Modulation of the Bacterial Defense Mechanisms by Various Chemical Structures

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Pharmaceutical active compounds (PhACs) have a remarkable successful effect on fighting animal and human diseases induced by bacteria, too. Over the time, PhACs treatments became victims of their own success, so their intensive use corroborated with their misuse resulted in PhACs treatment resistance microorganisms, becoming a serious health hazard and an environmental threat. In this study, we analysed the effect of four distinct pharmaceutical compounds such as quaternary ammonium compounds (benzenthonium chloride), tricyclic compounds (carbamazepine), fluoroquinolone (ciprofloxacin) and a derived compound from pyrimidine (trimethroprim) on five bacterial gram positive and negative strains which are naturally present in the environment. The results pointed out that carbamazepine had a specific inhibitory effect only on Delfia acidovorans and a border line effect on Staphylococcus warneri. On the other side, trimethroprim (80mg/L trimethropim) inhibited the growth of 3 out of 5 bacterial strains, the resistant strains were Citrobacter freundi and Pseudomonas aurantica. Benzenthonium chloride had an overall inhibitory effect with the exception of Staphylococcus warneri. Interestingly, ciprofloxacin had an inhibitory effect on all bacterial strains regardless of the concentration. This study clearly pointed out that various chemical structures affected specific structural and functional biological bacterial components and their presence into environment could create a massive ecological imbalance with direct impact on human health.

Keywords: Pharmaceutical active compounds, environment monitoring, bacteria

The term of pharmaceuticals is most commonly applied to designate various chemical structures used mainly in human and veterinary medicine for modulating biological pathways and especially controlling the microorganisms fate. The use of pharmaceutical compounds increased human life expectancy during the last decades [1], so they are very useful from the medical point of view. Unfortunately, their misuse induced resistant microorganisms, becoming one of the most global concerns in medicine. In order to keep under control highly resistant pathogens an *arm race* started between the production of new toxic chemical structures against bacteria and bacterial constant adaptation to chemicals. Nowadays, more and more new chemical structures have been developed so, at the beginning of XXI century, only US reached the usage of more than 16 thousands tones of antibiotics [2] and in the European Union 5400 tonnes were used only in veterinary medicine [3]. In addition, these pharmaceutical compounds were not entirely processed by human body and they reached, via used waters [4,5], the environment posing a new threat to the ecological balance. The sewage treatment plants were not designed to remove antibiotics or other pharmaceuticals. Moreover, micro-organisms have played an important role in the process of biological water treatment or soil bioremediation and the pharmaceutical could disturb bacterial growth and subsequently interfering with the ecological balance [6].

At the present, the evaluation of pharmaceutical toxic effects on environment become a priority and biological models belonging to all food chain from bacteria to fish were used. A particular emphasis has been put on bacterial microbiotests as an alternative method to vertebrate and invertebrate models used to detect the toxic effect of pharmaceuticals on the environment and its living organisms [7, 8].

In this study we assessed the ecotoxicity effect of four pharmaceutical on a bacterial model comprised of five gram positive and gram negative strains.

Experimental part

Growth medium

Lauryl sulphate broth were purchased from National Research and Development Cantacuzino (Bucharest, Romania).

Bacteria

Gram-positive and gram-negative [9,10] were purchased from ATCC: *Staphylococcus warneri* (ATCC 27836), *Pseudomonas aurantica* (ATCC 33663), *Delftia acidovorans* (ATCC 15668), *Comamonas testosteroni* (ATCC 11996) and *Citrobacter freundi* (ATCC 8090).

Chemicals

Benzethonium chloride (quaternary ammonium compound), Ciprofloxacin (fluoroquinolone compound), Carbamazepine (tricyclic compound chemically related to iminostilbene and structurally similar to phenytoin) and Trimethoprim (derivative of trimethoxybenzyl-pyrimidine) were purchased from Sigma Aldrich (table 1).

Growth inhibition assay

Every bacterial strain was initially grown on nutrient agar plates O/N at 37°C, then a single colony was grown in the nutrient broth to a density of 1 OD600nm. The bacterial growth inhibition test was performed in 96 wells plate in presence or absence of chemical compounds at various concentrations (ranging from 0 to 80mg/L). Bacterial growing rate was monitored by spectrometry at an absorbance of 600nm (A600nm).

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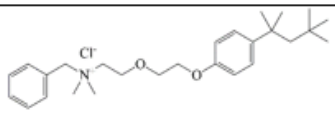
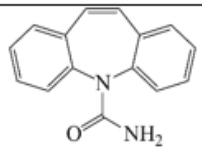
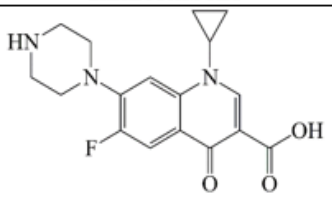
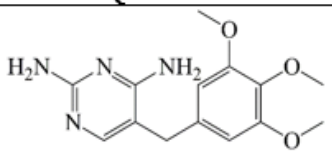
Name	Formula/ M* (Da)	Structure/ Chemical Classification	mp**(C)/ ρ*** (g/mL) (20°C)	Water Solubility
Benzethonium chloride	$C_{27}H_{42}NO_2Cl$ 448.1		162-164 0.998	1-5 g/100 mL at 18 °C
Carbamazepine	$C_{15}H_{12}N_2O$ 236.09	 Iminostilbene	189-192 1.296	insoluble in water
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$ 331.34	 Quinolone	253-257 1.5	insoluble in water
Trimethoprim	$C_{14}H_{18}N_4O_3$ 290.32	 Pyrimidine	238 – 240 1.3	slightly soluble

* M - molar mass ;** mp - melting point ;*** ρ - density

Table 1
INFORMATION ABOUT THE TESTED CHEMICALS

The toxic effect of chemical compounds was quantified in function of bacterial growing rate compared to control samples, no PhACs treatment. Each microbiological step had a positive and negative control to ensure quality outcomes and efficiency of working methods.

Results and discussions

Toxicity tests based on bacterial growth inhibition

The toxicity tests of various chemical compounds were performed on bacterial models.

Carbamazepine [5H-dibenz(b, f))azepine-5-carboxamide] is a tricyclic compound chemically related to iminostilbene and structurally similar to phenytoin encompassing two benzene rings, one seven-membered ring, one double bond and one amide group [11]. The amide functional group that is not included in heterocyclic ring and the lack the saturated carbon atoms that are characteristic to other antiepileptic drugs classify CBS as a tricyclic compound similar with other active agents such as chloropromazine or imipramine.

Carbamazepine has been extensively used as antiepileptic drug which unfortunately is not entirely processed by the human body, so it was detected not only in the sewage system but also in the groundwater at 5nmol/L [12].

We tested its environmental toxic effect on five bacterial strains. *D. acidovorans* (EC50 at 52mg/L) and *S. warneri* (EC50 at 71mg/L) where the only two bacterial strains sensitive to carbamazepine (able 2). The other three strains had a growth inhibitions around 20% induced by 80mg/L carbamazepine (fig. 1). *D. acidovorans* and *C. freundi* are two gram negative denitrifying bacteria and carbamazepine showed specificity only for *D. acidovorans* which suggested no specific interactions with the denitrifying processes.

Bacterial strains were grown in presence of up to 80mg/l carbamazepine and the inhibition growth rate was calculated by monitoring the bacterial growth at 600nm. All studies represent one of at least two independent experiments.

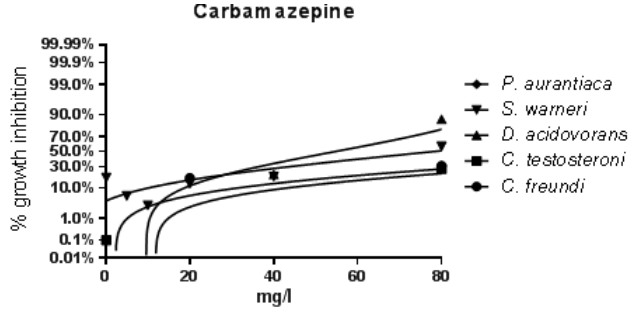


Fig.1.Carbamazepine effect on bacterial strains

Trimethoprim [5-(3,4,5-trimethoxybenxyl)-2,4-diaminopyrimidine] have a significant biological role due its amino pyrimidine and pyrimidine structures. Trimethoprim, used as antimicrobial and antiprotozoal agent, is a very potent antifolate drugs by inhibiting the bacterial enzyme dihydrofolate reductase.

The ecotoxicity tests showed that at very low concentrations trimethroprim (5 mg/L) was a very potent growth inhibitor (99% inhibition) for *D. acidovorans* (EC50 at 1mg/L *C. testosteroni* (EC50 at 7.5mg/L) (table 2). At the same time, *S. warneri* showed no growth inhibition at 40mg/l trimethroprim, then inhibition raised fast reaching an EC50 at 55mg/L (fig. 2). *P. aurentica* and *C. freundi*, two gram negative bacteria, reached a maximum growth inhibition of around 20% at 80mg/l trimethroprim (fig. 2.).

Bacterial strains were grown in presence of up to 80mg/L trimethropim and the inhibition growth rate was calculated by monitoring the bacterial growth at 600nm. All studies represent one of at least two independent experiments.

Ciprofloxacin is a synthetic broad spectrum antimicrobial agent of the fluoroquinolone class [13]. Fluoroquinolones have been used since the late 1980s in medical practice [14] as potent, broad-spectrum antibiotics for the treatment of severe or resistant

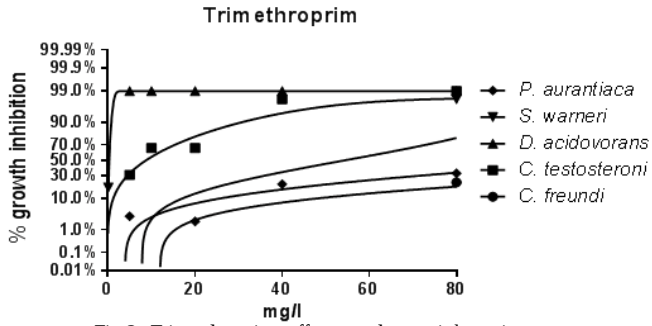


Fig.2. Trimethropim effect on bacterial strains

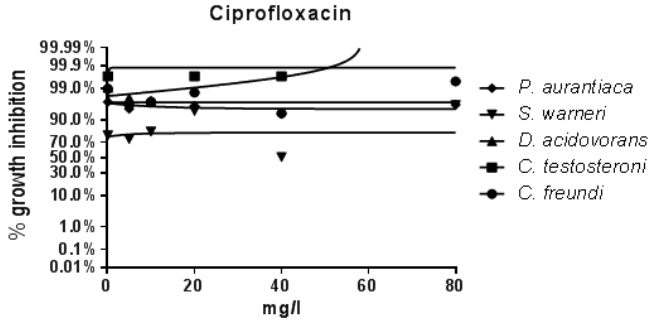


Fig.3. Ciprofloxacin effect on bacterial strains

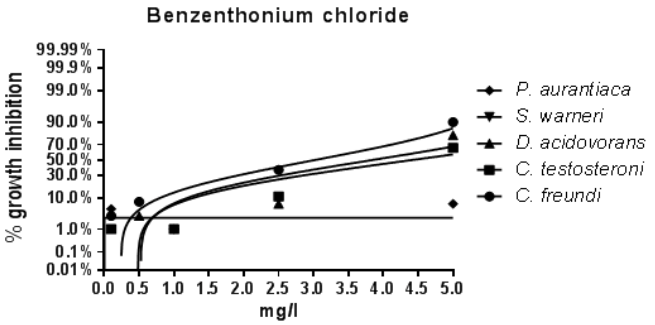


Fig.4. Benzenthonium chloride effect on bacterial strains

Bacterial strain	Carbamazepine EC50 (mg/l)	Trimethroprim EC50 (mg/l)	Ciprofloxacin EC50 (mg/l)	Benzenthonium chloride EC50 (mg/l)
P. aurantica	-	-	0.25	-
S. warneri	71	45	0.25	-
D. acidovorans	52	1	0.25	4.5
C. testosteroni	-	7.5	0.25	3.9
C. freundi	-	-	0.25	3.3

Table 2
EFFECTIVE CONCENTRATIONS (EC50) IN ACUTE TOXICITY TESTS

infections. They are derived from the quinolone family of antibiotics. Quinolones are synthetic constructs, developed by modification of 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid. Fluoroquinolones differ from quinolones by the replacement of the eighth carbon atom of the backbone with a nitrogen atom and the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity [15].

Their spectrum of efficacy against a wide range of gram positive and gram negative pathogenic, but it could lose its antibiotic potential by defluorination, decarboxylation, hydroxylation or oxidation of the amine moiety [16]. In spite of this, ciprofloxacin was persistent in the activated sludge under methanogenic conditions [17].

The mechanism of action of quinolones, including ciprofloxacin, is different from that of other antimicrobial agents such as beta-lactams, macrolides, tetracyclines, or aminoglycosides; therefore, organisms resistant to these drugs may be susceptible to ciprofloxacin. There is no known cross-resistance between ciprofloxacin and other classes of antimicrobials.

In our study, Ciprofloxacin proved to be a very potent bacterial growth inhibitor (table 2), so all five bacterial strains show an inhibition over 70% for a ciprofloxacin concentration less than 1mg/L (fig. 3).

Bacterial strains were grown in presence of up to 80mg/L ciprofloxacin and the inhibition growth rate was calculated by monitoring the bacterial growth at 600nm. All studies represent one of at least two independent experiments.

Benzethonium chloride is the most widely-used quaternary ammonium compound used as an antimicrobial agent. Quaternary ammonium compounds are any of a group of ammonium salts in which organic radicals have been substituted for all four hydrogens of the original ammonium cation. They has a central nitrogen atom which is joined to four organic radicals and one acid radical. The organic radicals may be alkyl, aryl or aralkyl, and the nitrogen can be part of a ring system. They are prepared by treatment of an amine with an alkylating agent. They show a variety of physical, chemical, and biological properties and most compounds are soluble in water and strong electrolytes. Benzethonium chloride is highly effective with short contact time against a large number of typical gram-positive and gram-negative bacterial organisms. This agent is soluble in water, lower alcohols and glycols. It is fully compatible with a wide variety of formulations as well as most types of cationic, non-ionic, and anionic systems.

It can be used effectively over a particularly wide *p*H range and can be added at both room and elevated temperatures in any phase of production. It has proven to be very stable and shows no change in composition over long periods of time.

We tested the toxic effect of Benzenthonium chloride on bacterial strains and the results showed to be very toxic at concentrations above 5mg/l (data not showed) so, its toxicity tests were performed at a concentration range below 5mg/L. BC showed a toxic effect on the bacterial growth only for a *S. warneri*, a gram positive bacteria and *P. aurantiaca* a gram negative bacteria, which suggested that its toxic effect wasn't related to the presence of not of the outer membrane (fig. 4). The BC treatment inhibited the growth of the other three bacteria (fig. 4), *C. freundi* (EC50 at 3.3mg/L), *C. testosteroni* (EC50 at 3.6mg/L) and *D. acidovorans* (EC50 at 4.3mg/L) (table 2).

Bacterial strains were grown in presence of up to 5mg/l benzenthonium chloride and the inhibition growth rate was calculated by monitoring the bacterial growth at 600nm. All studies represent one of at least two independent experiments.

Conclusions

Every particular bacterial strain used in this study showed a different resistance to the range of tested PhACs. Through the hydrophilic cationic region quaternary ammonium compounds (Benzethonium chloride) destabilized particularly denitrifying bacteria and *C. testosteroni* surface

by forming electrostatic interactions with negatively charged components.

The two phenyl groups separated by one C—C and two C—N bonds from the Carbamazepine structure seemed to affect only *S. warneri* and *D. acidovorans*.The carboxylic acid function at the 3-position and a basic piperazinyl ring (or another N-heterocycle) at the 7-position were very toxic for all five bacterial strains used in this study. On the other hand, the pyrimidine 2,4-diamine and 1,2,3-trimethoxy-benzene moieties linked by a methylene bridge had no toxic effect on two gram negative bacteria such as *P. aurantica* and *C. freundi*.

Bacterial resistance to PhACs could signify a bacterial defence mechanism mainly comprised by efflux pumps or by metabolizing the PhAcs (very important for biodegration and subsequently an efficient wastewaters treatment). For instance, no removal of Trimethroprim and Carbamazepine in the monoculture of nitrifying [18] and denitrifying bacteria (due to *C. freundi* and *D. acidovorans* sensibility to these PhACs), so the various removal efficiencies of Trimethroprim could be attributed to other heterotrophic bacteria which were not producing monooxygenase.

Overall, biodegradation of pharmaceutical compounds during biological wastewater treatment process involves a very complex microbial community structure (activated sludge composition) where heterotrophic bacteria as well as monooxygenase production bacteria such as ammonia monooxygenase is important for degradation of pharmaceutical compounds.

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