

Cyclodextrins in the Battle against Antibiotic Resistant Microorganisms: Mimicking Cyclic Peptide Antibiotics

Introduction

Nowadays, antibiotics are among the most frequently prescribed medications worldwide [1]. However, their indiscriminate use, improper prescriptions and their misuse by consumers led to the development of antibiotic-resistant bacterial strains. The established antimicrobial therapies are becoming less effective due to increasing emergence and prevalence of resistant bacteria. Conventional antibiotics are no longer the 'magic bullets' they were once thought to be and therefore there is an urgent need for development of new antibiotics and/or other novel strategies to combat the infections caused by antibiotic-resistant organisms. As resistance to antibiotics becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies, there has been a continued decline in the number of newly approved drugs. Because all of the so-called easily exploitable and already discovered bacterial binding sites for antibiotics have been exploited, drug development has become more complex than it was decades ago.

A World Health Organization report released April 2014 stated, "this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. Antibiotic resistance –when bacteria change so antibiotics no longer work in people who need them to treat infections– is now a major threat to public health"[2].

Cyclodextrins (CDs) offer several innovative strategies against microbial infection including not only reducing the resistance of the known antibiotics via complexation [3] but also decreasing the cell-to-cell communication (quorum sensing) [4], inhibiting bacterial and viral infections via cholesterol depletion [5] and plugging the pores of the pathogens by CDs [6]. The present issue of Cyclodextrin News gives an overview on one of the new alternative strategies where cyclodextrins are successfully used against antibiotic resistant microorganisms.

Cyclodextrin Derivatives as Membrane Disrupting Agents

Antimicrobial peptides have been recognized as promising agents against multidrug-resistant bacteria [7]. Many of these peptides owe their antibacterial activity to the formation of trans-membrane ion-channels resulting in cell lysis. They have cyclic structure and contain positively charged hydrophilic moieties that interact with negatively charged bacterial cell membranes, while their hydrophobic groups interact with fatty acid moieties in the lipid membrane to trigger membrane disruption. Nearly all known natural cyclic peptides display high antibacterial activity.

CDs have been modified in order to mimic the effect of these naturally occurring channel-forming peptides. Their diameter is nearly identical as the diameter of the antibiotic oligopeptides (ca. 1 nm), their structure also contain a rigid channel available for pore forming and they can be further modified due to their numerous hydroxyl groups in order to enhance their interaction with a bacterial cell membrane. This strategy has been followed recently by two research groups, the group of Hatsuo Yamamura from the Nagoya Institute of Technology and by the group of Troels Skrydstrup from the Aarhus University. Their work led to new CD derivatives capable of causing bacterial cell membrane disruption. Since these derivatives do not act on a specific cellular enzyme or receptor, but on the bacterial membrane itself, they exhibit broad-spectrum antibacterial activity. Therefore, compared to that of conventional antibiotics, the development of resistance to these membrane disrupting agents is difficult.

Research group of Hatsuo Yamamura is interested in the synthesis of regioselectively modified γ CD derivatives bearing polyalkylamino and polyarylamino derivatives, able to strongly interact with a bacterial cell membrane and cause cell lysis. The study of his research group relies on a structure–activity relationship study of Polymyxin B and Gramicidin S, which are membrane active cyclic polypeptides that strongly permeabilize both the outer and cytoplasmic membranes of Gram-negative bacteria. These polypeptides contain amino acid residues, whose side chain amino groups form a cationic region of an amphiphilic peptide structure (Fig. 1). Earlier studies demonstrated that the fatty acid chain in the molecule of polymyxin B has a high importance in the antimicrobial activity. The polymyxin B nonapeptide, a polymyxin B analogue lacking the fatty acid tail showed no significant antimicrobial activity, but it retained the high outer membrane-permeabilizing activity [8.]. These findings showed that the fatty acid portion is important in the antimicrobial activity because it dissolves in hydrophobic region of cytoplasmic membrane and disrupts membrane integrity. Binding of Polymyxin B causes leakage of cellular molecules and inhibition of cellular respiration. It also binds and inactivates endotoxins. It has bactericidal action only for Gram-negative bacteria species, since the cell wall in Gram-positive species is too thick to permit access to membrane.



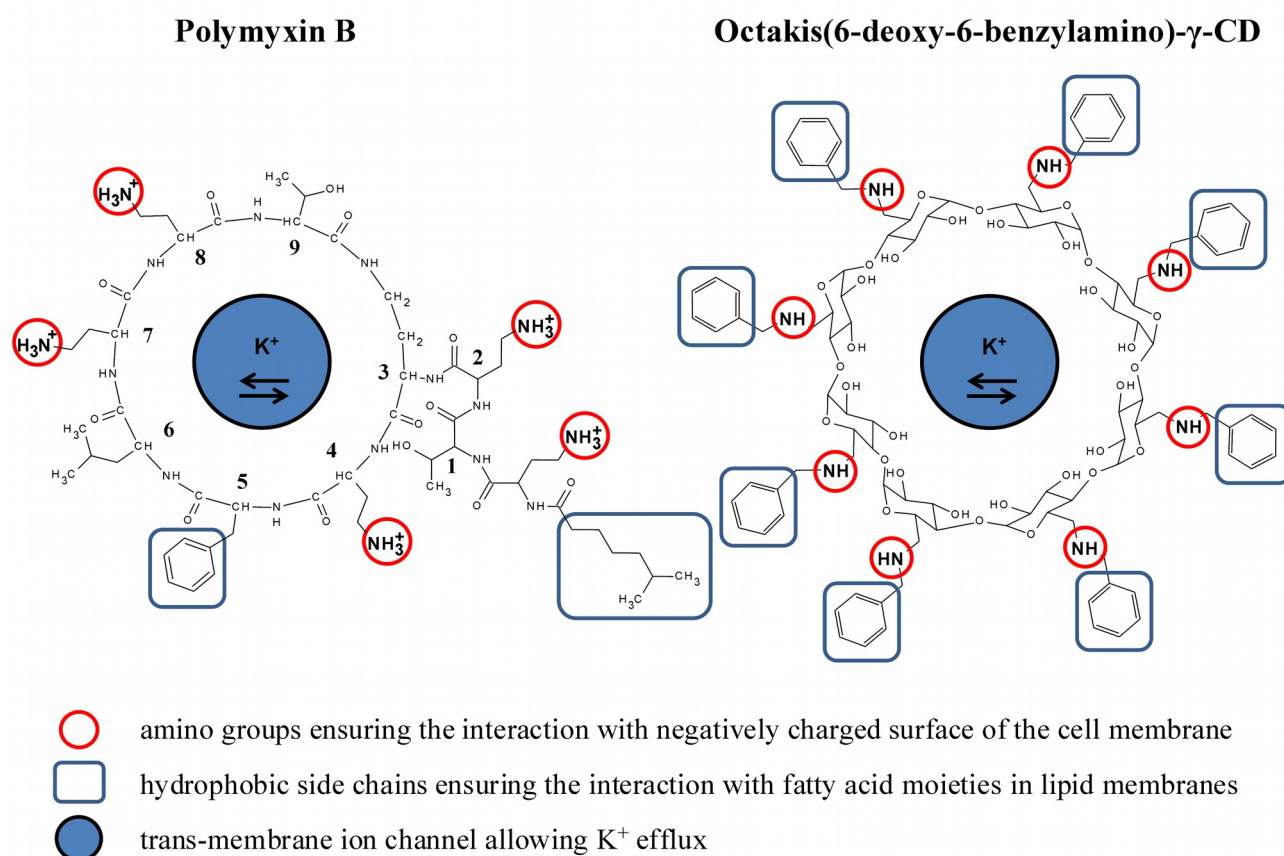


Fig. 1: Schematic representation of the structural relationship between the naturally occurring Polymyxin B and its CD based analogue, octakis(6-deoxy-6-benzylamino)- γ CD.

In order to prepare CD derivatives which are able to mimic the action of Polymyxin B, Yamamura and his coworkers prepared first a series of polyamino CDs where the amino groups were arranged on the primary side of the CD [9]. Activity of these derivatives to permeabilize bacterial membranes was examined by measuring the K^+ efflux from the bacteria. The measurement showed that the compound containing 8 amino groups, namely the octakis(6-deoxy-6-amino)- γ CD caused the highest K^+ efflux from both the Gram-positive strains of *Staphylococcus aureus* and from the Gram-negative *Escherichia coli*. The heptaamino derivative (heptakis(6-deoxy-6-amino)- β CD) showed only moderate membrane activity while the α CD derivative (hexakis(6-deoxy-6-amino)- α CD) containing six amino groups had no significant activity on the studied strains. These results demonstrated the importance of the number of the amino groups and the size of the CD cavity on the membrane activity. The observed K^+ efflux value from *E. coli* was smaller than that from *S. aureus*, which may be due to the differences in membrane structure between the Gram-negative and Gram-positive bacteria.

The most interesting result of this study is the observed synergic effect of polyamino CDs with probe antibiotics, such as novobiocin and erythromycin. These probes dosed alone have no effect on the growth of *E. coli* strains because the outer membrane of the Gram-negative



bacteria cell acts as a barrier for them (MIC > 120 $\mu\text{g}/\text{mL}$ for novobiocin, MIC > 80 $\mu\text{g}/\text{mL}$ for erythromycin). The polyamino CDs themselves have no antibiotic activity (MIC > 128 $\mu\text{g}/\text{mL}$) either, but the combination of the antibiotics with polyamino CDs showed surprisingly high antimicrobial activity (MIC < 10 $\mu\text{g}/\text{mL}$). Especially the octakis (6-deoxy-6-amino)- γ CD showed high synergic effect with both antibiotics. These observed effects and the MIC values of the polyamino- γ CD were comparable to those of polymyxin B nonapeptide.

After the successful demonstration of the fact that polyamino CDs can mimic the effect of the polymyxin B nonapeptide the researchers went further with altering the properties of the most promising derivative, the octakis(6-deoxy-6-amino)- γ CD. Their aim was to increase the amphiphilicity of the polyamino CDs because, as it was proved for polymyxin B and polymyxin B nonapeptide, the appropriate amphiphilicity of the membrane disrupting molecule is important in antimicrobial activity [8].

First, the octakis(6-benzylamino)- γ CD (6-BnAm₈- γ CD) derivative was prepared via the reaction of benzylamine as a nucleophile with a per-tosylated γ CD. This reaction resulted in moderate isolated yield of the product (17.3 %) mainly because the purification required an inefficient gel filtration chromatography. The prepared 6-BnAm₈- γ CD however showed much higher disruption of the bacterial membrane than the polyamino derivatives and exhibited surpassing antibiotic activity against Gram-positive strains (MIC = 4–8 mg/mL). The observed MIC values are comparable to the activity of the gramicidin S. It is interesting that the introduction of hydrophobic benzyl groups to polymyxin B nonapeptide-like amino CDs led to Gramicidin S-like membrane activity.

The direct nucleophilic substitution methodology used for the preparation of octakis(6-benzylamino)- γ CD was not effective in the synthesis of other alkylamino and arylamino γ CDs. To solve this problem Yamamura and co-workers developed an effective polyfunctionalization of γ CD via microwave-assisted Huisgen reaction [10]. Starting from octaazido γ CD they prepared a library of compounds varying the type of the alkyl chain or aryl moiety on the primary side of the γ CD (Fig. 2).

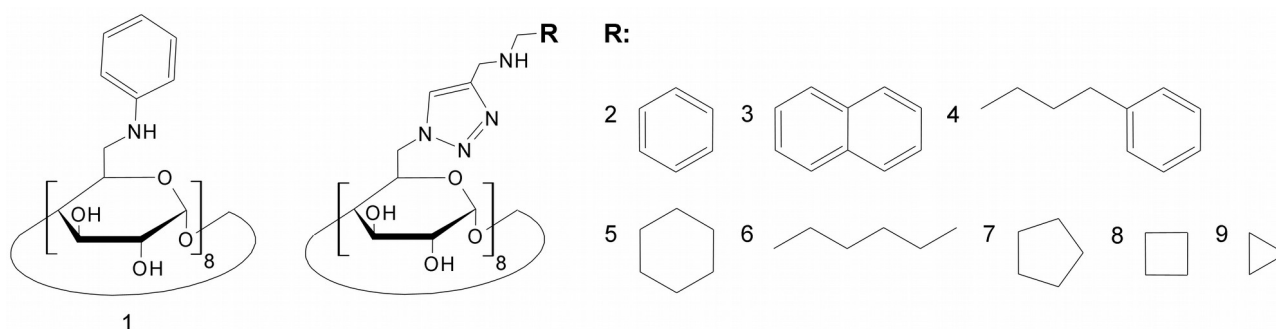


Fig. 2: Structure of polyalkylamino and polyarylamino γ CDs [9, 10]



Their bioactivity for the permeabilization of bacterial cell membranes depended on the substituents on the amino group. The results showed that the triazole ring in the structure did not decrease the membrane disrupting ability of these derivatives. The hemolysis of rabbit red blood cells by the prepared alkylamino and arylamino γ CDs was also examined in order to determine whether they disrupt the membranes of animal cells. It is worth pointing out, that although the benzylamino derivative prepared via direct substitution on the C6 carbon (compound 1, Fig. 2) showed excellent membrane disrupting activity on bacteria cells, it also permeabilized the red blood cells, therefore its selectivity is low. On the other hand, the benzylamino derivative prepared via the Huisgein reaction (compound 2, Fig. 2) exhibited significantly less hemolysis at the experimental concentration (50 μ M) while the MIC for the two compounds was found to be nearly the same (MIC = 4–8 μ g/mL) for both the Gram-negative and Gram-positive strains. These results suggest, that the triazole moiety works very well not only for effective functionalization but also for selective membrane disruption.

Slightly different approach towards CD based membrane permeabilizers was followed by the research group of Troels Skrydstrup from the Aarhus University, Denmark. Their main idea was to use cyclodextrin as a cyclic template for alamethicin oligomers [11]. Alamethicin is a subclass of pore-forming proteins called peptaibols. It has a hydrophobic α - conformation which can form channels in biological membranes. Although the precise structure of these channels is unknown, NMR and molecular dynamics investigations suggest that they are highly dynamic and nonuniform by nature and the most stable and predominant channel structure is helical consisting of 6–8 helices [12, 13].

In order to prepare a stable ion channel from alamethicin subunits, Skrydstrup and co-workers covalently attached 6 and 7 alamethicin molecule to α - and β CD respectively. These conjugates were prepared via click reaction from per-6-azidated CDs and alkyne conjugated alamethicin (Fig. 3). The permeabilizing ability of the conjugates was tested with calcein-release assay, in which calcein was entrapped in dioleoyl-phosphatidylcholine (DOPC) vesicles at a concentration high enough to cause self-quenching of its fluorescence. When permeabilization of the vesicle occurs, the released calcein dilutes and gives detectable fluorescent signal [14]. The ion channel forming ability of the alamethicin:CD conjugates was examined with voltage-clamp studies. The free alamethicin did not produce any detectable conductance at peptide concentrations below 10^{-3} mg/mL. However, with the alamethicin:CD conjugates, conductance could be measured at concentrations as low as 10^{-5} mg/mL. The membrane permeabilizing properties of the conjugates proved to be highly effective also, and lysis experiments revealed a 100-fold increase in activity for the most active alamethicin:CD conjugate in comparison to the free peptide.

These results suggest, that alamethicin:CD constructs are able to perturb cell membranes through the formation of stable ion-channels therefore are promising new semi-synthetic antimicrobial agents.



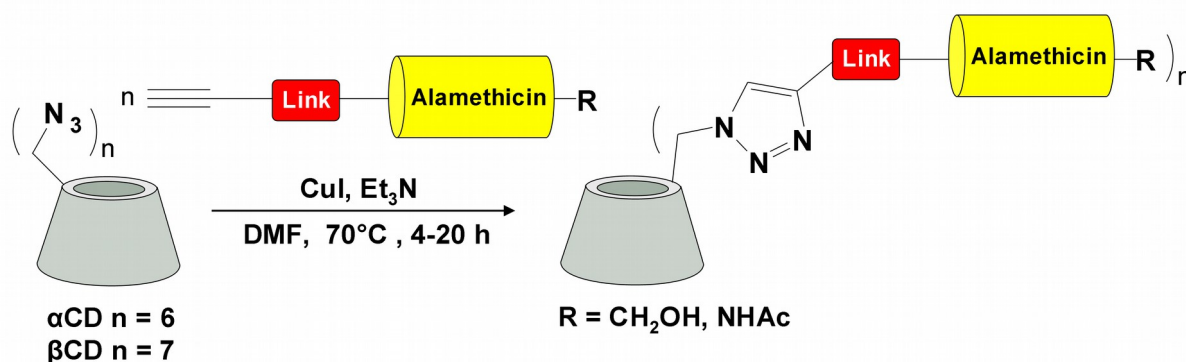


Fig. 3: Reaction scheme for the preparation of alamethicin appended CDs [11].

Conclusions and future perspectives

Because of the worldwide spread of antibiotic-resistant microorganisms the infectious diseases in the 21st century are again at the epicenter of a global dialogue capturing the attention of academics, governments, public health officials, and the general public alike. These diseases are evolving extremely fast, faster than the research in drug discovery. As Nobel laureate Joshua Lederberg stated that technology will never win this war permanently and we must be satisfied to merely stay one step ahead of the pathogens [15].

The works reviewed in the present issue of Cyclodextrin News show only a few of the many alternative ways in the treatment of antibiotic-resistant bacteria and emphasizes that CDs can be our very versatile weapons in this evolutionary battle between the host and the pathogen.

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Gabor Benkovics

CycloLab Cyclodextrin R&D Laboratory, Ltd.,
Budapest, HUNGARY



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Phosphates, Cyclodextrin-based coating, Targeting

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NMR, Computational interpretation

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Cerebrospinal fluid, System absolute bioavailabilities, Nasal formulations, Pharmacokinetic studies

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Cyclodextrin-assisted syntheses, Ionic liquids, GUMBOS, NanoGUMBOS, Size-Control

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Sand/kaolin blend, Surfactant delivery and carrier system, Surfactant adsorption inhibition, Surfactant/ β -cyclodextrin complexations, EOR surfactant: β -cyclodextrin inclusion complexes

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Ferrocene, Crosslinking catalase

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Benzothiazole, Thioflavin-T, γ -cyclodextrin

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Mesoporous materials, Multiple chromatographic separation functions, Chiral resolution, Liquid chromatography

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Homepage: www.cyclolab.hu
H-1525 P.O. 435, Budapest,
Hungary
Tel.: (+361)347-6060
Fax.: (+361)347-6068
e-mail: cyclolab@cyclolab.hu

