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ANTIBACTERIAL ACTIVITY OF NOVEL PRODRUGS OF AMOXICILLIN AND CEPHALEXIN

Rafik Karaman*[1,2], Samia Al-Kurd[1], Reem Yaghmour[1], Ahmad Amro[1], Donia Karaman[1]

[1]Bioorganic Chemistry Department, Faculty of Pharmacy, Al-Quds University, P. O. Box 20002, Jerusalem.

[2]Department of Sciences, University of Basilicata, Via dell'Ateneo Lucano 10, Potenza 85100, Italy.

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***Correspondence For Author**
Dr. Rafik Karaman
Bioorganic Chemistry Department, Faculty of Pharmacy, Al-Quds University, P. O. Box 20002, Jerusalem.

ABSTRACT

Two novel prodrugs of amoxicillin and cephalexin (amoxicillin ProD 1 and cephalexin ProD 1, respectively) were designed and synthesized to improve the stability and bitter sensation of their parent drugs. The in vitro susceptibility for both prodrugs was determined against *Escherichia coli, staphylococcus epidermidis,* staphylococcus *aureus, Klebsiella pneumonia, streptococcus group A* and *streptococcus group B,* and was compared to that of their active parent drugs.The antibacterial screening demonstrates that amoxicillin ProD 1 and cephalexin ProD 1 were found to be active and are considered among a small number of prodrugs that have therapeutic activity themselves before undergoing interconversion via enzymatic or chemical reaction to their corresponding active parent drugs. Both prodrugs exhibit their antibacterial activity against different types of bacterial strains due to the presence of β-lactam ring in their structures. In addition, it is expected that these novel prodrugs will be much more stable in aqueous media than their corresponding active parent drugs due to the fact that the chemically sensitive amine group contained in the active parent drug structures is replaced with an amide, more chemically stable group, in the corresponding prodrugs.

KEYWORDS: Antibacterials, amoxicillin, cephalexin, prodrugs, bitter sensation, cleavage.

1. INTRODUCTION

1.1 Historical Background

Infectious diseases are as old as lifetime itself. In 1910, Ehrlich synthesized salvarsan for treatment of syphilis to become the first antimicrobial drug in the world. In 1929 Fleming observed that bacterial growth was inhibited in the presence of *Penicillium notatum*. This observation makes penicillin the first broad antibiotic used in 1940s and led to its broad use during World War II. In 1935 Domagk developed sulfonamides, followed by the discovery of quinolones (e.g. Ciprofloxacin) in 1962 and oxazolidinones in 1979. [1-3]

Figure 1 shows the timeline discovery of antibiotics with natural and synthetic origins.

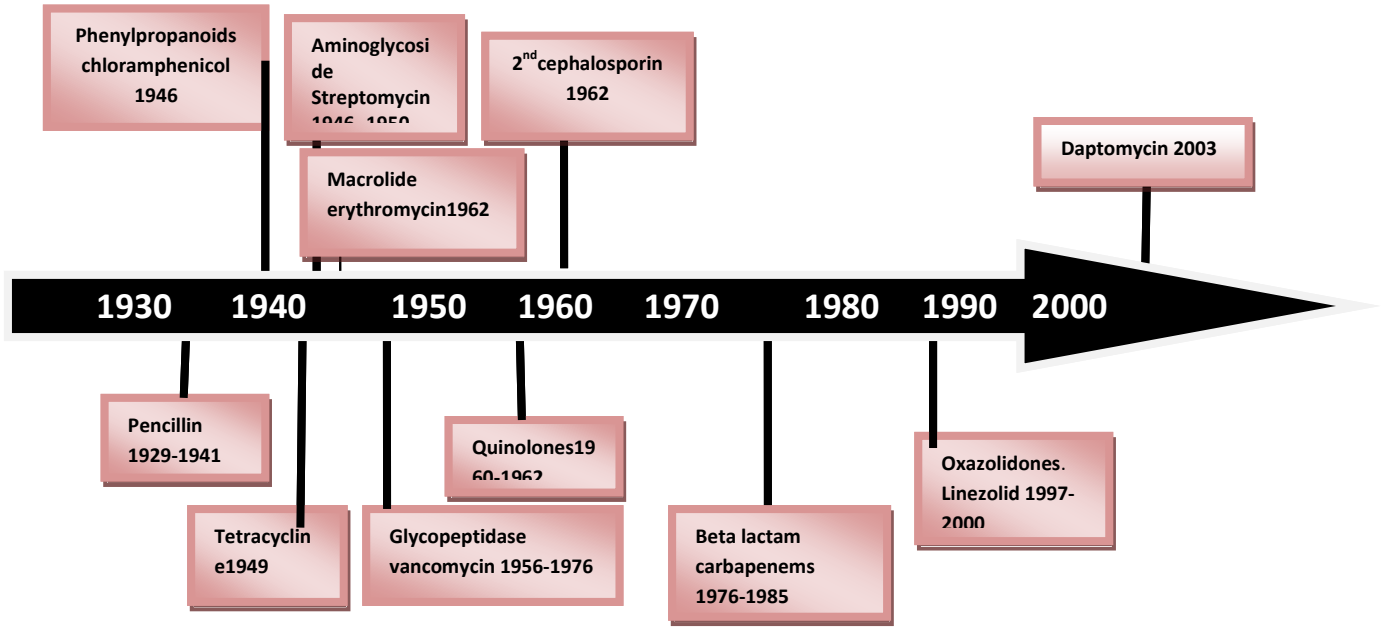

Figure 1. Timeline discovery of antibiotics[3]

1.2 Discovery of Penicillin

Sanderson and Robert independently noted that bacterial growth was prevented in the presence of fungi.[4] The same observation was made by Tyndall in 1876 upon surmising the antagonism of bacterial growth due to the low oxygen level, which presumably was consumed by fungi.

The first in vitro work was done by Cornil and Babes. Both scientists assessed the microbial inhibition and antagonism and explained this observation as a substance produced by one microorganism that may serve as an antagonist for the growth of another.[4, 5]

In 1887 Garre observed that the *staphylococcus pyogens* growth was inhibited in the presence of *Bacillus fluorescence*. Another observation which was noted by Duschesne in 1897 is the antagonism between *Penicillium* and *Escherichia* bacteria. In 1941, Waksman named these observations as antibiosis.[4] The true story began in 1928 by Fleming who observed an accumulation of *staphylococcus aureus* culture plates on one edge of his laboratory board and a colony of mold growing on the other side of the plate where *Staphylococcus aureus* around this area disappeared. Fleming was interested in this observation and he sub-cultured the mold and studied it. The culture of the mold was in nutrient broth and was for a period of eight days at room temperature. Fleming noticed that there was complete inhibition of growth of many bacteria. This fluid was first called mold juice and later Fleming named the active substance "penicillin".[6-8]

During this time period, Clutterbuck, Lovell and Raistrich extracted the active compound from the mold. They found that a pure compound could be separated by ether and watery acidic medium extractions. Upon evaporating the ether they recognized that the activity of the compound was diminished which led them to conclude that the active ingredient (penicillin) is unstable compound in acidic aqueous medium.[7]

1.3 β- lactam Antibiotics Structure

Structurally, β-lactam antibiotic molecules contain β -lactam nucleus, 6-amino penicillanic acid (6-APA) or 7-amino cephalosporinic acid (7-ACA), which provide a key for synthesis and modification (Figure 2).

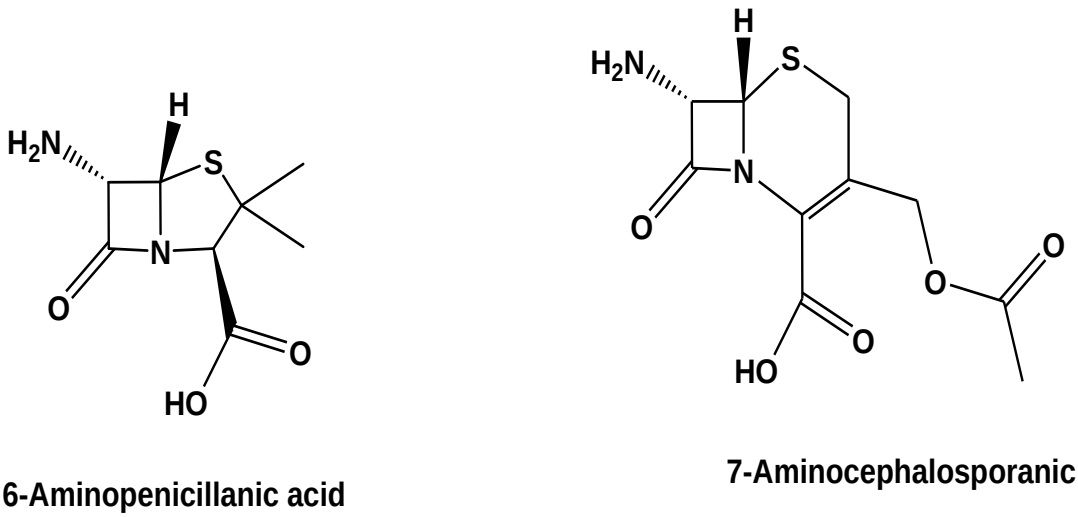

Figure 2. Chemical structures of 6-aminopenicillanic acid and 7-aminocephalosporanic acid.

Novel β-lactam agents can be synthesized by linking a unique side chain to 6-APA. Early work by Sheehan produced penicillin V by acylation of 6-APA. Thereafter, in 1960 methicillin was approved in the United States and became the first semisynthetic penicillin which is stable to enzymatic degradation, especially to penicillinase enzyme.

In addition, in 1967 carbenicillin was produced as semisynthetic compound by adding a carboxyl group instead of the amino group of ampicillin.

Abraham and Newton isolated a new family of β-lactam antibiotics from *Cephalosporium acremonium* called cephalosporin C which contains 7-ACA nucleus instead of 6-APA in penicillin.[5]

Chemical modification on β-lactam antibiotics provided many semisynthetic compounds. For example, various salts or esters of penicillin such as procaine and bezathine were synthesized and used for intramuscular injection due to their poor solubility in water.

The reactive β-lactam ring present in this class of antibiotics made these agents unstable and very labile. Therefore, a variety of modifications on the nucleus led to changes in their chemical properties such as an increase in their stability in acidic and basic media, a decrease in their degradation by enzymes and a broader spectrum of activity.[9]

Penicillanic acid (Figure 2) represents the core structure of penicillin which upon conversion to its Na^+ or K^+ salts provides soluble compounds and upon substitution with benzathine gives insoluble agents. The most important modification in the structure of the core is on the R group (Figure 3) because the β-lactam ring's reactivity and stability depend on the side chain substitution. This is essential for the action of β-lactam antibiotics to act as anti-bacterial agents.

The first semisynthetic modification was changing the side chain R in penicillin G with other side chains such as in phenoxyethyl, phenoxymethyl, where the β-lactam ring is less reactive to H^+ due to the change of the electron distribution and a creation of more stable entities.

In the basic chemical structure of cephalosporin which has a basic structure as penicillin, but it has six-member dihydrothiazine ring instead of the thiazolidine ring in penicillin both R_1 and R_2 provide opportunity for essential side chains modifications which result in changes of different properties of the agents (Figure 3).

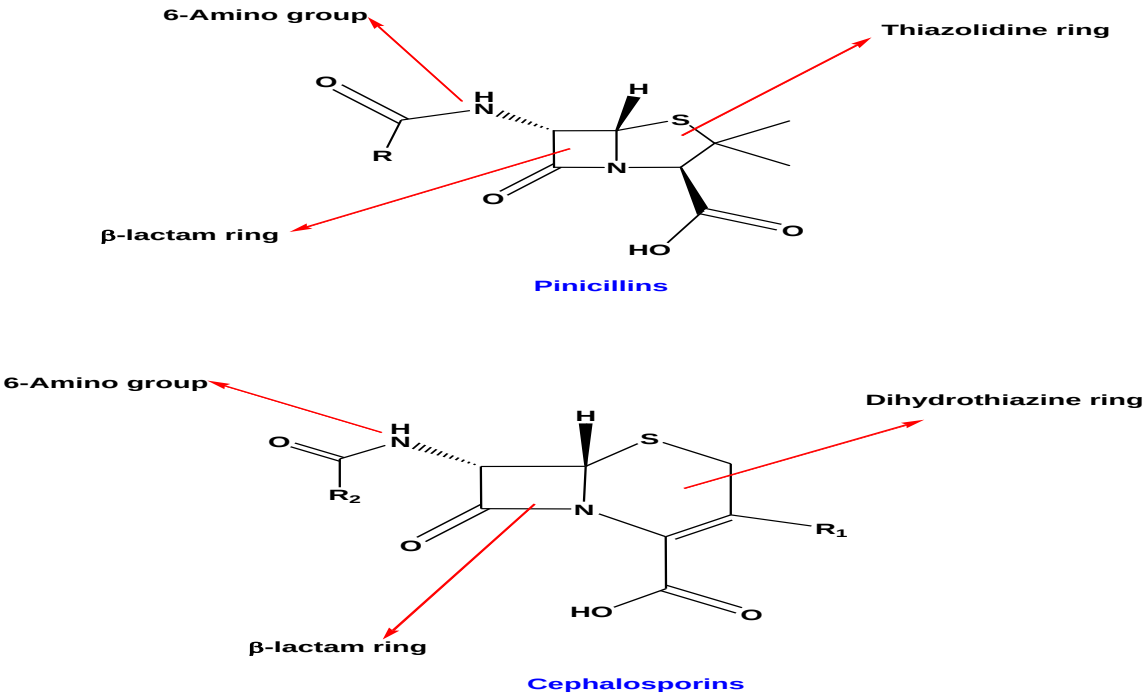

Figure 3. Penicillin's and cephalosporin's core structures, where the R, R_1 and R_2 groups are variable.[1]

The main entity contained in both structures, penicillins and cephalosporins, and essential for the antibacterial activity is the β-lactam ring. This entity interacts with active sites in the bacteria and produces the desired antibacterial effect. This happens when C—N bond in the β-lactam opens and binds to a carbon atom in the bacteria's site of action by a covalent bond, resulting in an acylation of an important group needed for cell wall synthesis.[1]

1.4 Mechanism of Action

Cell wall in bacteria is an important structure in both Gram positive and Gram negative bacteria because of stress bearing and shape maintaining function.[10] It is a complicated structure which is composed of multiple types of polymers, peptidoglycans, teichoice acid and lipopolysaccharides. However, the most important among these is peptidoglycan because it is essential for cells living under normal growth conditions.

Transpeptidase enzyme interacts with the peptide linkage contained in the pentapeptide chain of the uncrossed linked peptidoglycan (terminal D-alanine). This interaction results in D–alanine release and an acyl enzyme intermediate formation.

Penicillin behaves like terminal D-alanine in the pentapeptide chain. The CO-N bond in β-lactam structure crosses bond to the peptide bond during trans-peptidation. Thus new

transition state is formed and peptide bond is cleaved; when the enzyme cleaves the β-lactam ring forms a stable pencicilloyl-enzyme complex resulting in an inhibition of the transpeptidase enzyme.[11, 12]

1.5 Amoxicillin

In 1972 amoxicillin was synthesized in the UK. It has the same activity as ampicillin, but with higher bioavailability.[13] Later a combination of amoxicillin with clavulanic acid was developed to introduce better oral bioavailability and broad spectrum activity against a variety of pathogens that produce β-lactamase enzyme.[14]

As amoxicillin acts on cell wall of bacteria; it has bactericidal action against both gram positive and gram negative. Amoxicillin is used for many indications; treatment middle ear infection [15] laryngitis, bronchitis, pneumonia,[16] and typhoid fever.[15]

Amoxicillin is the most commonly prescribed antibiotic for children, it is well absorbed after oral administration, used for treatment in a variety of infections not only for broad spectrum also for outstanding advantage compared to other penicillins with higher bioavailability of 70-90%, and reaches C_{max} within 1-2 hours. [15] Amoxicillin is widely distributed in the body and the apparent volume of distribution is 0.26 - 0.31mL/kg, it has half-life 1-1.5 hours. [17] It is excreted by the renal route and approximately 10-25% of the drug is bio-transformed into penicillanic acid. [15]

1.6 Cephalosporin

Cephalosporins are related to penicillin β-lactam antibiotics; they act on cell wall of bacteria; interfering and lysing bacterial cell wall. This action is achieved by drug's crossing and binding to penicillin binding protein in the bacteria's cell wall (site of action).[18]

Cephalosporin has no activity against enterococcus due to low affinity on penicillin binding protein. However, it has different activities against *Pseudomonas aeruginosa* and Enterobacteriacea, because of differences in binding on the active site located on the bacteria's cell wall.[19]

Structure activity relationship and differences in side chain substitution at C7 position of the main core of cephalosporins can lead to various pharmacokinetics properties, spectra of activity, and β-lactamase stability.

It was reported that alteration of the substituent on C7 by the addition of methoxy group (cephamycin) or replacing the sulfur in dihydrothiazine ring with oxygen (moxalactam) leads to an increase in stability against enzymatic hydrolysis by β-lactamase.[18]

Conventional oral suspensions and solutions of antibiotics dispensed as powders need to be reconstituted with water at the time of use. The reconstitution process of penicillins and cephalosporins allows acceptable but short life of the antibacterial agent with storage in refrigerator.[20] The highly strained β–lactam ring that presents in both penicillin and cephalosporin structures is unstable in solution; hydrolysis occurs and as a result the antibacterial agent loses its activity.

The degradation process is an irreversible chemical change in the organic molecular structure of the antibacterial agent.[21] The degradation of penicillin can occur in different conditions; acidic or alkaline, in the presence of weak nucleophile as water and β-lactamase enzyme. Therefore, methods to increase the stability of penicillins and cephalosporins are crucially needed.

The palatability of the active ingredient of a drug is a significant obstacle in developing a patient friendly dosage form. Organoleptic properties such as taste are an important factor when selecting a certain drug from the generic products available in the market that have the same active ingredient. The problem of the bitter taste of drugs in pediatrics and geriatrics formulations still creates a challenge to pharmacists. Thus, different strategies should be developed in order to overcome this serious problem.[22-27]

In the past few years we have been engaging in studying intramolecularity and concluded that there is a need to research the mode and action by which intramolecular processes proceed in order to utilize them in the design of novel prodrugs. Unraveling the mechanism of intramolecular processes such as enzyme models would open the door widely for a precise design of chemical devices to be exploited as promoities to be covalently attached to commonly used drugs for providing prodrugs with better bioavailability and less adverse effects than their corresponding active parent drugs.

Among the intramolecular processes (enzyme models) that we have calculated using quantum mechanics and molecular mechanics methods are: (1) proton transfer between two oxygens and proton transfer between nitrogen and oxygen in Kirby's enzyme model; (2) acid-

catalyzed hydrolysis in Kirby's N-alkylmaleamic acids; (3) proton transfer between two oxygen atoms in Menger's rigid hydroxy-acids; (4) acid-catalyzed lactonization of hydroxy-acids as investigated by Cohen and (5) cyclization in dicarboxylic semi-esters as researched by Bruice and Pandit. Prodrugs in which the above mentioned enzyme models were utilized as linkers which covalently are attached to drugs having poor bioavailability or/and bitter sensation were designed and synthesized. The controlled (programmed) intraconversion rates of the novel designed prodrugs to release their active parent drugs are solely determined on the structural features of the linker and there is no need to an involvement of metabolic enzymes.[28-64]

For example, unraveling the mechanism for the intramolecular proton transfer in Kirby's acetals.[65-73] revealed a design and synthesis of novel prodrugs of aza-nucleosides for the treatment of myelodysplastic syndromes,[74] and statins to lower cholesterol concentration in the systemic blood circulation.[75] In these cases, the prodrug linker was covalently linked to the hydroxyl group in the active drug such that the prodrug has the capability to undergo a chemical cleavage upon reaching a physiological environment such as stomach, intestine, and/or blood circulation, with rates that are determined only by the structural features of the pharmacologically inactive linker (Kirby's acetal). Kirby's N-alkylmaleamic acids enzyme model.[65-73] was also studied as linkers in the design of tranexamic acid prodrugs for treating bleeding conditions [76] acyclovir prodrugs for the treatment of Herpes Simplex,[77] and atovaquone prodrugs as antimalarial agents.[78-80] The intramolecular proton transfer in Menger's Kemp acid enzyme model.[81-85] was also explored and used for the design of dopamine prodrugs for Parkinson's disease cases. [86] In addition, dimethyl fumarate prodrugs for treating psoriasis cases were also designed and developed..[87]

The novel prodrugs approach was also applied for masking the bitter sensation of the pain killer paracetamol, the anti-hypertensive agent atenolol, the decongestant phenylephrine, the anti-inflammatory agents, diclofenac and mefenamic acid and the bitter antibacterials cefuroxime, amoxicillin and cephalexin.[88-95]The role of the linker in the antibacterial prodrugs is to block the amine or/and hydroxyl groups which are believed to be responsible for the drug bitter sensation. The difference between the designed antibacterials prodrugs and their active parent drugs is that the free amine moiety in the active drug is replaced with an amide group. Replacing the amine with an amide eliminate the capability of the antibacterial

to form hydrogen bonds with the bitter taste receptor, thus masking the bitter sensation of the parent antibacterial drug.

Based on DFT calculations and experimental values obtained from intramolecular acid catalyzed hydrolysis in nine N-alkylmaleamic acids, we have designed and synthesized two prodrugs of amoxicillin (amoxicillin ProD 1) and cephalexin (cephalexin ProD 1) by reacting the antibacterial agent with a maleic anhydride linker (Figure 4) aiming to: (1) improve the stability and aqueous solubility of the antibacterial agent (2) provide antibacterial agents lacking bitter sensation.

The two synthesized prodrugs were designed such that the amine group in the active parent drugs is replaced with the more stable amide group.

In this manuscript we report the antibacterial spectrum of two novel prodrugs, amoxicillin ProD 1 and cephalexin ProD 1.

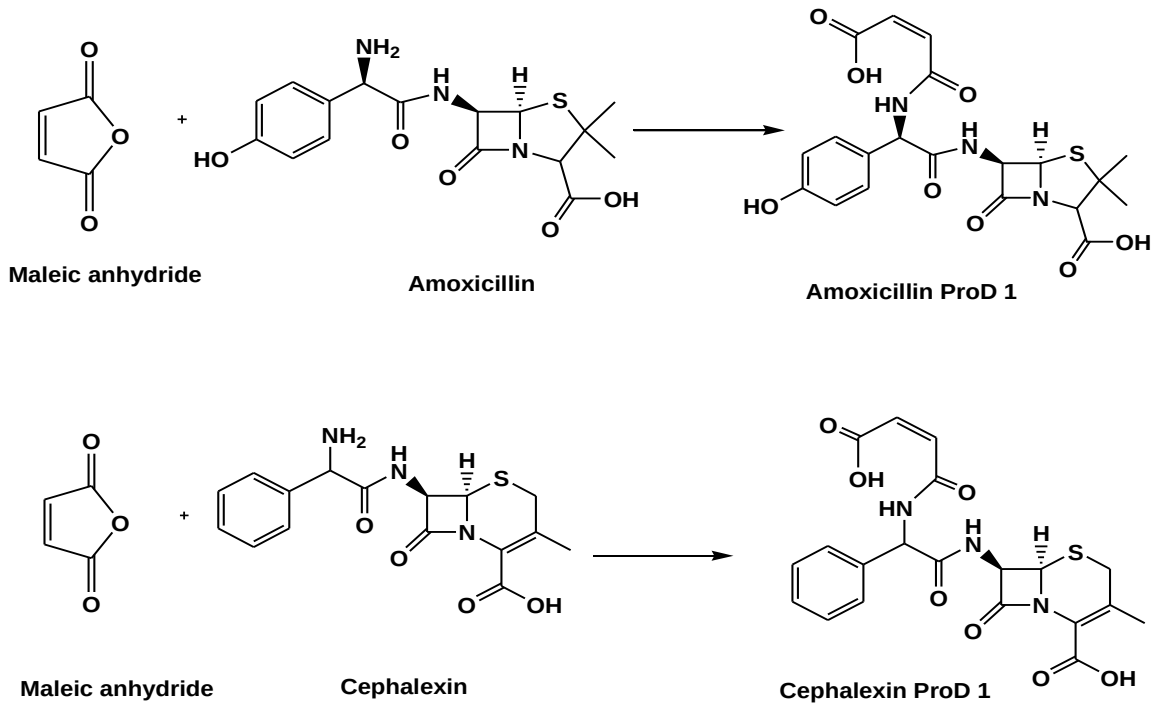

Figure 4. Synthesis of amoxicillin ProD1 and cephalexin ProD 1.

1.7 Antimicrobial Activity of Amoxicillin and Cephalexin

1.7.1 Amoxicillin Activity

Penicillins have been divided into classes based on their spectrum of activity; the first agent that was used clinically to treat infections is the natural penicillin (penicillin G), but after the

emergence of penicillinase in *staphylococci* penicillins became inefficient for these organisms. Therefore, development of penicillinase resistant-penicillins was initiated; this led to the development of three categories of penicillins: the aminopenicillins, carboxypencillins and ureidopencillin.[96]

Aminopenicillins was the first class of penicillin antibiotic that has activity to both gram positive and gram negative bacteria; ampicillin compared to natural penicillin has more activity against *enterococci,* but somewhat less activity against *pyogens, streptococcus pneumonia, and Neisseria species.* On the other hand, it has some activity against *E.coli, proteus Mirabella, salmonella, shigella, listeria,* which are gram negative bacteria.[96]

Amoxicillin has shown to be effective against a variety of infections, which are caused by gram positive and gram negative bacteria in humans and in animals. [97] Amoxicillin has a higher activity against gram positive than gram negative microorganisms.[98] In addition, it has greater efficacy relative to penicillinV and other antimicrobial such as ampicillin [15] and cefuroxime.[99]

Different study reports showed that amoxicillin was effective at MIC in the range of 0.06 µg/mL- 4 µg/mL against variety of microorganism, except *staphylococcus .epi* 64 µg/mL and *staphylococcus aureus* MIC up to 256 µg/mL.[100]

In one study, amoxicillin and ampicillin showed that the kill rates for amoxicillin was higher than ampicillin for *E.coli*, and the rate of killing was the same for both agents for *Staphylococcus Aureus,* but amoxicillin showed longer bacteriostatic phase which was not observed with ampicillin.[15]

In another study an investigation on the antibacterial activity of amoxicillin and ampicillin against 30 isolates of each of *proteus mirabilis, Klebsiella, E.coli, Enterobacter* and *idol positive proteus* was carried out, and the results obtained are as follows: 89% of the *E.coli* strains were inhibited by both drugs at 10 µg or less per mL, whereas only 5µg or less were sufficient in the case of *Proteus. mirabilas*. On the other hand, high response of resistance to amoxicillin and ampicillin was seen among strains of *Klebsiella, enterobacter and idol positive* species.[101]

In addition, other studies have demonstrated that amoxicillin was quite active against *group A hemolytic streptococci, penicillin G susceptible staphylococcus aureus* and *pneumococci,*

only 28% of *S. aureus* isolates which were resistant to 50 µg of penicillin G per mL were susceptible at 50 µg/mL or less to amoxicillin, 76% of *p. mirabilas* isolates were susceptible to amoxicillin at 1.56 µg/mL or less and 20% showed resistant to 12.5 µg/mL or more, 75% of *E.coli* isolates were susceptible to 6.65 µg/mL or less and most of the remaining isolates were resistant to 50 µg/mL or more. [102]

1.7.2 Cephalexin Activity

First generation cephalosporins are cefazolin, cephapirin and cephalothin for intravenous use and cephalexin, cephradine, and cefadroxil which are used orally. All of these cephalosporins are similar in spectrum of activity. They have high activity against gram-positive cocci. They have low activity against gram negative bacteria. In addition, most strains of *Escherichia coli*, *Klebsiella* species, and *Proteus mirabilis* are sensitive to this class of drugs. They have no activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-*resistant Staphylococcus epidermidis* (MRSE), as well as *enterococci*, *Listeria monocytogenes*, *Bacteroides fragilis*, *Citrobacter*, *Enterobacter*, *Proteus* (other than *mirabilis*), *Providencia*, *Pseudomonas*, and *Serratia* organisms. Gram-positive anaerobes like *Peptostreptococcus* and non-penicillinase producing *Bacteroides* species are usually sensitive.[18]

Cephalexin is used for the treatment of the upper and lower respiratory tract infections, genitourinary system, skin, soft tissue, bones, joints and many other infections due to susceptible organisms.[103]

EXPERIMENTAL

2.1. Media Preparation

Brain heart infusion agar, Muller Hinton agar (Becton, Disckinson and company sparks USA) and nutrient broth (hemedia laboratories Pvt. Ltd) were prepared in concentrations of 52 gm/L, 38 gm/L and 13 gm/L, respectively.

2.2. Preparation of the Buffer Solution

Buffer solution (pH=7.4) was prepared by dissolving 0.68 gm of potassium dihydrogen phosphate in100 mL water, then NaOH was added and the solution was stirred. pH meter model HM-30G: TOA electronics™ was used to measure the pH value for all buffers and reaction media used in this study.

2.3 Test Microorganisms

Reference strains obtained from American Type Culture Collection (ATCC) were used *(Staphylococcus aureus (ATCC 25923), Staphylococcus epidermidis (ATCC 12228), Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC 700603), Streptococcuspyogens (ATCC 19615),* and *Streptococcus group B* obtained from microbiological labs (Al-Quds University).

2.3.1 Preparation of Inocula

Part of an isolated bacterial colony was inoculated in 5 mL nutrient broth & incubated for 24 hours at 37^{o}C, the growth turbidity in nutrient broth was adjusted by further incubation or dilution with sterile physiological saline; after comparison with that of a McFarland nephlometer tube no. 0.5 (10^{8} cfu/mL) using spectrophotometer at 625 nm (optical density of 0.08-0.1)

2.4 Antimicrobial Activity Screening Methods

2.4.1 Disk Diffusion Method

With a sterile cotton applicator 10^{8} cfu/mL of each bacterial strain was swabbed on Muller Hinton agar (for *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Klebsiella pneumonia)* while brain heart infusion agar was used for *Streptococci spp*.) in the following manner:

1. The cotton applicator was dipped into the bacterial suspension, rotated several times and pressed against the inside wall of the tube to remove excess inoculum.
2. The agar plate was then streaked in three different directions and around the agar margin to ensure even distribution of the inoculum.
3. The plates were left to dry for 3-5 minutes.
4. Using sterile forceps the disks, which contain prodrugs, drugs, negative control were then distributed evenly on the surface of the agar plates.
5. The plates were incubated upside-down at 37 ^{o}C.
6. The inhibition zone around each disk was measured using a transparent ruler.

2.4.2 Broth Dilution Method

2.4.2.1 Preparation of Media

For each strain 13 tubes, each contains 9.9 mL Muller Hinton broth were prepared and autoclaved.

2.4.2.2 Preparation of the Active Ingredients Dilutions

The two prodrugs amoxicillin ProD 1 and cephalexin ProD 1 along with their active parent drugs, pure standards > 99% of amoxicillin and cephalexin, were commercially available from Sigma Aldrich and were used as positive control. 500 mg of each drug and prodrug were dissolved in 10 mL buffer solution pH 7.4, that has no effect on tested microorganisms and the prodrugs have maximum stability, to give a final concentration of 50 mg/mL. Then several dilutions of stock solution were prepared as shown in Table 1.[104]

Table 1. Dilutions of active ingredients

Tube No.	Volume taken from stock solution (50 mg/mL) in mL	Buffer (mL)	Final concentration mg/mL	Volume of broth in mL	Final volume added to each tube in mL
1	1	0	50	9.7	0.3
2	1	0	50	9.75	0.25
3	1	0	50	9.8	0.2
4	1	0	50	9.9	0.1
5	0.9	0.1	45	9.9	0.1
6	0.8	0.2	40	9.9	0.1
7	0.7	0.3	35	9.9	0.1
8	0.6	0.4	30	9.9	0.1
9	0.5	0.5	25	9.9	0.1
10	0.4	0.6	20	9.9	0.1
11	0.3	0.7	15	9.9	0.1
12	0.2	0.8	10	9.9	0.1
13	0	1	0	9.9	0.1

The experiment was repeated with *Klebsiella*, which required lower concentrations to find MIC and MBC. Broth tubes containing the active ingredients with different concentrations were prepared as shown in the Table 2.

Table 2. Dilutions of active ingredients used for Klebsiella

Tube No.	Stock solution 50mg/mL	Buffer (mL)	Final concentration mg/mL	Final concentration mg/0.1mL
1	1	0	50	5
2	0.9	0.1	45	4.5
3	0.8	0.2	40	4
4	0.7	0.3	35	3.5
5	0.6	0.4	30	3
6	0.5	0.5	25	2.5
7	0.4	0.6	20	2
8	0.3	0.7	15	1.5
9	0.2	0.8	10	1
10	0.1	0.9	5	0.5

11	0.05	0.95	2.5	0.25
12	0.025	0.975	1.25	0.125
13	0	1	0	0

2.4.2.3 Incorporation of Active Ingredients into Media

For the incorporation of the active ingredients into media, 13 broth tubes each contains broth volume as shown in Tables 3 and 4, into broth tube No. 1, 300 µL (0.3 mL) of stock solution was added into broth tube No. 2, 250 µL was added and 200 microliter into broth tube No. 3. The procedure was repeated for the remaining dilutions by adding 100 µL (0.1 mL) for each tube, the final concentrations of the active ingredients in broth are shown in Tables 3 and 4.

Table 3. Final concentration of the active ingredients, amoxicillin, cephalexin amoxicillin ProD 1 and cephalexin ProD 1 in the medium against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Streptococcus pyogens group A*, and *Streptococcus group B.*

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Concentration microgram/mL	150	125	100	50	45	40	35	30	25	20	15	10

Table 4. Final concentration of the active ingredients amoxicillin, cephalexin amoxicillin ProD 1 and cephalexin ProD 1 used for Klebsiella.

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Concentration microgram/mL	50	45	40	35	30	25	20	15	10	5	2.5	1.25

2.4.2.4 Determination of Minimum Inhibitory Concentration (MIC)

All tubes were inoculated with 10 µL of the tested bacterial suspension; the tubes were then incubated for 24 hours at 37^{o} C. After incubation, the tubes were examined for turbidity, indicating a growth of microorganisms; the organism will grow in the negative control tube (tube No.13) that does not contain antimicrobial agent to inhibit growth. The lowest concentration of the active ingredient (drug or prodrug) that inhibits a growth of the organism, as detected by a lack of visual turbidity is designated as the MIC (Figure 5).

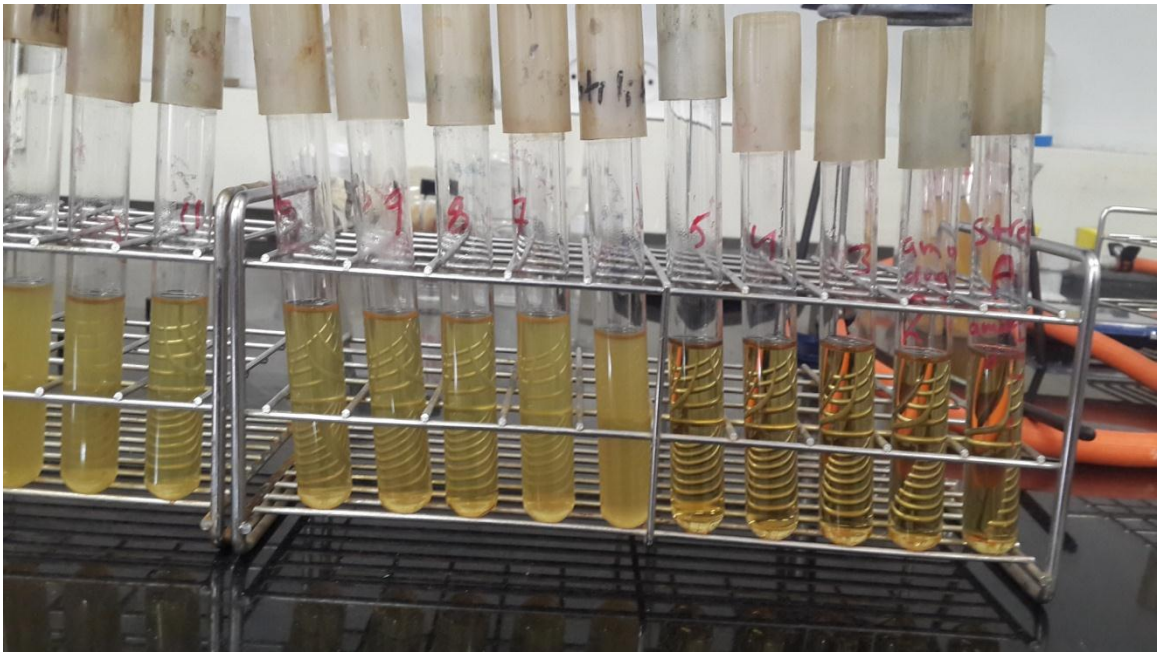

Figure 5. Broth dilution susceptibility test; the tube number 5 lacks of visual turbidity.

MBC is interpreted to be at a tube that shows no growth on the agar plate for example Figure 5 shows that MIC for the test illustrated in the figure in tube no. 5 is 45 microgram per mL; while MBC is checked by testing viable colonies in tubes 1-5.

Therefore, after reading the results of MIC by recording the lowest concentration that inhibits the organism growth, the following procedure is followed

1. Sub-culturing of all tubes which have no visible growth by spreading loop full over quarter of the agar plate.
2. Incubation at 37 °C for overnight then reading the result and recording as follows:

- Bacteriostatic if similar number of colonies are present
- Partial bactericidal if reduced number of colonies are found
- No growth indicates that the whole inoculums have been killed

3-RESULTS AND DISCUSSION

Table 5 and Figures 6-9 illustrate the bacterial inhibition by amoxicillin, cephalexin, amoxicillin ProD 1 and cephalexin ProD 1.

Table 5. Amoxicillin, cephalexin, amoxicillin ProD 1 and cephalexin ProD 1 inhibition of bacteria, showing zone of inhibition diameter in (mm).

Compound	Staph. Epidermidis (G+)	Staph. aureus (G+)	Streptococci. B (G+)	Streptococci. A (G+)	Klebsiella (G-ve)	E. coli (G-ve)
Amoxicillin	No inhibition zone	44 mm	30 mm	40 mm	No inhibition zone	33 mm
Amoxicillin ProD 1	No inhibition zone	30 mm	30 mm	26 mm	No inhibition zone	31mm
Cephalexin	25 mm	44 mm	33 mm	37 mm	26 mm	29 mm
Cephalexin ProD 1	20 mm	40 mm	32 mm	30 mm	21 mm	24 mm

Amoxicillin drug and amoxicillin ProD 1 (P value =.141)

Cephalexin drug and cephalexin ProD 1 (P value=.003)

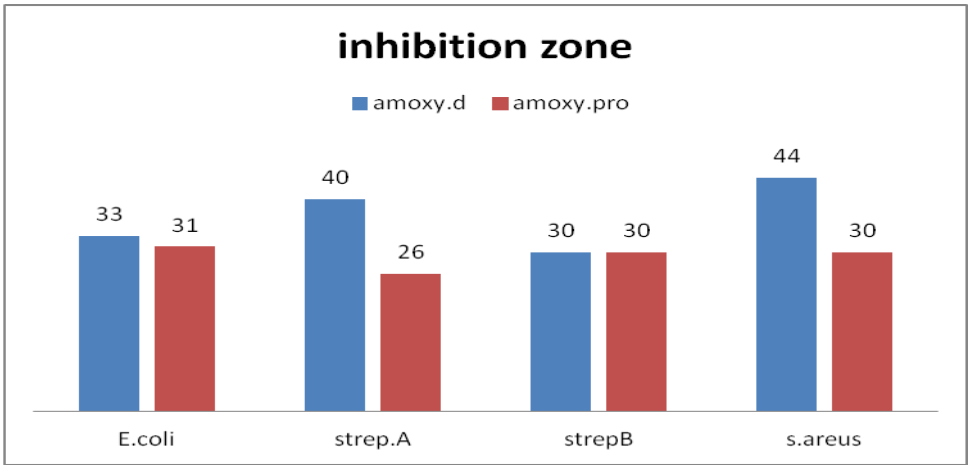

Figure 6. Antibacterial activity of amoxicillin drug and amoxicillin ProD 1 against bacterial strain.

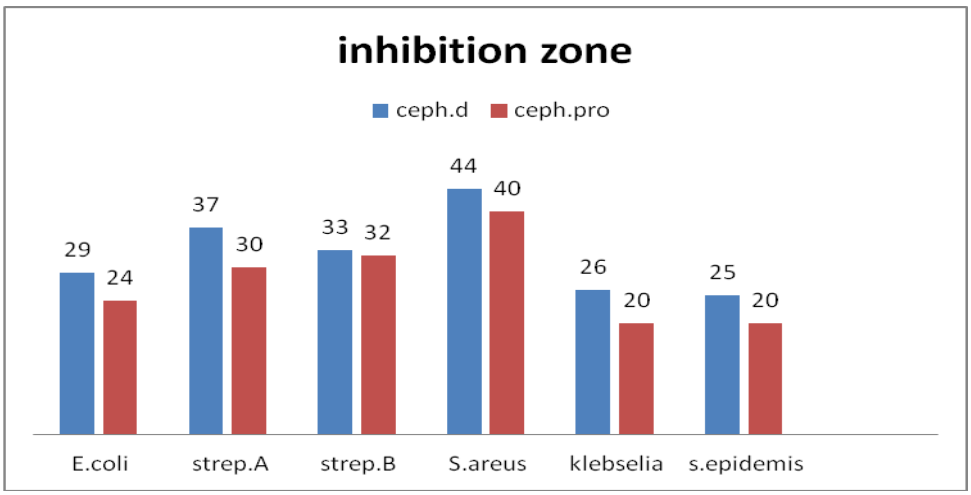

Figure 7. Antibacterial activity of cephalexin drug and cephalexin ProD 1 against bacterial strain.

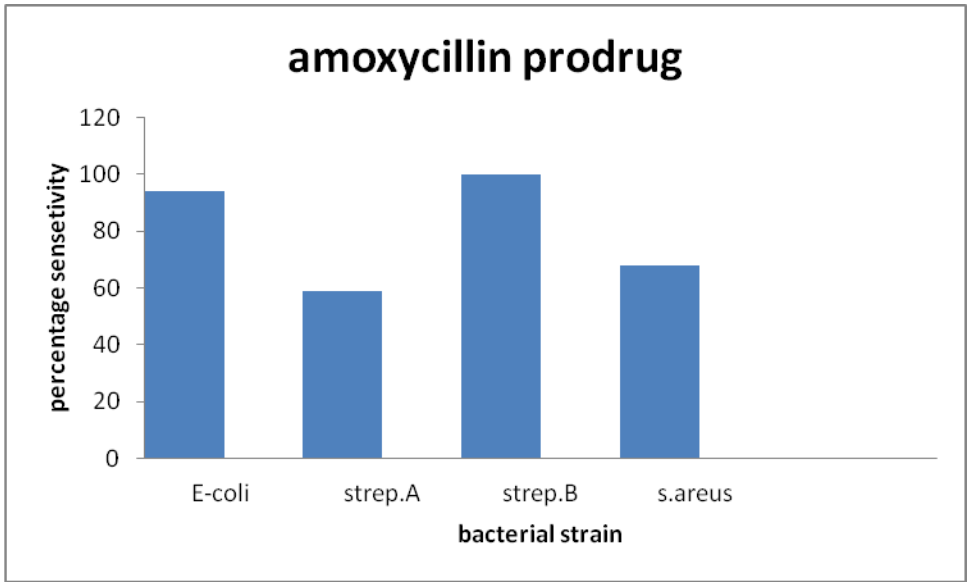

Figure 8. Percentage of amoxicillin ProD 1 to amoxicillin drug vs. bacterial strain.

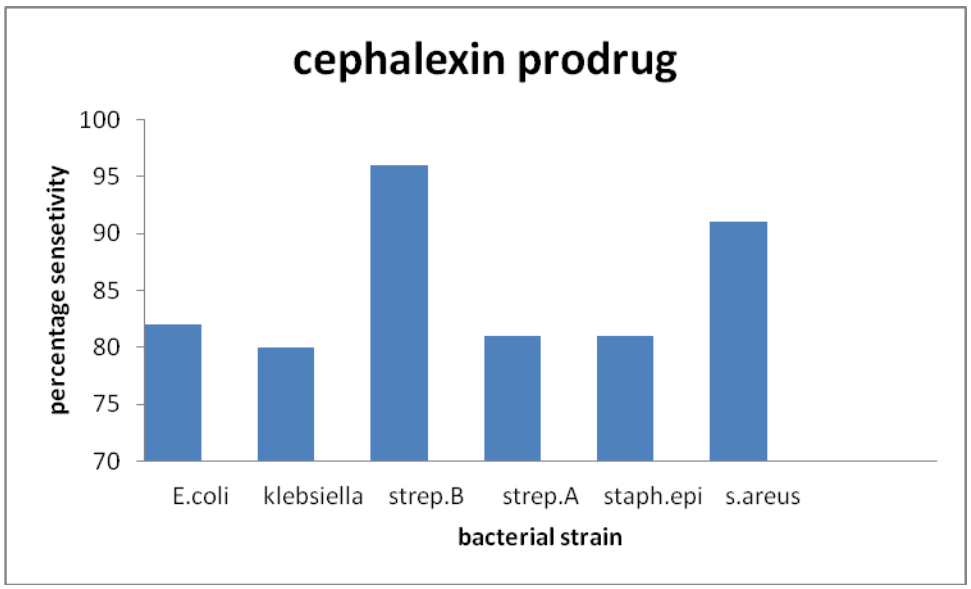

Figure 9. Percentage of cephalexin ProD 1 to cephalexin drug vs. bacterial strain.

The combined results shown in Table 5 and Figures 6-9 revealed that the novel two prodrugs, amoxicillin ProD 1 and cephalexin ProD 1 have antibacterial activity against most bacterial strains tested with about the same potency as their active parent drugs, amoxicillin and cephalexin.

Klebsiella showed resistance to amoxicillin drug and its prodrug amoxicillin ProD 1, since *klebsiella* is a gram negative bacteria, which exhibits resistance to amoxicillin drug, [101] and there is a need of clavulanic acid to overcome its resistance.

Staphylococcus epidermidis also showed resistance to amoxicillin drug and its prodrug, amoxicillin ProD 1 since it is β-lactamase positive. Cephalexin drug and its prodrug, cephalexin ProD 1 showed inhibition against all bacterial strains tested including *Klebsiella pneumonia* and *E.coli*, since cephalexin drug has a broader spectrum than amoxicillin.

Figures 8 and 9 demonstrate the percentage sensitivity of the two novel prodrugs, amoxicillin ProD 1 and cephalexin ProD 1 to antibacterial parent drug, amoxicillin and cephalexin, respectively, against bacterial strains tested in this study. The results demonstrate that for amoxicillin ProD 1/amoxicillin drug: 94% against *E.coli*, 59% against strep. A, 100% against strep. B and 68% against *staph. arues.* (Figure 8). For cephalexin ProD 1/cephalexin drug: 82% against E.coli, 80% against Klebsiella, 96% against *streptococcus group. B*, 81% against strep. A, 81% against *staphylococcus. Epidermidis* and 91% against *staphylococcus. areus* (Figure 9).

3.1 Minimum Bactericidal Concentrations (MBC) and Minimum Inhibition Concentrations (MIC)

In this study the MIC and MBC values for both amoxicillin ProD 1 and cephalexin ProD 1 were determined and compared with the values obtained for their active parent drugs, amoxicillin and cephalexin, respectively.

The MIC and MBC values obtained for the activity on *strep. B*, staph *arues and strep. A* demonstrated that amoxicillin drug is more potent than its prodrug, amoxicillin ProD 1, as indicated by the lower concentrations of the parent drug needed to inhibit and kill the bacteria compared to that of its prodrug.

Moreover, it was found that amoxicillin drug has similar MIC value as its prodrug, amoxicillin ProD 1 against *E.coli* 10 µg/mL. This indicates that both drug and prodrug are equal in potency as they have the same MIC and MBC. [101-103] Further, the study revealed that amoxicillin drug has different MIC value than its prodrug against staphylococcus. Areus, for the parent drug the MIC value was 50 µg/ml which is similar to that reported in previous studies.[101-103]

On the other hand, cephalexin drug was found to be slightly more potent than its prodrug, cephalexin ProD 1, as indicated by the MIC and MBC values obtained for both. Tables 6 and 7 demonstrate that the prodrug of cephalexin, cephalexin ProD 1, is more potent on all

bacteria tested than the prodrug of amoxicillin, amoxicillin ProD 1 as evident by the MIC and MBC values obtained for cephalexin ProD 1 which were less or equal to 150 µg/mL, whereas the values obtained for amoxicillin ProD 1 were all above 150 µg/mL.

Table 6. MIC, MBC of amoxicillin drug and amoxicillin ProD 1.

Bacteria	Amoxicillin Drug		Amoxicillin ProD 1	
	MIC	MBC	MIC	MBC
Streptococcus. Group B	100	100	>150	>150
E-coli	10	10	10	10
Staphylococcus. areus	50	100	>150	>150
Staphylococcus. Epidermidis	---	----	----	----
Klebsiella	-----	----	----	----
Streptococcus. group A	45	100	>150	>150

MIC (P value =.004) and (P value =.098). MIC and MBC in µg/mL.

Table 7. MIC, MBC of cephalexin drug and cephalexin ProD 1.

Bacteria	Cephalexin Drug		Cephalexin ProD 1	
	MIC	MBC	MIC	MBC
Streptococcus. Group B	45	50	50	100
E-coli	10	10	15	20
Staphylococcus. areus	35	40	35	40
Staphylococcus. Epidermidis	45	125	50	150
Klebsiella	5	5	10	15
Streptococcus. group A	45	100	50	100

MIC (P value =.004) and (P value =.098). MIC and MBC in µg/mL.

4. CONCLUSIONS & FUTURE DIRECTION

Two novel prodrugs of amoxicillin and cephalexin were designed and synthesized such that the amine group in the parent drugs is replaced with an amide group. This alteration made the two prodrugs much more stable to chemical reactions than their corresponding active parent drugs as judged by the stability study at a wide range of pH.

The biological screening results revealed that the two novel prodrugs are among a small number of prodrugs having activity themselves prior to inter- or intraconversion via enzymatic or chemical reactions to release their corresponding active parent drugs. It is suggested that these novel prodrugs exhibit their antibacterial activity on different types of bacterial strains due to the presence of a β-lactam ring in their structures.

It is planned that in the near future the following steps will be taken: (1) determination of the exact MIC and MBC values for those experiments where the values were above 150 μg/mL, (2) an assessment of the antibacterial activity on other types of bacteria, and (3) in vivo testing of the two novel prodrugs.

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