противоречий, мы смогли создать новую парадигму борьбы с бактериями с множественной лекарственной устойчивостью. Наша синергетическая комбинация лекарств позволяет нам убивать то, что считалось «неубиваемыми» бактериями, устойчивыми к антибиотикам. Это также можно добиться результата, используя более низкие дозы антибиотиков, чем те, которые в настоящее время используются в качестве стандарта лечения. Таким образом, в результате изучения влияния активаторов (энхансеров) накопления цАМФ на микроорганизмы с множественной лекарственной устойчивостью мы установили, что быстрая стимуляция роста энхансерами во втором пассаже приводит к ингибированию роста бактерий при последующих пассажах до полного прекращения роста в пассажах 4-5. Мы также обнаружили, что энхансеры способствуют значительному увеличению чувствительности штаммов бактерий с множественной лекарственной устойчивостью к противомикробным препаратам.

Ключевые слова: MDR, ТРИЗ, S-образная кривая, принципы ТРИЗ, вирулентные факторы.

SYNERGETIC COMBINATION S-CURVE AND TRANSITION TO A HIGHER-LEVEL SYSTEM FOR MULTI DRUG RESISTANT(MDR) BACTERIA

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Abstract

In this article we described our approach of synergetic combination s-curve and transition to a higher-level system for saving mankind from multi drug resistant bacteria. The problem is:antibiotics do not fight multi drug resistant bacteria. We created a method and made R&D in our lab to fight MDR.

Keywords: MDR, TRIZ, S-curve, TRIZ principles, virulent factors.

1 Project

1.1 Introduction

After publishing our article "Creation of New Medical Drugs Based on TRIZ and Computer Mathematical Modeling" [1,2], we received hundreds of comments and questions. In that article we described some results of our research in biotechnology and pharmacology, based on the fundamental science and TRIZ tools such as: contradictions; laws of development; technical systems; and sets of inventive principles. In our projects we used even more tools, some of them with our "know-how."

To better answer these questions, we decided to show one of our approaches in order to solve a particular problem in biotechnology and pharmaceuticals, based on S-Curve analysis and a transition to a higher-level system applicable for Multi-Drug Resistant (MDR) bacteria.

Why is this issue so important? Well, at the present time, antibiotic-resistant infections cost the U.S. economy nearly $20 billion in healthcare costs and $35 billion each year in loss of productivity.

According to the World Health Organization, as early as 2050, more than 300 million people will die from a multi-drug resistant (MDR) bacteria! In comparison, the total losses in World War II throughout the World, according to various estimates, range from 50 to 80 million deaths, including both military and civilian populations.

Why did we not begin to discover new antibiotics or modify existing ones? Creating a new antibiotic costs billions of dollars and will take many years to receive FDA approval. In the interim we will lose millions of human lives.

Since we have been working for many years by overlapping many areas, one of which is biology, we always consider analyzing in parallel S-curves: in TRIZ, J. P. Martino [2], and in Biology/ Medicine [3, 4,5,6,7].

Near the end of the late 1970's, I began building trends in bioengineering for developing prosthetics and orthotics. Part of that research was published in Novosibirsk in 1984. [8,9,10]

S-curve analysis usually helps to get information about system trends. However, it is not easy to determine the exact evolutionary stage of a System. This is why, when we analyze S -curve in bacteria growths, combined with fundamental science and other tools of TRIZ, we decided to make it as a synergetic tool to solve particular strategic tasks, which is extremely important for mankind. While we are solving a tactic problem improving Polymyxin-the last resort of antibiotic for fighting MDR, we understand that, since microbes are very flexible and dynamic, they also stop working in the nearest future. Therefore, we started working on strategic problems to find a more efficient approach fighting MDR.

What do we know about the growth curve of bacteria so far? According to the classical explanation, in a liquid nutrient medium has different stages. Briefly, the bacterial growth curve represents the number of

live cells in a bacterial population over a period of time.

There are four distinct phases of the growth curve: lag, exponential (log), stationary, and death.

The bacterial growth curve represents the number of live cells in a bacterial population over a period of time.

Lag Phase (W): This initial phase is characterized by cellular activity but not growth. A small group of cells are placed in a nutrient rich medium that allows them to synthesize proteins and other molecules necessary for replication. These cells increase in size, but no cell division occurs in the phase.

Exponential (Log) Phase (X): After the lag phase, bacterial cells enter the exponential or log phase. This is the time when the cells are divided by binary fission and are doubling in numbers after each generation. Metabolic activity is high as DNA, RNA, cell wall components, and other substances necessary for growth are generated for division. It is in this growth phase that antibiotics and disinfectants are most effective as these substances typically target bacteria cell walls or the protein synthesis processes of DNA transcription and RNA translation.

Stationary Phase (Y): Eventually, the population growth experienced in the log phase begins to decline as the available nutrients become depleted and waste products start to accumulate. Bacterial cell growth reaches a plateau, or stationary phase, where the number of dividing cells equal the number of dying cells. This results in no overall population growth. Under the less

favorable conditions, competition for nutrients increases and the cells become less metabolically active. Spore-forming bacteria produce endospores in this phase and pathogenic bacteria begin to generate substances that help them survive harsh conditions.

Death Phase (Z): As nutrients become less available and waste products increase, the number of dying cells continues to rise. In this phase, the number of living cells decreases exponentially and population growth experiences a sharp decline. As dying cells lyse or break open, they spill their contents into the environment making these nutrients available to other bacteria.

The death phase is characterized by an exponential decrease in the number of living cells.

In order to represent images of inner mechanism for describing the S-curve we use the Method of Smart Little People. The Method of Smart Little People utilizes a ‘pictorial’ idea generation approach. It engages a user in sketching ‘troops’ of smart little people represented bacteria that solve the problem.

Phase W - Imagine that hundreds of smart little people settled on a new planet (colony), where there are many resources, but there are also many aggressive predators and poisonous plants. At the same time, these smart little men lived in close quarters and competition, they have a lot of different weapons and survival techniques. The first thing they do, using these weapons and tricks, is to begin to clear the area of aggressive predators and poisonous plants releasing toxins and virulence factors into the environment to clear the area. And a sign that the territory is cleared is the absence of attacks on the colony. As soon as the territory is cleared and fenced, the second stage begins.

Phase X - when humans (bacteria) are protected and comfortable, they begin to multiply rapidly. Why do they need a gun or a camouflage in bed? This is the phase of rapid growth and reproducingbacteria. They stop producing virulence factors, including Toxin formation.

Phase Y- when in the “city” of smart little men, there will be overcrowding and will not be enough resources (food, energy). They cease to multiply, put on camouflage, take out their weapons again and start fighting with each other. The number of new smart little people (microbial cells) is approximately equal to the number of dead.

Phase Z - when resources are depleted, the smart little men are fully armed and continue to fight each other, the “city” starts dying. Part of the heavily armed men leaves the colony and “flies to a new planet”, and everything begins again from phase W.

Research took place in our lab in order to test those assumptions that we have explained above (based

on the method of small smart people). We studied the diphtheria toxin accumulation dynamics during the whole life cycle using the ELISA method (double probe by detection of dynamics of toxin's production). We also fixed the adhesion properties of bacteria.

As a result of our research, we found that in phase W, the synthesis of the maximum number of bacterial virulence factors is observed (toxin's production and adhesive properties). In the world of bacteria, such a sign is the disappearance of the aggression factors - blood components; living lymphocytes; and serum with antibodies.

However, only in phase X, no synthesis of exotoxins by bacteria was observed. In the remaining phases, the bacteria produced an exotoxin, the concentration of which was maximum in the presence of aggression factors.

In order to make the analysis stronger, based on a Transition to a Higher-Level System, we made the transition from a mono-system (one S-curve of bacterial growth), into multi-function bi-system, synergistically combining two graphs: S-curve of bacterial growths and curve of toxins.

What do the results of the analysis combined with curves of our bi-system demonstrate?

At phase W, when there are few bacteria, they just came from different medium, but they are very aggressive. In this phase, bacteria secrete the maximum amount of toxins and virulence factors in order to kill their competitors and clear the field for their further reproduction.

At the second phase X, when bacteria "feel" that there is a favorable environment around them and it is possible to multiply, they multiply rapidly, and the release of toxins and virulence factors is sharply reduced, because there is no need to secrete toxins and resistance factors.

When the medium is saturated with bacteria, they again begin to compete (in this case with each other) and again begin to release toxins and virulence factors (stationary phase Y). In phase Z, the nutrient medium is depleted, there are many inhabitants (bacteria) in the test tube, they begin to compete with each other through the production of toxins and special enzymes and eventually die - because they destroy and liquify each other.

Our task is to deceive bacteria and synchronize them in phase X, when they "feel" the need for reproduction and lose their virulence factors, thus becoming sensitive to antibiotics. The composition developed by us constantly synchronizes bacteria in the X phase.

The fight against multi-drug resistant (MDR) nosocomial microorganisms has become increasingly important in recent years [11].

Multi-drug resistant strains have arisen over the years due to overuse of antibiotics in hospitals and in the veterinary space [12]. 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 [13,14].

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 [15,16]. It is important to change the paradigm to combat multi-drug resistance of microorganisms [17].

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* [18]. 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 [19,20]. They would also stop synthesizing biofilm and other resistance factors [21]

2 TRIZ relation

To solve this problem we face a classic TRIZ contradiction [22,23 ,24 ,25] and modified our version of S-curve analysis. 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 [26,27 ,28 ,29] and studying bacterial growth [30,31 ,32].

1. TRIZ principle [33] of "Inversion" (#13, belongs to the group of methods for resolving contradictions due to structural changes within the system [34].) 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.

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 [35]. 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 [36].

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.

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 [37] 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 10fold MIC.

3 Results

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 [38]. The inoculum was added at initial concentration of 5 x 10^3 CFU/ml from an exponentialphase 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 ц1) 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 ц1) 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.

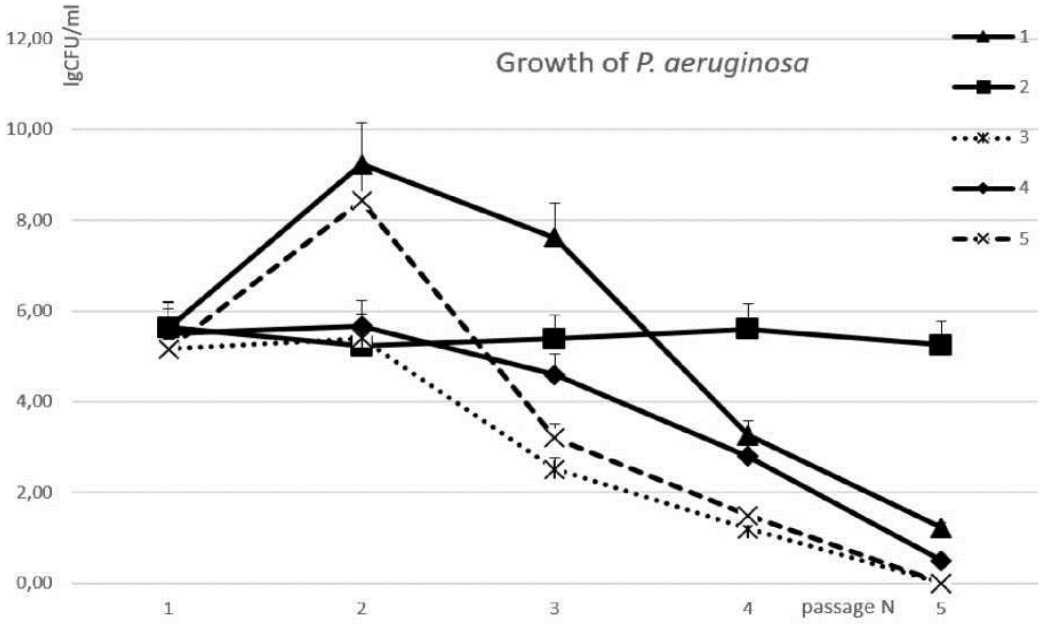

Fig.1. Relationship between the passage number of strain *P. aeruginosa* MDR Kharkov IMI1 and lg CFU/ml parameter in different media and with different antibacterials: 1 -growth on MHB with enhancers; 2 - growth on MHB without enhancers; 3 - growth on MHB with enhancers and polymyxin (0.5 gg/ml); 4 -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

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 gg/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 gg/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 gg/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.

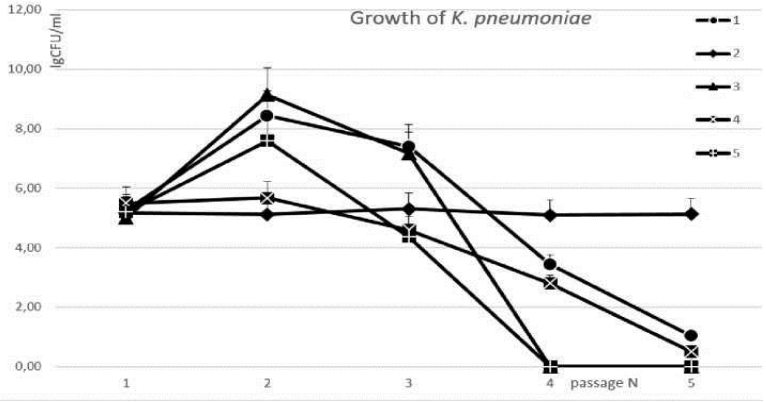

Fig. 2. Relationship between passage number of strain *K.* pneumoniae MDR Kharkov IMI1 and lg CFU/ml parameter in different media and with different antibacterials: 1 – growth on MHB with enhancers; 2 – growth on MHB without enhancers; 3 – growth on MHB with enhancers and polymyxin (0.5 μg/ml); 4 – growth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

Amikacin addition in the dose 8 gg/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.

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 gg/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).

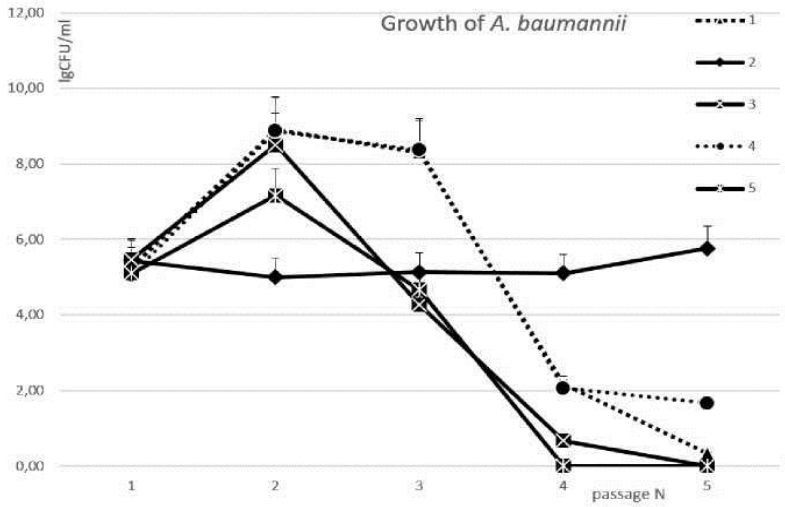

Fig. 3. Relationship between passage number for strain *A. baumannii* MDR Kharkov IMI1 and lg CFU/ml parameter in different media and with different antimicrobials: 1 – growth on MHB with enhancers; 2 – growth on MHB without enhancers; 3 – growth on MHB with enhancers and polymyxin (0.5 μg/ml); 4 – growth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

After 1 gg/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 gg/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 gg/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

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 gg/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 gg/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 gg/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.

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