JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 291–300

Received September 18, 2002
Accepted November 27, 2002
DOI 10.1211/002235702649
ISSN 0022-3573

Antimicrobial activity of β-lactam antibiotics against clinical pathogens after molecular inclusion in several cyclodextrins. A novel approach to bacterial resistance

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Abstract

Recognition and uptake by specific cellular receptors and transport systems for cyclodextrins have been demonstrated. Based on this concept, natural and synthetically modified cyclodextrins were used as drug carriers. Several β-lactam antibiotics were selected and their inclusion complexes with different cyclodextrins were prepared (molar ratio ranging from 1:1 to 1:3). The complex formation, in aqueous solution, was monitored and optimum complexation conditions were selected. The inclusion of the active molecules in the cyclodextrin cavity was confirmed by ^{1}H NMR spectroscopy. Specific HPLC methods for the quantitation of antibiotics in the presence of cyclodextrins were developed and their chemical stability under complexation conditions was confirmed. Antimicrobial activity of drug–cyclodextrin complexes, in terms of minimum inhibitory concentration (MIC), were compared with the corresponding values of uncomplexed free molecules. A wide range of clinical pathogens and known β-lactamase-producing strains were tested. The activity of the cyclodextrin-included antibiotics was increased, particularly against Gram-negative clinical strains. The nature and degree of substitution on cyclodextrin macromolecules may be the predominant factor in the observed improvement in antimicrobial activity. We believe that the proposed methodology is a novel approach to the microbial resistance problem and will trigger research towards the development of new cyclodextrin derivatives bearing the ability to increase the uptake of included antimicrobial molecules through intensification of the corresponding molecular recognition phenomena.

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Acknowledgement and funding: The authors would like to thank the Greek Secretariat of Research and Development for the financial support (PENED 1999 no. ED134).

Introduction

Over the past decades, a considerable number of antimicrobial agents have been developed. Despite this, the successful treatment of nosocomial infections still remains a serious problem (Neu 1992; Ash 1996). Bacteria that demonstrate in-vitro susceptibility to a chosen antibiotic may be able to develop resistance during therapeutic treatment because of the selection of a more resistant subpopulation or the appearance of derepressed mutants (Bennett & Chopra 1993). Toxicity considerations may limit our ability to increase doses of available agents to achieve serum concentrations that might adequately treat these infections.

Cyclodextrins are potential candidates for drug carriers because of their ability to alter physical, chemical and biological properties of guest molecules through formulation of inclusion complexes. Natural cyclodextrins (α-, β- and γ-) have been widely used for complexation because of their principal advantages as drug carriers, namely the availability of cyclodextrins of different cavity sizes, low toxicity and low pharmacological activity and the protection of the included drug molecules from biodegradation (Uekama et al 1998).

To optimize the physicochemical properties and inclusion capacity of natural cyclodextrins, various synthetic cyclodextrin derivatives have been prepared, and are commercially available, aiming at the ultimate requirement for a drug carrier: the ability to deliver a drug molecule at a target site (Szente & Szejtli 1999).

Recently the existence of a specific transport system for cyclodextrins in the bacterial cell wall has been revealed (cym transport system), initially in *Klebsiella oxytoca* (Böck

Table 1 Comparative in-vitro activity of three antibiotic compounds and their equivalent complexes with different β-CDs.

Organism (no. of strains) Antimicrobial agent and equivalent complex	MIC ($\mu g\ mL^{-1}$) for tested strains		
	Range	[a]MIC_{50}	[b]MIC_{90}
Staphylococcus spp (10)			
Ampicillin	0.25–32	2[a]	32[b]
Ampicillin–βCD (1:2)	0.25–16	1	16
Ampicillin–HPβCD (1:2)	0.25–16	1	16
Amoxicillin	0.50–32	2	32
Amoxicillin–βCD (1:2)	0.25–32	1	16
Amoxicillin–βCD (1:3)	0.25–32	1	16
Cefadroxil	2–256	64	256
Cefadroxil–βCD (1:2)	0.5–256	64	256
Cefadroxil–HPβCD (1:2)	0.25–256	64	256
Cefadroxil–MethβCD (1:2)	0.25–256	64	128
Klebsiella spp (14)			
Ampicillin	32–4096	2048	4096
Ampicillin–βCD (1:2)	8–4096	1024	2048
Ampicillin–HPβCD (1:2)	8–512	512	512
Amoxicillin	64–4096	2048	4096
Amoxicillin–βCD (1:2)	64–2048	1024	2048
Amoxicillin–βCD (1:3)	64–2048	1024	2048
Cefadroxil	32–512	128	256
Cefadroxil–βCD (1:2)	32–512	128	256
Cef–HPβCD (1:2)	32–256	128	256
Cefadroxil–MethβCD (1:2)	8–128	32*	64*
Escherichia coli (10)			
Ampicillin	2–2048	1024	2048
Ampicillin–βCD (1:2)	2–1024	1024	1024
Ampicillin–HPβCD (1:2)	2–1024	512	1024
Amoxicillin	2–2048	1024	2048
Amoxicillin–βCD (1:2)	2–1024	1024	1024
Amoxicillin–βCD (1:3)	2–1024	1024	1024
Cefadroxil	32–512	64	64
Cefadroxil–βCD (1:2)	32–256	32	64
Cefadroxil–HPβCD (1:2)	32–256	32	64
Cefadroxil–MethβCD (1:2)	16–128	16*	32*
Pseudomonas aeruginosa (10)			
Ampicillin	256–2048	1024	2048
Ampicillin–βCD (1:2)	128–1024	512	1024
Ampicillin–HPβCD (1:2)	128–1024	512	1024
Amoxicillin	512–2048	1024	1024
Amoxicillin–βCD (1:2)	256–1024	512	1024
Amoxicillin–βCD (1:3)	256–1024	512	1024
Cefadroxil	128–2048	1024	1024
Cefadroxil–βCD (1:2)	128–1024	1024	1024
Cefadroxil–HPβCD (1:2)	128–1024	1024	1024
Cefadroxil–MethβCD (1:2)	128–512	512	512
Enterobacter spp (10)			
Ampicillin	128–2048	512	1024
Ampicillin–βCD (1:2)	32–1024	256	1024
Ampicillin–HPβCD (1:2)	32–512	256	512
Amoxicillin	256–2048	512	1024
Amoxicillin–βCD (1:2)	256–1024	256	1024
Amoxicillin–βCD (1:3)	256–1024	256	1024
Cefadroxil	>2048	2048	2048
Cefadroxil–βCD (1:2)	512–1024	1024	1024
Cefadroxil–HPβCD (1:2)	512–1024	1024	1024
Cefadroxil–MethβCD (1:2)	64–256	128*	256
Citrobacter spp (10)			
Ampicillin	4–2048	1024	2048
Ampicillin–βCD (1:2)	2–1024	512	1024
Ampicillin–HPβCD (1:2)	2–1024	256	512
Amoxicillin	2–2048	1024	2048
Amoxicillin–βCD (1:2)	2–1024	512	1024
Amoxicillin–βCD (1:3)	2–1024	256	512
Cefadroxil	32–512	64	256
Cefadroxil–βCD (1:2)	32–256	64	256
Cefadroxil–HPβCD (1:2)	32–256	64	256
Cefadroxil–MethβCD (1:2)	16–256	16*	128*

[a]MIC_{50}, 50% of tested strains exhibit an MIC ≤ 2. [b]MIC_{90}, 90% of tested strains exhibit an MIC ≤ 32. *$P < 0.05$, activity of complexed compared with free antibiotic.

et al 1996; Pajatsch et al 1998, 1999). There is convincing evidence that α- and β-cyclodextrins are taken up as intact entities via the components of this transport system (Pajatsch et al 1999). Moreover, cyclodextrin derivatives can be used as carriers for transporting drug molecules, because of their ability to recognize specific cellular receptors, such as lectins located at the bacterial cell surface (Attioui et al 1994; Parrot-Lopez et al 1993).

Gram-positive and -negative bacterial resistance to β-lactam antibiotics is associated with production of β-lactamases and decreased uptake across bacterial cell wall (Livermore 1995; Matsumura et al 1999). The protection of β-lactam molecules against the hydrolytic attack of β-lactamases and the improvement of transport across cell-wall barriers is a prerequisite to preserve the antimicrobial value of these drugs (Rolinson 1998). Considering the above, cyclodextrins may be important as carriers for the permeation of antibacterial molecules based on recognition and uptake ability by specific cellular receptors or specific transport systems for cyclodextrins.

Since there is solid evidence for the existence of novel mechanisms transporting cyclodextrins through the outer membrane and the cytoplasmic membrane of bacterial cells, we have prepared complexes of several β-lactam molecules with β-cyclodextrins or synthetically modified β-cyclodextrins with molar ratios ranging from 1:1 to 1:3. Furthermore the aim of this study was to investigate the possible enhancement of the existing phenomenon of cyclodextrin transportation by exploring the biomimetic action of appropriately modified cyclodextrins. The antibacterial activity of the complexes were compared with those of corresponding uncomplexed molecules against a wide range of clinical pathogens (Table 1) and known β-lactamase-producing strains.

Table 2 represents the resistance phenotype of the eleven β-lactamase producers. All strains produce enzymes that belong to the best-studied molecular classes A and C and express their action as active site serine β-lactamases (Matagne et al 1998; Crichlow et al 2001). All tested bacterial strains were resistant against ampicillin, ceftazidime, cefotaxime and amoxicillin–clavulanate.

Table 2 MICs of β-lactams and their equivalent complexes with different β-cyclodextrins against eleven Gram-negative β-lactamase producers.

Strains (strain no.)	β-Lactamase type	MIC (μg mL^{-1})									
		AMP	AMP–β CD 1:1	AMP–Meth βCD 1:1	AMP–Meth βCD 1:3	AMC	CAZ	CAZ–β CD 1:1	CAZ–Meth βCD 1:1	CAZ–Meth βCD 1:3	CTX
S. marcescens 21	TEM-1, SHV-5	2048	512	1024	1024	32	64	32	64	64	32
S. marcescens 25	SHV-5	128	32	32	64	32	64	32	32	32	32
S. marcescens 22	SHV-5	2048	512	1024	512	128	128	64	64	64	64
E. cloacae TSV-288	TEM-1, SHV-5	4096	512	512	512	128	256	128	128	256	128
E. cloacae TSV-239	IBC-1	4096	512	1024	128	128	512	256	256	512	32
E. cloacae IpT-58	IBC-1	2048	1024	1024	512	128	128	64	64	128	32
E. aerogenes EY-25	LAT-2	4096	1024	1024	256	256	512	32	64	256	256
K. pneumoniae EY-205	LAT-2	4096	512	1024	128	64	512	128	128	512	64
E. coli *MEL-2*	LAT-4	2048	1024	1024	1024	64	256	128	128	256	128
S. typhimurium *661*	CTX-M-4	4096	1024	1024	1024	32	64	16	32	32	256
S. typhimurum 893	CTX-M-5	4096	1024	1024	1024	32	16	8	16	16	256

AMP, ampicillin; AMC, amoxicillin plus clavulanate (2:1); CAZ, ceftazidime; CTX, cefotaxime.

Materials and Methods

Materials

Antimicrobial agents were purchased from Sigma (St Louis, MO) or kindly offered by ELPEN S.A. Pharmaceutical (Attica, Greece) as laboratory powders of known potency. The selected antibiotics were: ampicillin, amoxicillin, cefadroxil, ceftazidime, cefotaxime and amoxicillin–clavulanate. Stock solutions of the antimicrobial drugs were prepared and then stored at −70 °C. β-Cyclodextrin (βCD) was purchased from Cyclolab (Cyclodextrin Res. & Dev. Lab. Ltd 1525, Budapest, Hungary). Randomly methylated β-cyclodextrin (MethβCD) and hydroxypropyl-β-cyclodextrin (HPβCD) (molar degree of substitution = 0.8) were purchased from Aldrich Chemical Company. Müeller-Hinton broth was purchased from Difco Laboratories (Detroit, MI). All other chemicals were reagent grade and were used without further purification. Organic solvents were HPLC grade. The water used was deionized and filtered by a MilliQ-Plus water purifying system.

Microorganisms

For the comparative study of antimicrobial activity, 64 clinical isolates were tested: *Staphylococcus aureus* (n = 10), *Klebsiella* spp (n = 14), *Escherichia coli* (n = 10), *Pseudomonas aeruginosa* (n = 10), *Enterobacter* spp (n = 10), and *Citrobacter* spp (n = 10). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 11229 and *Escherichia coli* ATCC 35218 were used as controls. Eleven strains, chosen as representatives of commonly abandoned extended-spectrum β-lactamase producers, were also tested (Table 2). These strains, were kind gifts from L. S. Tsouvelekis (Department of Microbiology, School of Medicine, University of Athens, Greece).

Preparation of drug–cyclodextrin complexes

The appropriate quantity of antimicrobial agent was added to cyclodextrin aqueous solution and sonicated for 20 min, then the mixture was placed in a constant-temperature (30 °C) shaking bath. The complexation procedure was monitored using NMR spectroscopy. The equilibrium for the different complexes was achieved after 3–8 h of continuous shaking. The solution of the complex was sterilized by filtration through Millipore sterile filters (Stericup TM 0.22 μm, GV Durapore Membrane, pore size 0.22 μm). The drug concentration in solution before and after filtration was measured using an HPLC method. Recoveries in all cases exceeded 99.5% of the initial concentration, suggesting that filtration had negligible effect on the drug concentration.

Proton nuclear magnetic resonance spectroscopy

^{1}H NMR spectra of all selected antibiotics in the absence and presence of different cyclodextrins were recorded at 400.13 MHz on a Bruker DRX 400 spectrometer equipped with a direct and an inverse 5-mm broadband probe and B_0 gradients. All experiments were carried out using D_2O solutions containing 16 mM of the drug and the corresponding cyclodextrin molar concentrations (β-CD, HPβCD or MethβCD), in a 5-mm tube at ambient temperature (20 °C). Standard Bruker programs were used throughout.

Stability indicating method and testing

All the prepared solutions were tested for degradation products using stability indicating methods, specific for each antimicrobial agent. All HPLC methods applied were known methods, suitably modified and optimized for the purpose of the study and validated. The HPLC methods were performed on a Waters (Milford, MA) liquid chromatography instrument, consisting of a pump (Model 590), a Rheodyne 717S injector, fitted with a 20-μL loop, a Waters 996 Photodiode Array UV detector and Millenium 32 software. Nova pak C_{18} reversed-phase 3.9 × 150 mm (15 μm) columns were used. The degassed mobile phases employed in the HPLC protocols were vacuum-filtered through a 0.45-μm pore-size nylon membrane filter. Mobile phases used were as follows: for the stability assays of ampicillin, amoxicillin and its cyclodextrin complexes: acetonitrile–H_2O 39:61, acetic acid 1%, flow rate 2 mL min^{-1}, at 240 nm, RSD = 1.8%; for cefadroxil, ceftazidime, cefotaxime and their cyclodextrin complexes: methanol–H_2O 30:70, acetic acid 1%, flow rate 1 mL min^{-1}, at 254 nm, RSD 1.6%.

In all cases the cyclodextrin complex preparation process had no measurable effect on the chemical stability of the drugs.

Inoculum preparation

Single bacterial colonies from an overnight agar plate were touched with a sterile loop and added to a tube containing cation-adjusted Müeller-Hinton broth (CA-MHB; Difco Laboratories, Detroit, MI). The tubes were incubated at 35 °C to achieve bacterial logarithmic growth and a density equal to that of no. 0.5 McFarland standard. Dilution of the inoculum was achieved in CA-MHB. The final inoculum size was verified each time by colony counting by a spread-plate technique.

Minimum inhibitory concentration determination

Minimum inhibitory concentrations (MICs) for all tested bacteria were determined by the broth microdilution method in CA-MHB (National Committee for Clinical Laboratory Standards 1993). The final inoculum was prepared from logarithmic-phase bacteria and was approximately 5×10^5 colony-forming units/mL (CFU mL^{-1}). This inoculum was added to 0.1 mL of CA-MHB containing serial two-fold dilutions of the antimicrobial agents. Microdilution trays were incubated at 37 °C for

approximately 18–24 h. The lowest concentration of antibiotic that prevented visible growth was defined as the MIC. Each experiment was replicated three times by different technicians. The experimental values of MICs were found to be identical without any variability.

Statistical analysis

The medians of MICs at which 50% and 90% of clinical isolates were inhibited (MIC50 and MIC90) were calculated for each bacterial species. The Kruskal-Wallis nonparametric analysis of variance on ranks was used to determine whether there were significant differences among susceptibilities to free antibiotic and to the corresponding cyclodextrin complexes for each bacterial species. Pairwise comparison based on ranks was performed for specific differences between treatments (Mann-Whitney test). Differences were considered significant when P was ≤ 0.05.

Results

Characterization of the inclusion complex in D_2O solution by NMR

Proton NMR spectrometry is commonly used for investigating the nature of the complexes in solutions (Perly et al 1991). For our soluble complexes, the observed resonances were the time-average peaks of pure and complexed compounds (fast exchange regime on NMR time-scale at 293 K). The complexation of antibiotics with cyclodextrins is demonstrated by the detected modification of NMR spectra of both the guest and host molecules in aqueous solutions (D_2O). Under our conditions, only the shifts of the signals were observed and no new peaks appeared that could be assigned to the pure complex. This observation implies that the complexation of the used antibiotics is a dynamic process, the included guest being in fast exchange (relative to the NMR time-scale) between the free and bonded states. Protons located inside the cavity of cyclodextrins (H-3 and H-5) are expected to experience chemical shift variations on inclusion of the guest ($\Delta\delta = \delta_{free} - \delta_{complex}$), whereas those residing outside the cavity (H-1, H-2 and H-4) should undergo no or minimal changes.

In the ^{1}H NMR spectra of ampicillin–βCD and ampicillin–MethβCD complexes, signals corresponding to protons located in the internal surface of βCD (H-5) presented a 0.015 ppm upfield displacement upon complexation. At the same time, the multiplet assigned to the aromatic protons of ampicillin also exhibited a downfield shift of about 0.012 ppm in the case of both the ampicillin–βCD and the ampicillin–MethβCD complexes, indicating that the ampicillin complexation occurred by inclusion of the aryl group part of the molecule. In spectral data of amoxicillin complexes with βCD and MethβCD, ^{1}H chemical-shift changes between corresponding resonances were also observed. The resonances of H-3 and H-5 protons of the cyclodextrin cavity were displaced, indicating that the environment of these protons was changed and they became more shielded. The measured $\Delta\delta$ values were 0.012 ppm and 0.011 ppm respectively. In cefadroxil complexes, the H-3 and H-5 protons of βCD cavity exhibited upfield shifts of $\Delta\delta = 0.023$ ppm and 0.020 ppm, respectively. Similarly, the aromatic protons of cefadroxil were also found to be shielded upon complexation, with a $\Delta\delta = 0.013$ ppm for βCD, and $\Delta\delta = 0.010$ ppm for MethβCD, indicating the inclusion of substituent at position 7 of the cefadroxil molecule in the cyclodextrin cavity.

Antibacterial activity against clinical strains

The MICs at which 50% and 90% of the clinical isolates are inhibited (MIC_{50}, MIC_{90} values), the range of MICs of the tested antibiotics and the corresponding complexes with several cyclodextrins are shown in Table 1. All cyclodextrins tested against clinical and reference strains appeared microbially inactive at the corresponding concentrations used for complexation.

Staphylococcus aureus

For *S. aureus* strains tested, the complexes of ampicillin with βCD and HPβCD (molar ratio 1:2) exhibited a two-fold increase in activity, in terms of both MIC_{50} and MIC_{90}. Moreover, amoxicillin complexes with CD (in molar ratios 1:2 and 1:3) exhibited two-fold increase in activity. Cefadroxil complexes appeared to be equally active against staphylococcal strains compared with free compound (based on MIC_{50} and MIC_{90}). Nevertheless, one methicillin-sensitive *S. aureus* strain was 16 and 4 times more sensitive to cefadroxil–HPβCD (1:2) and cefadroxil–βCD (1:2), respectively, than free antibiotic. Both complexes showed four- and two-fold increase in activity against two other methicillin-resistant *S. aureus* strains. A two-fold decrease in the MIC_{90} of cefadroxil–MethβCD (1:2) was detected relative to free antibiotic.

Klebsiella *spp*

Against strains of *Klebsiella* spp, complexes of ampicillin (with βCD and HPβCD) showed two- and four-times higher activity than free ampicillin, in terms of both MIC_{50} and MIC_{90}. Amoxicillin complexes appeared twice as active as cyclodextrin-free antibiotic. Cefadroxil–βCD (1:2) and cefadroxil–HPβCD (1:2) complexes appeared to be as equally active as free cefadroxil against all tested *Klebsiella* strains. The MIC_{50} and MIC_{90} of cefadroxil–MethβCD (1:2) for *Klebsiella* strains were four times lower than those of free cefadroxil, corresponding to significant differences of medians ($P < 0.05$).

Escherichia coli

For the ten *E. coli* strains, the MIC_{50} and MIC_{90} were, respectively (μg mL^{-1}): 1024 and 2048 for ampicillin, 1024 and 1024 for ampicillin–βCD (1:2), 512 and 1024 for ampicillin–HPβCD (1:2), 64 and 64 for cefadroxil and 32 and 64 for both βCD and HPβCD complexes of cefadroxil. Cefadroxil–MethβCD (1:2) was four times and two times as active against tested *E. coli* strains in terms of MIC_{50} and MIC_{90}, respectively ($P < 0.05$). Two-fold reduction in the MIC_{90} of amoxicillin–βCD (1:2 and 1:3) was also observed.

Pseudomonas aeruginosa

In the case of *P. aeruginosa* strains, ampicillin complexes demonstrated a two-fold increase in activity in terms of

MIC_{50} and MIC_{90}, while the MIC_{90} of amoxicillin complexes exhibited a two-fold decrease. Cefadroxil complexes with βCD and HPβCD appeared to be equally active compared with free drug, but again cefadroxil–MethβCD (1:2) complex was twice as active against 50% and 90% of *P. aeruginosa* strains.

Enterobacter *spp*

For strains of *Enterobacter* spp, the MICs at which 50% and 90% of isolates were inhibited were ($\mu g\,mL^{-1}$): 512 and 1024 for ampicillin, 256 and 1024 for ampicillin–βCD (1:2), 256 and 512 for ampicillin–HPβCD (1:2). Amoxicillin complexes demonstrated the same antimicrobial pattern. Cefadroxil complexes with βCD and HPβCD exhibited a two-fold increase in activity. For cefadroxil–MethβCD (1:2) complex, 8 and 4 times lower MIC_{50} and MIC_{90} values were observed, respectively, corresponding to significant difference in activity between cefadroxil–MethβCD (1:2) and free antibiotic ($P < 0.05$).

Citrobacter *spp*

Against strains of *Citrobacter* spp, complexes of ampicillin and amoxicillin showed two and four times higher activity than free antibiotics, in terms of MIC_{50} and MIC_{90}. Cefadroxil–MethβCD (1:2) complex was, again, 4 and 2 times more active than the corresponding free compound in terms of MIC_{50} and MIC_{90}, respectively. Both differences in activity were statistically significant ($P < 0.05$).

Antibacterial activity against known β-lactamase producers

Table 2 represents the resistance phenotype of the eleven β-lactamase producers.

TEM-1 and SHV-5 β-lactamase producers

Three strains of *S. marcescens* (21, 22 and 25) and one strain of *E. cloacae* (TSV-288) were TEM-1 and SHV-5 producers. TEM and SHV are two groups of extended spectrum β-lactamases (ESBLs) conferring resistance to newer β-lactams such as oxyimino-cephalosporins. They are encoded by self-transmissible multi-resistant plasmids and share extensive functional and structural similarities (Heritage et al 1999; Labia et al 1999). Two- to four-fold increase in activity was observed when ampicillin–βCD (1:1) complex was tested against the three *S. marcescens* strains and an eight-fold increase against *E. cloacae* strain, compared with ampicillin. Ampicillin–MethβCD (1:1) and ampicillin–MethβCD (1:3) complexes showed two- to four-fold increase in activity against the three *S. marcescens* strains and, again, an eight-fold increase against *E. cloacae* TSV-288 strain. Ceftazidime–βCD (1:1) and ceftazidime–MethβCD (1:1) complexes were twice as active against *S. marcescens* strains 21, 22 and 25 and *E. cloacae* TSV-288 compared with uncomplexed ceftazidime. The MICs of ceftazidime–βCD (1:1) and ceftazidime–MethβCD (1:1) were equal to the MIC of amoxicillin–clavulanate against the above mentioned four stains. Complexes of ceftazidime with MethβCD (1:1 and 1:3) were also twice as active as free ceftazidime against *S. marcescens* 22.

IBC-1 β-lactamases producers

Two *E. cloacae* strains (TSV-239 and IpT-58) were IBC-1 producers. IBC-1 β-lactamase is a novel class A ESBL, encoded by an integron-associated gene harboured in a multidrug resistant transferable plasmid found in *E. cloacae* (Giakkoupi et al 2000). The enzyme confers resistance against both penicillins and cephalosporins. Although both strains were resistant to ampicillin and ceftazidime complexes, their susceptibility to those formulations was elevated when compared with uncomplexed antibiotics. There was a two-fold decrease in the MICs of ampicillin–βCD (1:1) and ampicillin–MethβCD (1:1), compared with the MIC of ampicillin against *E. cloacae* IpT-58 strain, and a four-fold decrease in the MICs of ampicillin–βCD (1:1) and ampicillin–MethβCD (1:3) against *E. cloacae* TSV-239 and IpT-58 strains, respectively. The MIC of ampicillin–MethβCD (1:3) against *E. cloacae* TSV-239 was 32-times less than the MIC of ampicillin, and equal to the MIC of amoxicillin against the same strain. Ceftazidime–βCD (1:1) and ceftazidime–MethβCD (1:1) complexes were twice as active against both *E. cloacae* TSV-239 and *E. cloacae* IpT-58 strains as uncomplexed ceftazidime. It is most interesting that the MICs of ceftazidime–βCD (1:1) and ceftazidime–MethβCD (1:1) against *E. cloacae* IpT-58 were equal to the MIC of ceftazidime–tazobactam and two-fold below the MIC of ceftazidime–clavulanate against the same strain (data not shown).

LAT β-lactamase producers

Three of the tested resistant strains (*E. aerogenes* EY-58, *K. pneumoniae* EY-205, *E. coli* MEL-2) were LAT β-lactamase producers. LAT is a plasmid-mediated group 1 β-lactamase with chromosomal cephalosporinase characteristics. The enzymes resemble chromosomal class C β-lactamases, because they confer resistance to cephalosporins, including cefoxitin, and they are not inhibited by clavulanate (Gazouli et al 1998). Ampicillin complexes with βCD (1:1) and MethβCD (1:1) were two to four times more active than ampicillin against *E. aerogenes* EY-25 and *E. coli* MEL-2 strains. Ampicillin–βCD (1:1) was 8 times more active against *K. pneumoniae* EY-205 strain, while ampicillin–βCD (1:3) was 16 and 32 times more active than ampicillin against strains *E. aerogenes* EY-25 and *K. pneumoniae* EY-205, respectively. Ceftazidime complexes with βCD (1:1), MethβCD (1:1) and MethβCD (1:3) appeared 2 to 16 times more active against the above mentioned strains than ceftazidime. Ceftazidime–βCD (1:1) was 16 times more active against *E. aerogenes* EY-25 strain, while ceftazidime–MethβCD (1:1) was 8 times more active against the same strain.

CTX-M β-lactamases producers

Two *Salmonella typhimurium* isolates were chosen as CTX-M β-lactamase producers. CTX-M type constitutes a novel group of class A β-lactamases. They are encoded by transferable plasmids and they hydrolyse, among others, the third generation oximino-cephalosporins. The best substrates for these enzymes are cefotaxime and ceftriaxone; they also hydrolyse ceftazidime but with lower

efficiency (Tzouvelekis et al 2000). All three different ampicillin–βCD complexes were four times more active against both strains than free ampicillin. Ceftazidime–βCD (1:1) complex was four and two times as active as free ceftazidime against *S. typhymurium* 661 and 893 strains, respectively. Ceftazidime–MethβCD (1:1) and (1:3) were twice as active as ceftazidime against *S. typhymurium* 661 strain and equal in activity to amoxicillin–clavulanate.

Discussion

As shown by MIC determination, a susceptibility elevation was observed, in terms of MIC_{50} and MIC_{90}, when cyclodextrin-included antibiotics were tested against the 64 clinical isolates. Increase in activity was more considerable against Gram-negative strains, particularly when the selected antibiotics were complexed with MethβCD.

In Gram-negative bacterial strains, β-lactamases are located in the periplasmic space and bacterial resistance due to β-lactamases is further enhanced by the outer membrane's permeability barrier.

Fick's law represents the rate of entry of β-lactam molecules across the outer membrane, utilizing outer membrane porins (OmpF, OmpC), since passive diffusion is the mechanism describing the above phenomenon (Knox 1995).

Eventually when equilibrium is established, the rate of drug diffusion equals the rate of drug hydrolysis by β-lactamases (Zimmerman & Rosselet 1977) and therefore:

$$C(S_o - S_p) = V_{max}S_p/(K_m + S_p) \quad (1)$$

where S_o and S_p are the external and periplasmic drug concentrations, C is the diffusion coefficient related to the permeability of the membrane, V_{max} reflects the amount of enzyme molecules produced and the number of drug molecules hydrolysed per unit time by each enzyme molecule and K_m is a kinetic constant, which is inversely proportional to the enzyme's affinity for the substrate. Consequently, MIC is the minimum required S_o to give an S_p just enough to inactivate penicillin binding proteins (PBPs) (Nikaido & Normark 1987). Rearrangement of equation 1 gives:

$$MIC = S_p\{1 + [V_{max}/C(K_m + S_p)]\} \quad (2)$$

Equation 2 suggests that the MIC is raised when a drug is hydrolysed rapidly (high V_{max}), when the enzyme exhibits high affinity for the drug molecule (low K_m) or when drug diffusion through the outer membrane is slow (low C). Conversely, low affinity for the substrate (high K_m) or rapid penetration of the antibiotic's molecules (high C) reduces the ability of the enzyme to protect against drug action, resulting in a reduction of the MIC and an increase of drug's activity.

Our experimental results demonstrate that several bacterial strains appeared much more sensitive when their viability was challenged by β-lactam molecules complexed with βCDs or synthetically modified βCDs. The presence of cyclodextrin molecules did not alter the amounts of enzymes produced by the examined strains (data not shown). Therefore, in terms of equation 2, it is reasonable to assume that increase in activity of complexed drug molecules can be attributed to decrease in V_{max}, increase in K_m or increase in C.

It is clearly documented that the affinity of β-lactamases for drug molecules is strongly related to the amino-acid sequence and the tertiary structure of the enzyme molecule (Giakkoupi et al 1999). Accordingly, if K_m elevation occurs, even though it may contribute to the increased activity of complexed drug molecules, it is not expected to be the predominant event. On the other hand, a decrease in V_{max} (i.e., decrease in the number of drug molecules hydrolysed per unit time by each enzyme molecule) is most likely to occur due to steric hindrance of complexed β-lactam molecules. Nevertheless, both V_{max} and K_m as kinetic parameters may be altered and contribute to the MIC decrease observed when using antibiotic–cyclodextrin complexes.

Class A β-lactamases interact with β-lactams in a similar mode. A well-ordered network of hydrogen bonds and electrostatic interactions aligns the substrate within the active site of the enzyme, facilitating a nucleophilic attack against the β-lactam ring by Ser-70 residue and the release of the inactivated product (Matagne et al 1998). More than fifteen other amino acids participate or contribute to the formation and functionality of the active site (Knox 1995).

The reactive Ser-70 residue is located at the N terminus of the enzyme's H2 α-helix. Between the N terminus of H2 and the edge of the B3 β-sheet, there exists a so-called oxyanion pocket. The purpose of the pocket is the polarization of the carbonyl group of the β-lactam ring, which is strongly attracted to this pocket by hydrogen bonding with backbone amides' NH groups at positions 70 and 237 (Knox 1995). The active site would contain a specific amino-acid side chain, which increases the nucleophilicity of the Ser-70 hydroxyl group, by acting as a general base catalyst. In the most prevailing hypothesis, this role is played by the conserved Glu-166 residue, through the formation of a hydrogen-bonding network between the Ser-70 hydroxyl group, the Glu-166 carboxylate and a tightly bound water molecule (W1). The conserved water molecule acts as a relay molecule in the transfer of the proton between the Ser-70 and Glu-166 side chains (Lamotte-Brasseur et al 1991). The activated γ-O of Ser-70 can then attack the β-lactam carbonyl carbon (acylation of the enzyme) and the abstracted proton is back donated to the nitrogen atom of the β-lactam ring through another hydrogen-bonding sub-network involving a second water molecule (W2), the ϵ-amino groups of Lys-73 and Lys-234 and the hydroxyl group of Ser-130, which acts as the last proton donor (Lamotte-Brasseur et al 1992).

The above mechanism requires the strong binding and coordination of the β-lactam molecule in the active site. In the case of TEM-1 β-lactamases, the γ-OHs of Ser-130 and Ser-235 residues interact with the carboxylate group in position C-3 or C-4 of penam or cepham molecule respectively (Masaji & Seiichi 1996; Atanasov et al

2000). The side-chain NH_2 group of Asn-132 and the carbonyl backbone of Ala-237 interact with the exocyclic CONH acylamido linkage in positions C-6 or C-7 of penam or cepham ligand. The backbone NH groups of Ser-70 and Ala-237 polarize the carbonyl group of the beta-lactam amide bond. Several other residues, in the vicinity of Ser-70, such as Glu-240, Glu-104 or Ser-238, interact with other β-lactam moieties and particularly the larger acylamido substituents of oximino-cephalosporins.

In complexation of β-lactam molecules with βCDs, as shown by NMR experimental results, the substituents at position 7 were included in the cavity of cyclodextrin molecules. The outcome of this procedure is the inability of complexed β-lactam molecules to fit into the active site in the way observed at the free substrate. Due to steric hindrance, and possibly displacement of the water molecules W1 and W2, both acylation of the enzyme and most possibly hydrolysis of the acyl-enzyme become most unfavourable, as expected from the above-described mechanism. A realistic approach would be that β-lactam–cyclodextrin complexes resemble the feature of imipenem and moxalactam inhibitors (Shoichet et al 2001). Complexed β-lactams are not complementary enough to the active site, compared with free β-lactam molecules, rather they trap serine β-lactamases in a covalent adduct due to unfavourable interactions between cyclodextrin substituents and several residues in the vicinity of the active site. These unfavourable contacts, as in the case of imipenem or moxalactam, lead to a catalytically incompetent conformation of the complexed β-lactam in the active site, blocking nucleophilic attack and deacylation of the acyl-enzyme. This approach would explain the higher activity of MethβCD complexes, where hydrophobic interactions between the methyl groups and several residues of the enzyme may account for inappropriate fitting of complexed antibiotic molecules in the active site of β-lactamases. Crystallographic studies will elucidate the above mechanisms.

Increase in diffusion rate of complexed molecules through the outer membrane is also a parameter that should be considered. The inevitable question is how the bulky molecules of those complexes traverse the permeability barriers and especially the outer membrane of Gram-negative bacteria. Because of their outer diameter of approximately 1.53 nm it is most improbable that they could use the lamB channel, which facilitates the entry of maltose and maltodextrins into the periplasm (Boos & Shuman 1998). The apparent existence (characterization and purification) of CymA porin, the outer membrane component of the Cym system, as was demonstrated (Böck et al 1996; Pajatsch et al 1998, 1999), might be a most prominent answer to the above question. CymA is a component of the outer membrane and functions as a porin specific for cyclodextrins. Although cyclodextrins are relatively rigid molecules, they were found to adapt their structure upon binding with proteins, as was demonstrated by crystallographic studies (Schmidt et al 1998). An equivalent to the Cym system has been recently identified in *Bacillus subtilis* (Kamionka & Dahl 2001). Several other outer-membrane bacterial proteins appeared structurally similar to CymA and may facilitate penetration of cyclodextrin–drug complexes to several bacterial strains (Balderman et al 1998; Daniel et al 1998; Henderson et al 1998). Increase in the diffusion rate of complexed molecules through the outer membrane is further supported by the following observation. Although the IBC-1 β-lactamase producers used in the study hydrolysed cefotaxime and ceftazidime at comparable efficiencies, as previously shown (Giakkoupi et al 2000), a higher level of resistance was conferred against the latter antibiotic in the strains harbouring the above enzyme. This is presumably due to the faster diffusion of cefotaxime through the enterobacterial outer membrane (Yoshimura & Nikaido 1985). The observed decrease in MICs of ceftazidime–βCD (1:1) and ceftazidime–MethβCD (1:1) compared with free antibiotic, when tested against the IBC-1 producers, strongly indicates a facilitation of ceftazidime diffusion through complexation with βCD and MethβCD.

Another approach might be an overall destabilization of the outer membrane by cyclodextrin molecules, which eventually leads to an increase in diffusion rate. It has been demonstrated that cyclodextrin molecules, complexed with several drugs, are able to interact and increase the fluidity of artificial lipidic membranes, altering the lipid packing density, and eventually enhancing in-vitro cell-membrane permeability and diffusion rates of various drugs (Castelli et al 1989; Antoniadou-Vyza et al 1996, Grosse et al 1997, Mavromoustakos et al, 1998).

Resistance phenomena have become increasingly widespread. Besides the modification of the permeability barrier, the synthesis of several β-lactamases represents the most efficient mechanism devised by bacteria to overcome the lethal action of β-lactam molecules. Today it appears that the therapeutic future of β-lactam antibiotics is seriously depressed. To preserve the antimicrobial value of these drugs, a solution to the problem is urgently needed. The problem is further complicated by the microbial genetic fluidity, which allows the dissemination of resistance genes.

Conclusions

In this study, the development of cyclodextrin–β-lactam complexes with increased antimicrobial activity compared with uncomplexed compounds was proposed. The proposed strategy constitutes a novel approach to the problem of microbial resistance. Further investigation is necessary for a better understanding of the implicated mechanism of antimicrobial activity of the above-mentioned complexes.

From the experimental results it can be concluded that the nature and degree of cyclodextrin modification may be the predominant factor of the observed improvement in antimicrobial activity.

We believe that the proposed approach will trigger the research towards the development of new cyclodextrin derivatives bearing the ability to increase the uptake of included antimicrobial molecules through intensification of the corresponding molecular recognition phenomena.

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