Real Science Review: Antibiotic Resistance

James, a Future Farmers of America student, asks his FFA teacher, “Mr. Mac, why does the government say that ranchers should not use antibiotics in cattle feed? I heard somewhere that antibiotics actually make cattle grow better, which is why ranchers want to use them in the cattle feed.”

Teacher McPherson: "The government does more than just recommend. They actually test cattle at slaughterhouses and reject them if antibiotics had been used. The reason is that bacteria can become resistant to antibiotics. A few bugs will survive because they are naturally resistant. They create a whole new population that is resistant. All descendants now have this defense against the antibiotic. A new strain of resistant bacteria evolves rapidly, on a time scale of a few months or years. As more and more bacteria become resistant, we find that many of our antibiotics are no longer effective to treat disease. Many of these now-resistant bacteria populations can infect animals as well as humans.”

Darwin’s Theory of Evolution helps explain how bacterial strains evolve to acquire resistance to antibiotics. Nature favors or “selects” organisms that possess unique adaptations that allow them to survive in an unsuitable or toxic environment. Some bacteria have developed genes that confer resistance to antibiotics. As antibiotic use has increased in both animals and humans, more resistant bacteria are developing.

Vocabulary Used in the Original Research Report

AcrAB-TolC multidrug efflux pump: a gene sequence that codes for the ability of certain bacteria to eject certain drugs. See “efflux pump” below.

Bacterial conjugation: Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells.

Drug resistance: ability of an organism, such as bacteria, to resist a drug that normally would damage or kill the organism.

Efflux pump: a biochemical system that operates like a pump to move molecules out of a bacterium. Bacteria that pump out antibiotics may be resistant to antibiotics because the intracellular concentration of drug is too low to be effective.

Gene Expression: genes are not necessarily “turned on” to perform their function. Those that are turned on to be active are called “expressed.”

Gram-negative bacteria: the common dye for staining bacteria is cresyl violet. A wide range of bacterial strains do not take up the stain, and these are called “gram negative.”

Horizontal gene transfer: passing or transferring genetic information “sideways” to a relatively unrelated organism (as opposed to a direct descendent)

MIC: abbreviation for “minimum inhibitory concentration.” It means the lowest concentration of an antimicrobial that will inhibit the growth of a microorganism after overnight incubation.

.
MDR: abbreviation for “multidrug resistance” or “multidrug-resistance. (In this research report, it refers to bacteria that are resistant to many different kinds of antibiotics.)

Plasmid: a segment of DNA independent of the chromosomes and capable of duplication. Used in recombinant DNA procedures to transfer genetic material from one cell to another.

Tetracycline: an antibiotic that is effective against a wide range of bacterial types. The drug is commonly used in livestock feed and in animal and human medicine. Tetracycline-resistant bacteria may infect animals or humans.

TetA: name for the tetracycline efflux pump.

Original report: Kuete, Victor, et al. (2010). Efflux Pumps Are Involved in the Defense of Gram-Negative Bacteria against the Natural Products Isobavachalcone and Diospyrone. Antimicrobial Agents and Chemotherapy. May 2010, p. 1749–1752. DOI: 10.1128/AAC.01533-09

Adapting author: W. R. Klemm

Efflux Pumps Are Involved in the Defense of Gram-Negative Bacteria against Two New Antibiotics

Abstract

We evaluated the antibiotic activity of two naturally occurring new antibiotics, isobavachalcone and diospyrone, on normal and multidrug-resistant (MDR) Gram-negative bacterial cultures. The two new antibiotics exhibited antibacterial activity against several Gram-negative bacteria and their activities. The antibacterial effect increased when we added an efflux pump inhibitor to the bacterial culture.

Also, the pump inhibitor even increased the antibiotic effect in bacterial strains in which the pump gene sequence was incomplete. The overall results indicate that the new antibiotics could be candidates for the development of new drugs against MDR strains. Combining them with efflux pump inhibitors could reinforce their activity.

Introduction

Antibiotics are drugs that kill bacteria, but not viruses. When exposed to antibiotic drugs, bacteria that escape destruction may evolve resistant strains. Drug-resistant bacteria can become dominant, and the infections they cause may no longer respond to antibiotics treatment (10, 27). The problem is worldwide in both animals and humans. There is growing challenge in healthcare to find ways to combat resistant organisms (10, 27, 31).

Approaches to avoid development of drug resistance include improving control of early infections, using antibiotics appropriately, preventing the spread of strains that do become antibiotic resistant, and development of new antibiotics (31). One reason for antibiotic resistance, is that bacteria have biochemical transport systems that eject antibiotics. These systems are called efflux pumps (15, 25). If the genes that make these pumps are fully expressed, they make it easier for the bacteria to survive antibiotics.

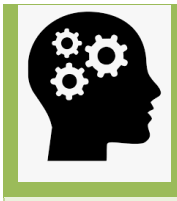
Efflux pumps have been identified in Gram-negative bacteria and enterobacterial strains (16, 19, 28). Several chemicals can inhibit these efflux pump mechanisms and restore bacterial killing levels of antibiotics. A gene sequence called AcrAB-TolC (16, 19, 28) helps to create efflux pumps. Thus, any

Introduction: Questions to Answer

1. If there was a hypothesis, either stated or implied, what was it?
2. How well did the authors justify doing this study?
3. What are some other elated ideas that they did not test?

chemical that inhibits expression of this gene would disable the pumping and allow antibiotic to become effective (29).

We tested here the ability of various bacteria strains to develop resistance to two new antibiotics for ability to develop resistance to the two new antibiotics, tetracycline, and various other antibiotics. We also wanted to know if any resistance they develop could involve efflux-pumping mechanisms.



Think About It!

In your notebook, state:

- **What antibiotics are and what "antibiotic resistance" means.**
- **Explain the idea of efflux pumps.**
- **Explain why antibiotic resistance might result from over-activity of genes that make efflux pumps.**

Methods

Chemicals for antimicrobial assays. The new antibiotics were obtained from the chemical stock bank of the Laboratory of Organic Chemistry, University of Yaoundé I, Yaoundé, Cameroon.

As a basis for comparison, we used several established antibiotics: chloramphenicol and norfloxacin (Sigma-Aldrich), tetracycline hydrochloride (Merck), imipenem-cilastatin (500/500 mg; Merck), and cefepime (Bristol-Myers), We also tested. *p*-Iodonitrotetrazolium chloride (INT), phenylalanine arginine ß-naphthylamide (PAßN), and 1,3,5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich).

Bacterial strains and culture media. The microbial species used included antibiotic-resistant and reference strains of *Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Pseudomonas aeruginosa, and Klebsiella pneumoniae. E. cloacae* strains Ec0769 and *Ec1194* were from the laboratory collection (UMR-MD1, Université de la Méditerranée, Marseille, France). Strains and their features are shown in a table that is omitted here. Prior to any assay, all strains were precultured overnight on Mueller-Hinton. Mueller-Hinton broth (MHB) was used as the liquid culture medium for antibiotic susceptibility tests (13, 20).

Bacterial susceptibility determinations. The drugs and compounds were tested in the absence (−) or in the presence (+) of the efflux inhibitor PAßN at a final concentration of 20 μg/ml. We determined minimum effective concentrations (MICs) of the new and comparison antibiotics with a colorimetric assay (8, 13). Briefly, the test sample and selected antibiotics were first dissolved in dimethyl sulfoxide. The solution obtained was serially diluted twofold in a 96-well microplate. We then added one hundred microliters of bacterial inoculum (1.5 × 106 CFU/ml) prepared in MHB. The plates were covered with a sterile plate sealer and then agitated with a shaker to mix the contents of the wells. Plates were then incubated at 37°C for 18 hours. Wells containing MHB, 100 μl of inoculum, and DMSO at a final concentration of 2.5% served as the negative control.

A dye that would indicate surviving bacteria was added (iodonitrotetrazolium chloride, 0.2 mg/ml). These samples were incubated at 37ºC for 18 hours.

Surviving bacteria were indicated by a pink color from the dye. The MIC of the antibiotics was defined as the lowest sample concentration that prevented this color change. The samples were tested alone and in the presence of an efflux-pump inhibitor, phenylalanine arginine ß-naphthylamide (PAßN), 20 µg/ml, as described previously (11). We independently repeated each assay three times.

Methods: Questions to Answer

1. What acts as a control group by receiving no treatment? What is the purpose for having this group and how well does it serve that purpose?

2. What factors (variables) that might affect the results are not taken into account?

3. What are the advantages and disadvantages of the procedures and equipment used?

Results and Discussion

When used by itself, the pump inhibitor had no effect on the bacteria.

Antibiotic activities of the two new antibiotics.

We tested various normal and antibiotic-resistant bacterial strains for their susceptibilities to the two new antibiotics and to reference antibiotics (norfloxacin, chloramphenicol). The two new antibiotics were more effective on some bacterial strains than the two common antibiotics. Isobavachalcone is nontoxic to healthy eukaryotic cells (21), suggesting that it might has clinical use.

We compared antibiotic effect with and without the antibiotics in the presence of the efflux inhibitor, PAßN. Table 1 compares the effectiveness against all strains of the two new antibiotics and the two commonly used ones. Interestingly, the activities of the two new antibiotics against resistant MDR strains (EA5 and KP63), were better than those of the commonly used antibiotics.

Table 1

MICs of the various antibiotics on normal and antibiotic resistant bacterial strains. The drugs and compounds were tested in the absence (-) or in the presence (+) of the efflux inhibitor, PAßN.

Bacterium and strain	MIC (µg/ml)[b]							
	New Antibiotics				Common Antibiotics			
	Isobavachalcone		Diospyrone		Chloramphenicol		Norfloxacin	
	–	+	–	+	–	+	–	+
E. coli								
ATCC 10536	128	2	32	2	1	0.5	0.06	0.03
ATCC 8739	256	8	128	4	4	1	0.12	0.12
AG100	64	0.5	64	1	4	0.25	0.12	0.12
*AG100A	16	0.25	4	0.12	0.5	0.25	0.03	0.007

Bacterium and strain	MIC (μg/ml)[b]							
	New Antibiotics				Common Antibiotics			
	Isobavachalcone		Diospyrone		Chloramphenicol		Norfloxacin	
	−	+	−	+	−	+	−	+
*AG100A$_{Tet}$	64	8	16	0.24	32	2	1	0.25
AG102	64	8	64	2	32	2	1	0.25
E. aerogenes								
ATCC 13048	256	16	128	32	4	1	0.25	0.25
*EA-CM64	>256	256	128	16	256	8	4	2
EA289	256	16	128	8	>256	128	128	128
EA294	32	0.5	128	8	64	16	64	32
EA298	8	0.5	32	16	64	16	8	8
EA27	256	8	128	16	>256	128	256	128
EA3	128	32	128	64	>256	128	128	64
EA5	64	16	128	64	>256	32	256	128
K. neumoniae								
ATCC 11296	32	4	32	2	2	2	1	0.5
KP55	32	4	64	8	32	4	16	8
KP63	16	0.5	32	4	>256	128	16	4
P. aeruginosa								
PAO1	64	16	64	4	128	8	2	1
PA124	64	4	64	1	256	8	64	32
E. cloacae								
Ec07769	128	8	128	8	>256	256	>256	>256

Bacterium and strain	MIC (μg/ml)[b]							
	New Antibiotics				Common Antibiotics			
	Isobavachalcone		Diospyrone		Chloramphenicol		Norfloxacin	
	−	+	−	+	−	+	−	+
Ec1194	64	1	32	2	2	1	32	32

* Strains that over-expressed efflux pump genes. Table cells shown in pink reveal data for strains resistant to the common antibacterial drugs used here.

Role of efflux pumps Inhibitor.

The efflux pump inhibitor PAßN significantly increased the antibacterial activities of the two new antibiotics, with all MICs decreasing to below 10 μg/ml for the *E. coli, K. pneumoniae*, and *E. cloacae* strains (Table 1). In addition, this enhanced activity was observed against various strains of *E. coli, E. aerogenes, K. pneumoniae, P. aeruginosa,* and *E. cloacae*.

All of the E. aerogenes strains except 298 were resistant to both new and common antibiotics. Even so, the pump inhibitor made them susceptible to the new antibiotics.

The pump inhibitor even increased the antibiotic susceptibilities of strains that lacked a complete pump gene sequence (AG100A and EA294) (Table 1, yellow highlight). This suggests that there may be an additional undiscovered pump mechanism that the inhibitor also inhibits.

The efflux mechanisms clearly appear to be the first line of bacterial defense against antibiotics, as has been demonstrated for other natural compounds (1, 5). This study provides evidence that efflux pump inhibition makes antibiotics effective against antibiotic MRD MDR strains. Both strain KP55 and strain KP63 were reported to be resistant to most of the commonly used antibiotics, showing high levels of resistance to ampicillin, ceftazidime, and aztreonam (MIC values, up to 512 μg/ml) (3). Here, we observed that all those antibiotic-resistant bacteria were susceptible to the two new compounds studied, especially in the presence of the efflux pump inhibitor.

The study also demonstrates that the efflux pump mechanism is one of the primary active defense mechanisms of bacterial cells against these molecules. This indicates that medical treatment with antibiotics might be improved if given jointly with efflux pump inhibitors.

References: Identification of the references can be found in the original report and are not necessary for our purposes here.

Results: Questions to Answer

1. Do the results support the hypothesis or not? How convincing is that support?
2. Do you notice anything of possible importance in the data that authors failed to mention?
3. Is the variation in data large enough to suggest that some unknown variables interfere with reliable results? What might these be?
4. How big is the 'treatment' effect? Is it large enough to be of much practical importance?