Asian Journal of Chemistry Vol. 19, No. 4 (2007), 3145-3151

Activity of Bisbenzimidazoles Derivatives to *Staphylococcus epidermidis*

OZTEKIN ALGUL* and NIZAMI DURAN†
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mersin University Yenisehir Campus, 33169, Mersin, Turkey
E-mail: oztekinalgul@mersin.edu.tr

Biomaterial-associated infections, most frequently caused by *Staphylococcus epidermidis* and *Staphylococcus aureus*, are of increasing importance in modern medicine. The most important factor in the pathogenesis of biomaterial-associated *staphylococcal* infections is the formation of adherent, multilayered bacterial biofilms. The aim of this study was to synthesize *bis*(benzimidazole) derivatives and evaluate their *in vitro* antimicrobial activity against the growth of gram positive (*Enterococcus faecalis*, methicillin resistant *S. aureus*, methicillin sensitive *S.aureus*, *S. epidermidis* and *S. epidermidis* RP12 (slime producing), gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and pathogenic fungi (*Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata*). The antimicrobial activity of *bis*(benzimidazole) derivatives was higher in gram positive bacteria. *Bis*(benzimidazole) derivatives were inhibited gram positive bacteria at the concentration range of 12.5-100 μg/mL. All compounds were shown to be bacteriostatic as well as bacteriocidal for cultures of *S. aureus* and *S. epidermidis* strains, regardless of their antibiotic susceptibility profile. This was demonstrated by using simultaneously the optical density measuring method and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-reduction assay. The highest activity was shown by 1,2-di(1H-benzo[d]imidazol-2-yl)ethane which demonstrated interesting activity regarding its effect on 24 h old staphylococcal biofilm cells viability.

Key Words: *Bis*(benzimidazole), *Staphylococcus epidermidis*, Antimicrobial activity, Biofilm.

INTRODUCTION

Staphylococcus epidermidis, the most common member of coagulase-negative *staphylococci*, is an opportunistic pathogen, habitual inhabitant of the human epithelia and the most prevalent and persistent species on skin and mucous membranes[1]. Despite of its low virulence, this organism

†Department of Microbiology, Faculty of Medicine, Mustafa Kemal University, 31100, Hatay, Turkey.

has evolved as one of the leading causes of nosocomial sepsis, the most frequent causal organism isolated in infections of implanted medical devices such as intravascular and peritoneal dialysis catheters, cerebrospinal fluid shunts, prosthetic heart valves and prosthetic joints, vascular grafts, cardiac pacemakers and intraocular lenses[2-5]. The major virulence factor of this organism is its ability to adhere to devices and form biofilms, which is responsible for a greater resistance to antibiotics.

The therapy of *S. epidermidis* in prosthetic infections is problematic due to frequent multiple antibiotic resistances to this microorganism. The repeatedly clinical ineffectiveness of antibiotics tested *in vitro*, regularly requires removal of the implanted prosthetic device for successful therapy[6,7]. Moreover, microbial biofilms which are described as polymer-dipped communities of cells, being responsible for a number of diseases of chronic nature, demonstrate extremely high resistance to antibiotics and host defence systems[8,9]. For this reasons many research groups investigate potential strategies, which could be accessory or alternative to antibiotic therapy.

Benzimidazole and *bis*(benzimidazole) derivatives are key components in many bioactive compounds of both natural and synthetic origin. These ligands and their derivatives display a wide range of pharmacological activity and their inhibitory properties in regard to replication of polio viruses, adenosine deaminase, and casein kinase have been fully demonstrated[10,11]. In addition, other members of this group are important potent antiviral agents and many of them are active components of biocides such as fungicides and insecticides[12-14]. There have been no reports in the literature on the interaction of biofilm structure of *S. epidermidis* and *S. epidermidis* inhibitions. Therefore, in present studies the antimicrobiological activity of these ligands towards many bacterial and yeast isolates have been investigated. In the present study, the three *bis*(benzimidaozole) derivatives were investigated with respect to their bacteriostatic, bactericidal and fungistatic activities. Initially, all compounds were tested for their bacteriostatic, bactericidal and fungistatic activities and it was found that these compounds were effective. The susceptibility of microorganisms cultured as suspension cells or as adherent biofilm, to these compounds was tested.

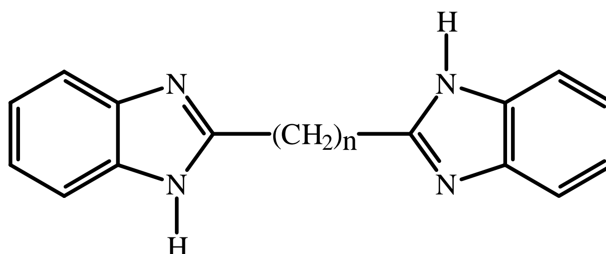
EXPERIMENTAL

Melting points were measured on an Electrothermal 9200 melting point apparatus and were uncorrected. Infrared spectra were recorded in KBr pellets on a Mattson 1000 FTIR spectrometer in the range 4000-400 cm^{-1}. Proton magnetic resonance (^{1}H NMR) spectra were recorded in DMSO-d_6 (Merck) on a Bruker 400 MHz spectrometer. All chemicals and solvents

used were of reagent grade (Aldrich, Merck, Sigma) and were used without further purification. Thin-layer chromatography (TLC) was performed on pre-coated aluminum plates (Silica gel 60 F_{254}, Merck). Plates were visualized by UV light, Dragendorff reagent and iodine vapour.

General procedure for the synthesis of *bis*(benzimidazoles) (I-III)

A mixture of oxamide, malonamide or succinic acid and *o*-phenylenediamine (in 1:2 ratio) was placed in the flask and a thick paste was made with polyphosphoric acid. Extra polyphosphoric acid was then added to give smooth slurry. The temperature of flask was raised very slowly until 250°C. This temperature was kept constant for a specific period. After completion of the reaction the thick slurry was cooled to about 80°C and added slowly in a fine stream with rapid stirring to cold water. Stirring was continued for a further 1 h. The resulting solution was filtered and neutralized with dilute alkali to obtain the product. The product was filtered, well washed with water, dried and recrystallized (Fig. 1).



n	Compound No.
0	**I**
1	**II**
2	**III**

Fig. 1. Chemical structure of *bis*(benzimidazole) derivatives

In addition, the purification by recystallization from ethylene glycol was not totally efficient and an extra purification step was introduced. The solid was dissolved in boiling NaOH, 0.25 M and refluxed for 2 d. Some insoluble material was filtered off and the solution was neutralized with concentrated HCl. The solid was filtered, transferred into water and refluxed for 1 h to remove any ionic form of the molecule. Finally, the solid was filtered, washed with water, ethanol and diethyl ether and dried in air. All *bis*(benzimidazoles) were characterized by elemental analysis, infrared and NMR measurements.

2,2'-Bi(1H-benzo[d]imidazole) (I): Oxamide (2 mmoles) was reacted with *o*-phenylenediamine (4 mmoles) at 250°C for 5 h and worked up as above. The product was recovered as bright yellow needles for a yield of 24 % and m.p. 395°C. [>300°C, 396°C][15-17]. Anal. calcd. for $C_{14}H_{10}N_4$: C 71.79, H 4.27, N 23.93; Found C 71.60, H 4.33, N 23.97. ^1H NMR ([d$_6$]DMSO): δ = 7.1 (4H, d, J = 6 Hz), 7.5 (4H, d, J = 6 Hz). 13.70 (br, s, 2 NH). IR (KBr, cm^{-1}): 3280-2500, 1620-1585, 1530, 1480, 1445, 1400, 1010, 950, 840-740.

Di(1H-benzo[d]imidazol-2-yl)methane (II): Malonic acid diamide (2.5 mmoles) was reacted with *o*-phenylenediamine (5 mmoles) at 250°C for 7 h and worked up as above. The product was recovered as a colourless needless for a yield 30 % and decomposed at 390°C. [>300°C, 390°C][15,17]. Anal. calcd. for $C_{15}H_{12}N_4$: C 72.5, H 4.8 N 22.6 found C 72.6, H 4.9, N 22.7. ^1H NMR ([d$_6$] DMSO): δ = 4.50 (s, 2H), 7.20 (m, 4H), 7.50 (d, 4H) IR (KBr, cm^{-1}): 3300-2500, 1620, 1590, 1528, 1485, 1450, 1430, 1030, 990, 850, 730.

1,2-di(1H-benzo[d]imidazol-2-yl)ethane (III): Succinic acid (5 mmoles) was reacted with *o*-phenylenediamine (10 mmoles) at 250°C for 8 h and worked up as above. The product was recovered as a colourless needless for a yield of 55 % and m.p. 327°C. [>300°C, 327°C][15,17,18]. Anal. Calcd. for $C_{16}H_{14}N_4$: C 73.3, H 5.3, N 21.4; Found C 73.0, H 5.0, N 21.3. ^1H NMR (250 MHz, CD_3COOD): δ = 4.05 (s, 4H), 7.40-7.80 (m, 4H), 8.20 (d, 4H). IR (KBr, cm^{-1}) : 3440, 3180-2500, 1620-1570,1535, 1482, 1450, 1430, 1030, 930, 880, 750.

Biological tests

Microorganisms (bacteria and yeasts): 4 Gram-positive bacteria: *S. aureus* (ATCC 29213; MSSA), *S. aureus* (ATCC 43300; MRSA), *S. epidermidis* (ATCC 12228) and *S. epidermidis* RP12 (slime producing), *Enterococcus faecalis* (ATCC 29212), the reference methicilin susceptible (MSSA) strain; 2 Gram negative *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and yeast *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 22019) and *C. glabrata* (ATCC 32554) (from the Refik Saydam Hifzisihha Institute, Ankara, Turkey) were used.

Antimicrobial susceptibility testing: The susceptibility of microorganisms to *bis*(benzimidazole) derivatives was determined by the standard NCCLS microdilution method (National Committee for Clinical Laboratory Standards, M7-A5, 2000). Sterile stock solutions of each compound at the concentration of 1600 μg/mL were prepared in DMSO. The agent concentration range used in the antimicrobial tests was 1.56-1600 μg/mL prepared for bacteria in Mueller-Hinton broth (Difco) and for yeast in RPMI-

1640 medium supplemented with L-glutamine and $NaHCO_3$. To specify the minimal inhibitory concentrations (MIC), tubidometric (OD_{600}) studies were carried out using the multifunction counter Victor2 (Wallac, Finland).

Biofilm bacterial cultures for antimicrobial test: The overnight cultures of *S. aureus* and *S. epidermidis* strains diluted 1:40 in tryptone soya broth (TSB) (Oxoid) + 0.25 % glucose were added (200 μL) to each well of a 96-well tissue culture plate (Nunclon^{TM} Surface, Nunc). In order to allow bacteria to form biofilms, the plates were incubated for 24 h at 37°C. After the incubation and removing of the medium, the biofilms were treated either with various concentrations of amikacin or *bis*(benzimidazole) derivatives for 24 h at 37°C. The viability of the biofilm remaining on the surfaces of the wells or chamber slides was stained with 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) was used for staining of live and adherent bacteria as described earlier[19].

RESULTS AND DISCUSSION

The aim of this study was the evaluation and comparison of bacterio-static and bacteriocidal activity of *bis*(benzimidazole) derivatives. As an indication of the bacteriostatic effect, minimal inhibitory concentration (MIC) of these three *bis*(benzimidazole) derivatives (Fig. 1) were deter-mined for gram-positive and gram-negative bacteria as well as for fungi, using the standard microdilution susceptibility test (Table-1). It was found that the growth of gram-positive bacterial strains (*S. aureus*, *S. epidermidis*, *E. faecalis*) was inhibited by three *bis*(benzimidazole) derivatives tested at the concentrations of 12.5-100 μg/mL (Table-1). The MIC values of the compounds obtained for gram-negative *E. coli* and *P. aeruginosa*, as well as *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* exceeded 100 μg/mL. Moreover, these compounds were revealed to be not only bacteriostatic but also bacteroiocidal for the cultures of *S. aureus* and *S. epidermidis* strains, regardless of the antibiotic susceptibility profile of these bacteria. This was demonstrated by using simultaneously the optical density measuring method and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)-reduction assay. Inhibition of bacterial growth/ killing, established by both methods, ranged from 12.5 to 400 μg/mL. The highest activity was expressed by compound (**III**), the MIC for *S. aureus* ATCC 29213 was 50 μg/mL, for *S. epidermidis* ATCC 12228 was 12.5 μg/ mL. It also prevented bacterial adhesion and biofilm formation being bactericidal at the same concentration range. Compound (**III**) at a concen-tration of 50 μg/mL was also bacteriostatic for other gram-positive bacte-ria- *E. faecalis* ATCC 29212.

TABLE-1
ANTIMICROBIAL ACTIVITY OF *BIS*-BENZIMIDAZOLE DERIVATIVES

Microorganism	Compound (MIC μg/mL)					
	I	II	III	Flucanazole	Ampicillin	Amikacin
S. aureus (ATCC 29213)	50	25	50	Nt*	Nt	6.25
S. aureus (ATCC 43300)	200	200	100	Nt	Nt	12.5
S. epidermidis (ATCC 12228)	50	50	12.5	Nt	Nt	1.56
S. epidermidis PR 12	>100	>100	50	Nt	Nt	3.12
E.faecalis (ATCC 29212)	100	50	50	Nt	6.25	Nt
E. coli (ATCC 25922)	100	200	100	Nt	6.25	Nt
P. aeruginosa (ATCC 27853)	100	100	100	Nt	3.12	Nt
C. albicans (ATCC 90028)	>100	400	100	6.25	Nt	Nt
C. krusei (ATCC 6258)	>200	>100	>100	3.12	Nt	Nt
C. parapsilosis (ATCC 22019)	>100	200	>100	3.12	Nt	Nt
C. tropicalis (ATCC 20336)	>100	>100	100	6.25	Nt	Nt
C. glabrata (ATCC 32554)	400	400	>100	3.12	Nt	Nt

MIC values were determined by microduliton assay, according to NCCLS recommendations, Nt*, not tested.

A very interesting activity was demonstrated by compound (**III**), when its effect on 24 h old biofilm cells viability was examined. Two *S. aureus* strains: reference ATCC 29213 (methicillin sensitive *S. aureus*; MSSA), ATCC 43300 (methicillin resistant *S. aureus*; MRSA) and *S. epidermidis* ATCC 12228 were selected for this part of the study. Using MTT-reduction assay it was demonstrated that biofilms of these *S. aureus* as well as of *S. epidermidis* strains were highly resistant to amikacin but unexpectedly susceptible to compound (**III**). The absorbance of the antibiotic-treated biofilms were the same after 24 h as at the time of antibiotic application (OD_{550} 2.7-3.0), whereas the absorbance (OD_{550}) of compound (**III**) treated biofilms dropped by 40-65%. However, none of biofilms was completely eradicated.

Conclusion

The present report is the first one which demonstrates that the inhibitory activity of *bis*(benzimidazole) derivatives against *staphylococcal* biofilms. It can be speculated that a small molecular mass of compound

(**III**) as well as high antimicrobial activity may be sufficient to destabilize biofilm matrix and allow their detachment. Another explanation for biofilm-limiting activity of compound (**III**) can be surfactant properties affecting bacterial cell surface hydrophobicity or its interference with biofilm formation. In addition, it was known that amikacin is one of the most effective agents against gram-positive bacteria. Although, compound (**III**) was not as effective as amikacin, it is proposed that it may a lead compound in this area.

ACKNOWLEDGEMENT

The authors are thankful to Prof. Dr. Fatma Gumus, Faculty of Pharmacy, Gazi University, Turkey for her valuable support.

REFERENCES

1. W.C. Noble, Staphylococcal Carriage and Skin and Soft Tissue Infection, in eds.: K.B. Crossley and G.L. Archer, The Staphylococci in Human Disease, Churcill Livingstone, New York (1997).
2. M.E. Rupp and G.L. Archer, *Clin. Infect. Dis.*, **19**, 231 (1994).
3. W.E. Kloss and T.L. Bannerman, *Clin. Microbiol. Rev.*, **7**, 117 (1994).
4. T.G. Emori and R.P. Gaines, *Clin. Microbiol. Rev.*, **6**, 428 (1993).
5. M.A. Pfaller and L.A. Herwaldt, *Clin. Microbiol. Rev.*, **1**, 281 (1988).
6. I. Raad, A. Alrahman and K. Rolston, *Clin. Infect. Dis.*, **26**, 1182 (1998).
7. G.L. Archer and M.W. Climo, *Antimicrob. Agents Chemother.*, **38**, 2231 (1994).
8. K. Lewis, *Antimicrob. Agents Chemother.*, **45**, 999 (2001).
9. C.A. Fux, P. Stoodley, L. Hall-Stoodley and J.W. Costerton, *Expert Rev. Anti-Infect. Ther.*, **1**, 667 (2003).
10. M.P. Groziak, Z.-W. Huan, H. Ding, Z. Meng, W.C. Stevens and P.D. Robinson, *J. Med. Chem.*, **40**, 3336 (1997).
11. R. Szyszka, N. Grandkowski, K. Felezak and D. Shugar, *Biochem. Biophys. Res. Commun.*, **208**, 418 (1995).
12. S. Saluja, R. Zou, J.C. Drach and L.B. Twonsend, *J. Med. Chem.*, **39**, 881 (1996).
13. R. Zou, K.R. Ayres, J.C. Drach and L.B. Twonsend, *J. Med. Chem.*, **39**, 3477 (1996).
14. A.S. Demir, H. Hamamci, O. Sesenoglu, R. Neslihanoglu, B. Asikoglu and D. Capanoglu, *Tetrahedron Lett.*, **43**, 6447 (2002).
15. B. Cakir, E. Buyukbingol, U. Ucucu, U. Abbasoglu and N. Noyanalpan, *Gazi Ecz. Fak. Der.*, **5**, 71 (1988).
16. G. Kaupp and M.R. Naimi-Jamal, *Eur. J. Org. Chem.*, **8**, 1368 (2002).
17. P.C. Vyas, C.K. Oza and A.K. Goyal, *Chem. Industry*, **7**, 287 (1980).
18. K. Aghapoor, F. Mohsenzadeh and H.R. Darabi, *Z. Naturforsch.*, **60B**, 901 (2005).
19. E. Walencka, B. Sadowska, S. Rozalska, W. Hryniewicz and B. Rozalska, *Polish J. Microbiol.*, **54**, 191 (2005).

(*Received*: 12 October 2006; *Accepted*: 30 January 2007) AJC-5372